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Abstract

Intracranial Self-Stimulation (ICSS) at the medial forebrain bundle consistently facilitates learning and memory in rats when administered post-training or when administered non-concurrent to training, but its scope regarding remote memory has not yet been studied. The present work aims to test whether the combination of these two forms of ICSS administration can cause a greater persistence of the facilitating effect on remote retention and affect neurogenesis in the dentate gyrus (DG) of the hippocampus. Rats were trained in active avoidance conditioning and tested in two retention sessions (10 and 90 days) and later extinction. Subjects received an ICSS session after each of the five avoidance acquisition sessions (post-training treatment) and half of them also received ten additional ICSS sessions during the rest period between retention tests (non-concurrent treatment). All the stimulated groups showed a higher performance in acquisition and retention sessions, but only the rats receiving both ICSS treatments showed greater resistance to extinction. Remarkably, at seven months, rats receiving the non-concurrent ICSS treatment had a greater number of DCX-positive cells in the DG as well as a higher amount of new-born cells within the granular layer compared to rats that did not receive this additional ICSS treatment. Our present findings significantly extend the temporal window of the facilitating effect of ICSS on active avoidance and demonstrate a neurogenic effect of rewarding medial forebrain bundle stimulation.

Keywords	Intracranial self-stimulation; Medial forebrain bundle; Active avoidance; Long-term memory facilitation; Extinction; Neurogenesis

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HIGHLIGHTS

Post-training ICSS facilitates acquisition and 10d-retention of active avoidance

Post-training ICSS maintains a favourable effect on 90d-remote retention test

Additional ICSS sessions strengthen remote retention and hinder extinction

10-session ICSS treatment improves neurogenesis in DG in 7-month-old rats

TITLE

Rewarding deep brain stimulation at the medial forebrain bundle favours avoidance conditioned response in a remote memory test, hinders extinction and increases neurogenesis

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Running title:
ICSS potentiates memory maintenance and neurogenesis

Keywords:
Intracranial self-stimulation; Deep Brain stimulation; Medial forebrain bundle; Active avoidance; Long-term memory; Extinction; Neurogenesis

ABSTRACT

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Rats were trained in active avoidance conditioning and tested in two retention sessions (10 and 90 days) and later extinction. Subjects received an ICSS session after each of the five avoidance acquisition sessions (post-training treatment) and half of them also received ten additional ICSS sessions during the rest period between retention tests (non-concurrent treatment). All the stimulated groups showed a higher performance in acquisition and retention sessions, but only the rats receiving both ICSS treatments showed greater resistance to extinction. Remarkably, at seven months, rats receiving the non-concurrent ICSS treatment had a greater number of DCX-positive cells in the DG as well as a higher amount of new-born cells within the granular layer compared to rats that did not receive this additional ICSS treatment.

Our present findings significantly extend the temporal window of the facilitating effect of ICSS on active avoidance and demonstrate a neurogenic effect of rewarding medial forebrain bundle stimulation.

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1. INTRODUCTION

Long-term memory plays a key role in the psychological well-being of individuals and increases their adaptive possibilities in the future. Memory consolidation can be affected by multiple variables, including the subject's motivational state. Activation of neuroanatomical areas of the reward system through deep brain stimulation (DBS), not only manages to generate appetitive responses, but it also modulates memory systems, affecting both their physiology and the behavioural outcome associated to their function. In fact, activation of the lateral hypothalamus at the medial forebrain bundle (MFB) via intracranial electrical self-stimulation (ICSS), a form of rewarding DBS regulated by the subjects themselves, has been proven to consistently facilitate learning and memory, in both implicit [1],[2] and explicit [3] tasks, in rats. However, the anatomical structures mediating this memory-improving action are not yet fully specified.

Effects of MFB-ICSS in two-way active avoidance (TWAA), an emotional conditioning task that involves an implicit memory, have been extensively studied. It has been shown that post-training ICSS enhances TWAA memory consolidation and improves retention evaluated at a few days to a few weeks after the treatment. Thus, using a distributed TWAA paradigm and five ICSS treatment sessions, the facilitated memory was maintained for up to 30 days [4]. Data from several post-training ICSS studies agree with the idea that the temporal extent of the memory facilitating effect could depend on the amount (number of trains) as well as the distribution (number of sessions) of the treatment [4],[5],[6],[7],[8],[9]. However, as far as we know, it has not been possible to demonstrate maintenance of the facilitating effect beyond 30 days. For instance, a single ICSS session was unable to maintain its memory facilitation effect after 60 days [10]. Nevertheless, a different study found long-lasting (60 days)

structural changes in memory-related structures after a longer ICSS treatment [11], suggesting that longer-term effects on retention could be achieved by adjusting treatment parameters. In that study, ICSS treatment consisted of 1h daily over a period of 10 days, which was administered alone, without another learning task. Furthermore, other studies showed that this form of non-concurrent to training administration of ICSS also facilitates later learning –after 10 days– in operant and spatial tasks [12],[13], but their longer-term effects on memory have not yet been determined. Altogether, ICSS at the MFB seems to be able to facilitate learning and memory whether it is administered alone, or as a post-training treatment, but the number of rewarding trains must be taken into account when considering its long-term effects.

The capacity of the MFB-ICSS to modulate different types of tasks and memory processes is supported with its extensive connective network found therein, which causes a widespread state of arousal and simultaneous activation of many areas, some of which are associated with various memory systems [14]. It has been consistently found that MFB-ICSS leads to increased levels of c-Fos and Arc proteins [15],[16],[17] and mRNA of some synaptic plasticity-related genes in memory-related regions [18],[19], as well as morphological changes of the dendritic tree and the spines of hippocampal pyramidal neurons [20],[21]. Overall, these data argue in favour of the ability of ICSS to induce neurophysiological and structural changes similar to those underlying learning and memory. In addition to these mechanisms, some data also go in favour of an effect of ICSS in neurogenesis, another relevant form of structural change related to learning and memory. In a previous study, we have shown that an acute ICSS treatment induces expression of neurogenesis and neuroprotection related genes [18]. Similarly, Takahashi et al. (2009) observed that the activation of the reward pathway via ICSS at the MFB (1h/day for 3 days) appears to enhance cell proliferation in the hippocampal DG of mice

and rats. Moreover, these authors showed that, even if low, a percentage of newly generated cells mature to become functional after a spatial learning experience [22]. However, DBS to other brain targets, when also being used as a treatment for cognitive and behavioural disorders, does not always have an effect on neurogenesis. Thus, while DBS in regions such as the anterior nuclei of the thalamus, ventromedial prefrontal cortex or the entorhinal cortex promotes neurogenesis [23],[24],[25], in others such as the fornix there are completely contradictory results [26],[27]. Moreover, no effects on neurogenesis were observed after DBS in reward system regions other than MFB [28]. These data show that a deeper knowledge of the neurogenic capabilities of the diverse DBS treatments for improving memory, and of MFB-ICSS in particular, is needed.

The present study aims to test whether a new form of ICSS treatment administration can promote remote retention in a TWAA task, as well as to verify if this treatment affects neurogenesis in the dentate gyrus of the hippocampus (DG). In order to do so, we have tested the combined action of ICSS administered at two different times of the procedure: post-training –immediately after each acquisition session of the task – and alone – in the rest period between retention sessions (non-concurrent to training). We examined the persistence of a memory facilitated by the post-training ICSS to a remote retention test at 90 days; and on the other hand, we explored whether additional sessions of ICSS during the rest period before the remote retention test can favour the maintenance of the previously facilitated memory, and protect against forgetting. Moreover, DG hippocampal neurogenesis was analysed 19 days after the start of the non-concurrent ICSS treatment by means of doblecourtin (DCX) -as a marker of young neurons- and 5-bromodeoxyuridine (BrdU) –as mitotic marker of cell proliferation and a possible index of survival effect of the non-concurrent ICSS along the differentiation process.

2. MATERIAL AND METHODS

2.1. Subjects

A total of twenty-seven Wistar male rats from the laboratory of Psychobiology breeding stock (Universitat Autònoma de Barcelona; registered number B99-00021), aged between 13-14 weeks and weighted 363.94 g (SD=39.66) were used. Three days before the stereotaxic surgery they were singly housed in individual cages (50 x 22 x 14 cm). The animals were maintained on a 12hr light/dark cycle, and food and water were available ad libitum throughout the whole experiment. All procedures were approved by the Ethics Committee at the Autonomous University of Barcelona (protocol 2022).

2.2. Stereotactic surgery

Under general anaesthesia induced by 110 mg/Kg Ketolar® *Ketamine chlorhydrate* (Parke-Davis S.L. Pfiezer, Madrid, Spain) and 0.08 ml/100 g Rompun® *Xylazin* 23 mg/ml; i.p. (Bayer, Barcelona, Spain), all rats were implanted with a monopolar stainless steel electrode (150 µm in diameter, PlasticsOne, Roanoke, Va, USA, ref. 008inSW) aimed at the lateral hypothalamus, into the fibers of the MFB according to coordinates from the stereotaxic atlas [29]: AP = −2.3 mm; L = 1.8 mm (right hemisphere) and P = −8.8 mm. ICSS electrodes were anchored to the skull with three jeweller's screws and dental cement (vertex self-curing, Dentimex, Netherland). The animals were weighed and handled daily in the post-surgery recovery period (7 days). Rats were assigned to three independent groups according the ICSS treatment they would receive: T1 (n=9, post-training ICSS only), T1+T2 (n=8, post-training and non-concurrent ICSS) and Sham (n=10, without ICSS).

2.3. Establishment of ICSS behaviour

Rats in the experimental groups that would at some point receive ICSS treatment (T1 and T1+T2 groups) were taught to self-stimulate by pressing a lever in a conventional Skinner box (24 x 27 x 30 cm; LE 850, Letica Scientific Instruments, Panlab, Barcelona, Spain). Electrical brain stimulation consisted of 0.3 s trains of 50 Hz sinusoidal waves at intensities ranging from 30 to 300 µA (CS2-10 Cibertec, Madrid, Spain). The ICSS behaviour was shaped for each subject to establish the range of current intensities that would support responses on a continuous reinforcement schedule. The optimum intensity (OI), or the mean of the two current intensities that gave rise to the highest response rate (responses/min), was obtained during two consecutive days of training and applied in treatments (ranging from 50 to 140 µA).

2.4. TWAA acquisition, retention and extinction

TWAA was conducted in two 50 x 24 x 23 cm identical automated two-way shuttle-box (AccuScan Intruments Inc. Columbus, Oshio, USA), enclosed in two separate sound-attenuating boxes ventilated by an extractor fan and controlled by Fusion software, in a no-door version. The conditioned stimulus (CS) was 80 dB and 1 kHz tone of 3 s duration. The unconditioned stimulus (US) was a 0.6 mA electrical foot shock, presented for 10 s at maximum. The inter-trial interval varied randomly from 50 to 70 s. TWAA conditioning consisted of the CS introduction followed by the US, according to a delay procedure. Crossing into the opposite compartment during the first 3 s of the trial, i.e. before the onset of the US, was registered as an avoidance response; otherwise, both stimuli terminated simultaneously when the rat crossed (escape response), or after 13 s in the case that the rat did not respond (no response).

Fig. 1 depicts timeline of experimental design to better follow the procedure.

Habituation. Three days before the first acquisition session, each rat was allowed 10 min of free ambulation in the shuttle box to be familiarized with the learning environment. Crossing responses were recorded as a measure of control of locomotor activity.

Acquisition. This phase consisted of one daily 10-trial session for 5 consecutive days. Number of avoidance responses was considered as level of conditioning, and inter-trial crossing responses as a measure locomotor activity.

Retention. Two TWAA retention sessions, RT10 and RT90, were performed 10d and 90d after the last acquisition session, respectively. These sessions were identical to the acquisition ones, except they consisted of 20 trials. No group received post-training ICSS treatment (T1) immediately after retention sessions. With the aim of being able to differentiate between the process of memory recovery, which is more evident in the first trials of the retention session, and relearning due to the additional trials, the subjects performance was analysed separately for the first half (1 to 10 trial) and the second half (11 to 20 trial) of each of the retention sessions.

Extinction. The day after the RT90 session, rats were tested in a 30-trial extinction session (EXT). Extinction is a progressive decrease in the frequency of a conditioned response that is no longer followed by the US. It has been used as a measure of the strength of learning and memory, since resistance to extinction is stronger when the learning experience is also stronger [30]. The procedure was the same as in the retention sessions but, independently of the subject response, the CS never was followed by the presentation of the US. In addition to the number of avoidance responses, number of escape responses, non-responses and the response latency were also registered, for the 3

blocks of 10 trials and for the full session.

2.5. ICSS treatments administration

Post-training ICSS (T1). The rats in the T1 and T1+T2 groups were allowed to self-administer 2500 trains at the OI of each subject immediately after each acquisition session. The rats in the Sham group were also placed in the ICSS box for 45 min, but they did not receive any stimulation (sham treatment). Then, all rats were returned to their home cages. Treatment duration (min) and total number of lever pressings (total responses) were recorded for each T1-ICSS session.

Non-concurrent to training ICSS (T2). On day 90, nine weeks (63 days) after the RT10, rats in the T1+T2 group were allowed to one daily hour of ICSS set at the IO of each animal, for a total of 10 sessions. The sessions were distributed in two blocks of 5 consecutive days, separated by 2 days of rest. Rats in T1 and Sham groups received a sham treatment of 45 min. Total number of lever pressings (total responses) and stimulation trains were recorded for each T2-ICSS session.

2.6. BrdU administration

On the day 90, in order to analyse survival of a concrete pool of new generated cells all rats received a single dose of BrdU (200 mg//Kg, i.p.) 2 hours before to the first session of T2 (or sham) treatment. The brain samples were obtained 19 days after the BrdU marker infusion.

2.7. Tissue collection

24 hours after EXT session, rats were anesthetized with a lethal dose of pentobarbital (200 mg/kg body weight, i.p.) and perfused transcardially with a solution of 0.1M

phosphate buffer saline (PBS), pH 7.4, followed by a solution of 4% paraformaldehyde in PBS. Brains were post-fixed in 4% paraformaldehyde in PBS solution for 4 hours and then placed in 15% sucrose in PBS for 3 days and 30% sucrose in PBS at 4ºC until they sank. 30 µm coronal frozen sections were cut serially in a cryostat (Cryocut 1800, with 2020 JUNG microtome) at -25ºC, from the coordinates -2.56 to -4.80 anteroposterior to Bregma, and stored at -80ºC until immunohistochemistry staining.

2.8. Immunohistochemistry

For DCX detection, after 0.3% H_2O_2 in TBS incubation, sections were transferred to the blocking solution 0,1% BSA, 0.1% Triton-X diluted in TBS (TBS-T) for 30 min at RT. Sections were then incubated with sc 8066 anti-DCX (1:300, Santa Cruz) in 0.1% BSA TBS-T, the first 1 hour at RT and ON at 4ºC. The next day the secondary antibody donkey anti-goat biotin (1:500, Jackson Immunosearch) was applied 1hour at RT followed of incubation with SA-HRP (1:1800, Perkin Elmer) for 2 hours at the RT and visualized with diaminobenzidine (DAB) using a DAB substrate kit (Vector, Burlingame, USA). Finally, sections were mounted onto slides, dehydrated and coverslips were placed with Pertex mounting medium (Sigma, Alddrich).

For BrdU detection, sections were incubated in 0.3% H_2O_2 in TBS for 30 min. DNA was denatured by incubating the sections in 2X saline sodium citrate (SSC) containing 50% Formamide for 2h a 65ºC and in 2N HCl for 30 min at 37 °C and 0,1M Borate Buffer (pH 8.5) for 10 min at room temperature (RT) to neutralize acidic effect. Tissues were transferred to 0.1% BSA in TBS-T for 30 min. Sections were incubated overnight with sc-56258 rat Anti-BrdU, (1:75, Santa Cruz Biotech, Santa Cruz, USA) in 0.1% BSA TBS-T for 1 hour at RT and then placed ON at 4ºC on a constant shaker. The secondary antibody, Alexa Fluor-488 anti-rat (1:500; Thermo Fisher Scientific,

Rockford, USA), was applied for 1 hour at RT. Finally, sections were mounted onto slides and coverslips were placed with fluorescence mounting medium (Dako, Glostrup, Denmark).

2.9. Image acquisition and analysis

Images were obtained with a digital camera (OlympusXC-50) coupled to an epi-fluorescence microscope OlympusVanox-T. For DCX analysis, images were obtained with a 4X objective and DCX positive cells were recorded in the crest, suprapyramidal blade (SP) or infrapyramidal blade (IP) of the DG, both in the ipsi and contralateral hemispheres. A 20X objective was used to analyse cell details. DCX labelling was measured as number of labelled cells/mm along the corresponding DG layers. The number of BrdU labelled cells was recorded in the granular and subgranular layers of the DG with a 40X objective. Cells incorporating BrdU were counted along the entire SP blade or IP blade of the DG, both in the ipsi and contralateral hemispheres to the electrode. The number of cell counting from BrdU or DCX labelling was averaged from 5 or 6 sections between -3.15 and -4.30 anteroposterior to Bregma from each rat.

2.10. Design and Data analyses

Analysis of the conditioning in TWAA task was conducted with general linear model (GLM) for mixed design: *GROUP* × *SESSION* for the acquisition phase and *GROUP* × *BLOCK* for RT-10, RT-90 and EXT sessions. When the effect of the SESSION factor was statistically significant, polynomial contrasts explored the presence of linear and/or quadratic trends in the performance. A survival Kaplan-Meyer analysis was also done for the EXT session, where the *time event* was defined as the trial in which rats showed a "significant behavioural change" in relation to their individual performance in the RT90 session (defined in the results section). As we had no assumption about the shape

of hazard function, the Kaplan-Meier nonparametric procedure was used. Pairwise comparisons between groups were computed with a Log rank (Mantel Cox) test, by weighting all time points equally.

Quantitative analyses of BrdU and DCX immunopositive cells were also performed with GLM, considering the variable *GROUP* and the intra-group *HEMISPHERE*, *BLADE* or *LAYER* variables, as specified in the results section. Additionally, Spearman's correlation coefficient Rho (rs) was used to explore possible specific relationships between the parameters of ICSS behavior, the TWAA performance and neurogenesis values. The α level for all tests was set at .05. Statistical analysis was carried out with SPSS 23 (SPSS Inc., Chicago, IL, USA; SPSS Inc., 2006).

3. RESULTS

3.1. ICSS parameters

All rats receiving ICSS treatment rapidly learned to press the lever. The mean values (±SD) of ICSS parameters are summarized in Table 1 (see Table S1 for more detail). There was no difference between T1 and T1+T2 groups regarding the common ICSS parameters.

3.2. Effects of post-training ICSS on TWAA acquisition

Results showed that post-training ICSS facilitated TWAA acquisition when considering the number of avoidance responses (Fig. 2A). Longitudinal analyses throughout sessions showed that performance of groups was adjusted to an ascendant linear function (*SESSION*: $F_{(1,24)}=184.919$, $p< 0.001$) but with different slopes

(*GROUP* × *SESSION*, polynomial contrast: $F_{(2,24)}$= 5.387, p= 0.012); in particular, both the T1 and the T1+T2 groups, which had received exactly the same ICSS treatment at this experimental phase, showed a similar and more accentuated slope (between the first and the last acquisition sessions) than the Sham group (*difference* contrast, p=0.046). Differences between groups in the number of avoidance responses increased progressively over the training sessions, reaching a peak on the fifth acquisition session (p= 0.027), where both ICSS-treated groups showed a significant higher number of avoidance responses compared to Sham group (T1: p= 0.038; T1+T2: p= 0.013). There were not statistical differences between groups neither in weight nor in average number of inter-trial crossings in any TWAA session.

3.3. Effects of post-training ICSS on long-term retention (RT10)

As stated in methods section and attempting to appraise any proactive effects of the ICSS treatment on memory or on the possible relearning caused by the retention session itself, performance in both retention sessions was studied separately for each of the two 10-trial blocks in which they were divided.

The *GROUP* factor was statistically significant in the RT10 session ($F_{(2,24)}$= 4.77, p= 0.018). As it can be observed in Fig. 2B, the two groups that until then had received the T1 treatment showed significantly greater number of avoidance responses in each of the two blocks of the RT10 session, compared to Sham group (first block/retrieval, T1: p= 0.028; T1+T2: p= 0.011) (second block/relearning, T1: p=0.020; T1+T2: p= 0.036). Instead, all the experimental groups increased performance between the first and the second block of trials ($F_{(1,24)}$= 7.467, p= 0.012) and with similar evolutions (*GROUP* × *BLOCK*: $F_{(2,24)}$= 0.716, p= 0.687).

Comparison between the last acquisition session and the first block of trials in RT10

showed that all groups maintained their respective levels of avoidance response (*SESSION*: $F_{(1,24)}$=0.268, p= 0.609; *GROUP* × *SESSION*: $F_{(2,24)}$= 0.077, p= 0.926). Otherwise, the facilitating effect of post-training ICSS treatment remained 10 days after the last acquisition session.

3.4. Effects of ICSS on remote retention (RT90)

After 60 days of rest since RT10, half of the rats previously treated with post-training ICSS received 10 additional ICSS sessions alone, non-concurrent to any training (T1+T2 group), while the remaining half (T1 group) and the Sham group were placed into the ICSS box without being allowed to self-stimulate (sham treatment).

In the RT90 (Fig. 2C), differences among groups were detected depending on the block of trials (*GROUP* × BLOCK: $F_{(2,24)}$= 11.316, p= 0.001). Differences were observed in the second block of trials (p= 0.039), but not in the first (p= 0.626). The T1+T2 group showed a significantly higher number of avoidance responses than the Sham group (p= 0.017), while T1 group only showed a tendency (p= 0.056). Moreover, the analysis of the simple intra-group effects pointed out that both the T1 (p= 0.002) and the T1+T2 (p< 0.001) groups increased the number of avoidance responses between blocks. The Sham group did not vary their performance between blocks (p= 0.694).

Looking at the first block of the RT90 in relation to the last block of the RT10 session, the *GROUP* × *BLOCK* factor was significant ($F_{(2,24)}$= 7.344, p= 0.016), revealing that the Sham group was the only one that maintained their performance level (p= 0.718), while both T1 and T1+T2 groups showed significant decreases in the number of avoidance responses (p= 0.005, p= 0.002, respectively). Thus, we could state that retention levels of the three groups are equalized in the first block of trials in the RT90 session (p= 0.626).

3.5. Effects of non-contingent ICSS on Extinction

The following day after the RT90, all rats were tested in an extinction session of thirty trials. In order to study extinction with greater detail, avoidances, escape responses, and non-responses were analysed for the 3 blocks of 10 trials and for the total session. As expected, the number of avoidance responses (Fig. 3A and Fig. 3D) declines sharply between the last block of trials in the RT90 and the first block of the EXT session (Sham: from 50% to 20%; T1: from 80% to 33%, and T1+T2: from 90% to 46%), and no significant differences between groups were observed in any block of trials (*GROUP*: $F_{(2,24)}$= 1.653, p= 0.213; *GROUP* × *BLOCK*: $F_{(2,24)}$= 1.009, p= 0.412). In contrast, there were significant differences among groups in escape responses (Fig. 3B and Fig. 3D) regardless of the block, specifically when considering the total session (*GROUP*: $F_{(2,24)}$= 3.516, p= 0.046; *GROUP* × *BLOCK*: $F_{(2,24)}$= 0.927, p= 0.456). There were also differences between groups in the number of no-responses (Fig. 3C and Fig. 3D) (*GROUP*: $F_{(2,24)}$= 3.402, p= 0.05). As the interaction factor *GROUP* × *BLOCK* tended to significance ($F_{(2,24)}$= 2.288, p= 0.073), the study of simple effects was carried out and showed differences between groups specifically in blocks 1 and 3 of the extinction session ($F_{(2,26)}$= 3.543, p= 0.045; $F_{(2,26)}$= 3.515, p= 0.046). Contrast analyzes were performed based on the assumption that group T1+T2 would have greater difficulty to extinguish the previous conditioning, which would result in a greater number of conditioned responses -avoidance and escape- and therefore fewer non-responses (one sided post-hoc test of Dunnet). Indeed, T1+T2 rats showed higher number of escape responses in the total session compared to both the T1 (p= 0.031) and the Sham (p= 0.019) groups. They also had a fewer number of no responses than Sham ones in each of the 10-trial blocks (block 1: p= 0.013; block 2: p= 0.042; block 3: p= 0.014), as well as in the total extinction session (p= 0.016). No differences were

observed between the T1 and the Sham groups.

A survival analysis was also carried out to compare the number of trials that each group of treatment would need to show a significant change in their response to the CS, as a sign that they had acquired the contingencies of the new conditioning (CS signals no-US) (Fig. 3E). The *time event* was defined for each subject as the first trial in which it showed, during 3 consecutive trials, a latency of response to the CS greater than the average latency in the RT90, plus one standard deviation. According to a Kaplan-Meier analysis, the T1+T2 group presented an inferior and slower process of extinction of the conditioned response than the T1 (χ^2= 3.910; p= 0.048) and the Sham (χ^2= 4.467; p= 0.035) groups, which had not received the additional T2 treatment. No differences were observed between T1 and Sham groups.

3.6. Correlations between TWAA performance and ICSS parameters

Correlation analyses into each treatment group showed a strong and positive relationship between latencies in the RT10 and RT90 in all of them: T1 (rs= 0.929, p=0.001), T1+T2 (rs= 0.929, p= 0.001) and Control (rs = 0.915, p< 0.001). The only group in which there was a relationship between the performance in the extinction session and in RT90 was group T1. Specifically, positive and significant correlations were observed between latencies in RT90 and latencies and total number of non-responses in the extinction session (rs= 0.750, p= 0.02; rs= 0.783, p= 0.013, respectively).

In addition, Because T1 affected RT10 and T2 affected RT90 and extinction performance, correlation studies between the treatments parameters and TWAA performance were conducted. No significant correlations were found between any parameter of T1 and execution in RT10. On the other hand, a positive correlation was

observed between the total number of stimulation trains self-administered in the T2 treatment and number of avoidance responses in RT90 (rs= 0.975, *p*= 0.005). It is important to note that T2 favoured a wide range of stimulation (between 2162 and 4026 trains), whereas T1 was set at 2500 trains of stimulation. We also observed a correlation between the number of trains received in some specific T2 sessions (from 2th to 7th) and the avoidance responses in the third block of extinction (rs= 0.894, *p*= 0.0041, for all).

3.7. Effects of ICSS on number of DCX positive cells in the DG

Immunodetection showed DCX labelled young neurons in DG in rats sacrificed one week after the last session of T2 treatment and 13 weeks after T1 treatment (Fig. 4A). ICSS effects into each hemisphere and blade were analysed (*GROUP* × *HEMISPHERE* × *BLADE* ($F_{(2,19)}$ = 2.475, p=0.065) (Fig. 4B). In the ipsilateral side to the electrode, increased number of DCX positive cells were observed in T1+T2 compared to the T1 group in both the SP and IP blades (Dunnet, p= 0.03 and p= 0.036, respectively). No significant differences were observed between T1 and Sham groups, pointing to no prospective long-term effects of the treatment on T1. No differences were observed in the ipsilateral crest in either region in the contralateral hemisphere.

The correlation study did not show any significant relationship between the number of DCX labeled cells and T2 treatment parameters, TWAA performance in RT90 or extinction.

3.8. Effects of ICSS on number of BrdU positive cells in the DG

New-born BrdU labelled cells were observed 19 days post BrdU injection, which was administrated prior to the first T2 session. Comparison between T1 and T1+T2 groups,

which had received the same treatment until BrdU injection, can show the effects of non-concurrent ICSS administration on the survival of the pool of new generated cells. Moreover, the comparison between T1 and Sham groups would assess if the previous ICSS treatment, administered concurrently to the TWAA acquisition, had a long-term influence on neurogenesis. The GLM tests do not revealed statistically significant differences in the number of BrdU positive cells of the DG (granular plus subgranular cells), regardless of hemisphere, blade or treatment. Even so, the T1+T2 group exhibited great variance, statistically significant in the IP blade, as demonstrated by the Levene test (F=3.850, p=0.045). Subsequently, cells incorporating BrdU in the DG were analysed separately for the granular and subgranular layers (Fig. 5A).

In the granular layer (Fig 5B), the hemisphere does not seem to be a decisive factor for the expression of BrdU, as no differences between hemispheres were detected ($F_{(1,19)}$= 0.531, p= 0.475), nor was there any statistically significant interaction between *HEMISPHERE* and the other factors (× *GROUP*: $F_{(1,19)}$= 0.531, p= 0.475; × *BLADE*: $F_{(1,19)}$= 0.038; p= 0.233; × *GROUP* × *BLADE*: $F_{(2,19)}$= 0.007; p= 0.764). Thus, we proceeded to adjust the model by removing this factor. Data from hippocampal sections with unknown hemisphere from SP and IP were included in the analysis. For this adjusted model, neither differences were observed depending on the blade (*BLADE*: $F_{(1,19)}$= 0.008, p= 0.930; *GROUP* × *BLADE*: $F_{(2,19)}$= 0.457, p= 0.640). Instead, the GROUP factor was significant ($F_{(2,19)}$= 5.638, p= 0.012), and the Dunnett post-hoc test of showed that the T1+T2 group had a higher number of BrdU positive granular cells compared to T1 (p= 0.004) and Sham groups (p= 0.032). No statistically significant difference was observed between T1 and Sham groups (p=0.406). By contrast, no statistically significant effects were observed in the number of BrdU positive cells in the subgranular layer of the DG, neither due to the treatment

(*GROUP*: $F_{(2,19)}$= 0.018, p= 0.982), nor to its interaction with other variables (× *HEMISPHERE*: $F_{(2,19)}$= 2.285; p= 0.129; × *BLADE*: $F_{(2,19)}$= 0.347; p= 0.711).

Spearman correlation analysis showed a positive correlation between the total number of trains received during T2 sessions and the number of BrdU labeled cells that reached the granular layer in the IP DG (rs = 0.90, p = 0.037). Moreover, the total number of DG granular BrdU labeled cells correlated with the total number of avoidance responses in the RT90 (rs = 0.72p=0,043). No other consistent correlations were observed.

4. DISCUSSION

The present study tested the combined action of two procedures of MFB-ICSS administration – post-training and non-concurrent to TWAA conditioning – on memory facilitation and its long-term persistence. Results show that additional non-concurrent ICSS sessions in the rest period between retention tests can enhance the long-term maintenance of a previously ICSS facilitated conditioned response. In addition, the additional ICSS sessions have a positive effect on neurogenesis in the DG.

4.1. Effects of post-training ICSS on TWAA acquisition, long-term memory and remote maintenance

Our results are consistent with our previous findings showing that post-training ICSS, administered immediately after each acquisition session, facilitates active avoidance conditioning in a distributed paradigm and that this facilitated performance is maintained in a retention test after 10 days [31],[32],[33]. These results confirm once again the enhancing effect of ICSS on the consolidation of emotional memory as seen in other learning paradigms [1],[20].

Remarkably, this is the first study revealing that the enhancing effects of the ICSS on an active avoidance task can last for up to 3 months. Although both T1 and Control groups showed similar retention level in the first trials of the retention test at 90 days, the T1 group achieved a marked improvement throughout the session compared to the Control group, matching the high levels of conditioning presented in previous sessions. Thus, present result suggests that ICSS could have facilitated late relearning rather than long-term memory facilitation maintenance. As of the moment, the longest ICSS had maintained its effects on retention was 30 days [4]. In fact, in the only known study looking at the effects of post-training ICSS on a TWAA task beyond 30 days – 60 days –, the treatment ceased to have enhancing effects on memory [10]. Nevertheless, this apparent disagreement could be explained by the use of a massed training paradigm which allowed for only one ICSS session. Thus, while massed training paradigms are very effective at 24h [8][10] or at 48h [9][15] - regardless of the number of trials -, they become less efficient in longer periods of retention [10]. Contrarily, distributed paradigms seem to allow for greater maintenance of the memory over time. Since the number of post-training ICSS treatment sessions varies according to the number of conditioning sessions, it can be suggested that the binomial "number of ICSS sessions-quantity and distribution of training" seem to be relevant variables for the maintenance of memory potentiation by stimulation of the MFB.

Furthermore, we believe that these differences between groups can be attributable to the ICSS treatment, given that the different performances of the groups could not be explained neither by differences in weight of the rats nor by their motor activity. Therefore, we can suggest that the post-training ICSS treatment facilitates memory consolidation and long-term (10 d) retention, and that its facilitative effect is maintained in a remote retention test (90 d), manifesting itself especially in the improved ability of

stimulated subjects to relearn the conditioned response.

4.2. Effects of non-concurrent to training ICSS treatment on TWAA remote retention and extinction

Contrary to our expectations, no differences between both ICSS stimulated groups were observed in the first trials of the retention test at 90 days. Thus, it can be suggested that ICSS administered during the rest period between both retention tests does not seem to affect long-term memory persistence. In fact, neither T1+T2 nor T1 groups differ from control animals, suggesting that some trials are necessary to prime the conditioned response in a remote test of memory, even in animals that receive ICSS. Even though no direct differences were observed between both stimulated groups in the last trials of the test at 90 days, some results lead us to suggest that the additional ICSS treatment could have had a positive effect on the retention test. On the one hand, only the animals in the T1+T2 group reached a statistically better performance than the non-stimulated animals at the end of the retention test. On the other hand, there is a very significant relationship between the total amount of stimulation received during T2 and performance in terms of total conditioned responses in the remote retention test. The lack of differences between both stimulated groups may be due to a ceiling effect; i.e. the high number of correct responses shown by the T1 subjects (82%) could not leave enough room for improvement as a result of adding the effects of T2 treatment. Even though it has been demonstrated that non-concurrent ICSS predisposes subjects for a better acquisition of future learnings [34],[35], this is the first time that its effectiveness is proposed to affect the recall of a previously facilitated conditioned response 90 days later.

Moreover, the resistance to extinction shown by the T1+T2 group is consistent with this idea. Extinction after fear conditioning is considered a new learning experience as a

result of replacing the previously acquired CS–US association to a CS–no US pairing and not only a simple process of progressive erasure or attenuation of the initial learning, even though these mechanisms are not mutually exclusive [36],[37]. In TWAA, extinction also affects the instrumental response and results in a CR reduction. These new associations would coexist with the previous ones and compete with the initial conditioning when there is a new encounter, or expectation of encounter (according to the cognitive model) with the CS [38]. This means that a stronger conditioning could result in a change in the contingencies between stimuli, or perhaps it could prevent the inhibition of the conditioned voluntary response, which could in turn increase the interference between the first and second learning. Thus, the greater difficulty in extinction shown by the subjects who were subjected to the additional non-concurrent ICSS treatment could be interpreted as a sign of strengthened conditioning 90 days after its acquisition. In support of this idea, other memory-enhancing treatments –such as Rolipram– also slowed down extinction of conditioned fear [39]. It is noteworthy that molecular changes related to neural plasticity caused by this drug, such as the expression of Arc protein in the hippocampus, are also induced by ICSS [40],[41].

4.3 Effects of ICSS on neurogenesis and cell survival

Here, we provide evidence for MFB-ICSS treatment in regulating adult rat hippocampal neurogenesis in 7 months old rats. The detection of young neurons by DCX immunolabelling shows that the non-concurrent ICSS treatment, initiated 19 days before tissue collection, increased neurogenesis in the DG neurons of the stimulated hemisphere, but not in the contralateral hemisphere. The hemispheric specificity of the neurogenic effect reinforces the idea that the stimulation is primarily responsible for the increase in neurogenesis, and not any other elements that could potentially affect brain

activity, such as motor or arousal factors. Instead, the initial post-training ICSS treatment did not exert any long-term effects on forthcoming neurogenesis three months after its administration.

Despite self-stimulation and DBS treatments to different targets applied for memory enhancement having increased neurogenesis in 2-3 months old rats [22],[23],[24],[42]), this is the first report showing that brain stimulation could potentiate neurogenesis at more advanced age, at 7 months old, and therefore ameliorate neurogenic decline. A decline of neurogenesis and maturation ratio of granule cells has been reported in humans and rodents as they grow old [43] and it has been associated to aging-related cognitive deficits [44] or AD [45]. There is evidence from a post-mortem study of Parkinson disease patients which reveals that DBS leads to increased neuronal precursor cell proliferation [46]. In addition, MRI scans have shown that DBS managed to reduce hippocampal volume loss associated to AD [47]. No neurogenic effects have been reported in AD patients so far, and there is growing interest in the metabolic and cellular effects of DBS in clinical trials for AD [48],[49],[50]. In this context, the present study demonstrates that structural plasticity in cognitive brain regions adversely affected by age or AD, such as the hippocampus, could be enhanced by MFB-ICSS in middle-aged, 7 month old rats.

Considering that granule cells born in the adult brain are mostly positioned in the subgranular layer [51], it is interesting to note that MFB-ICSS treatment applied after a BrdU injection increased the number of new-born DG cells that were located within the granular cell layer. Hence, in addition to enhancing neurogenesis, ICSS treatment seems to have altered the distribution of BrdU positive cells in favour of the granular layer. Moreover, this increase of cells in the granular layer correlates with the number or stimulating reinforcements received. Since this increased migration was observed

bilaterally, we could not rule out that it may be due to learning experience in the TWAA task, rather than a specific ICSS effect. Although delayed TWAA is considered to be a hippocampus-independent task and experimental data suggest that only hippocampus-dependent learning tasks stimulate neurogenesis [52],[53], TWAA learning is capable of affecting the hippocampal formation by increasing postsynaptic plasticity [54] and, therefore, it could have also affected neurogenesis in this region. However, even though the T1+T2 group (with more BrdU positive granular cells at the end of the process) was the group with better retention of the TWAA task, the T1 group also improved TWAA retention performance relative to Sham. Notwithstanding, the latter group did not have an increased number of new-born cells positioned in the middle-upper granular layer. It has been previously reported that neither proliferation nor survival is affected by the TWAA learning task [55]. Overall, our data seem to indicate that any effects on the new-born cells were due to the ICSS treatment and not directly related to the acquisition of the task. This is supported by the absence of correlation between acquisition or retention and any of the neurogenic measures at ten days. On the other hand, since we also observed that the increase of BrdU positive cells within the granular layer positively correlated with performance of long-term retention and negatively correlated with extinction, a relationship between amounts of stimulation, neuronal differentiation and improved retention could be assumed. More research is necessary to unravel the degree of causality of the effects of stimulation or/and enhanced neurogenesis on reinforcement of long-term memory.

Surprisingly, while the overall increase in neurogenesis took place only in the stimulated hemisphere, the facilitation of migration of new-born cells was found to be bilateral. However, this apparent disagreement would be in accordance with our previous studies reporting either ipsilateral or bilateral effects of ICSS depending on the

neural plasticity marker analysed and the brain zone considered [15],[17]. For instance, ICSS has been found to increase c-Fos expression in the DG and CA1/CA3 of the hippocampus in both hemispheres; however, while Nurr1 expression was also increased bilaterally in CA1/CA3, it was only increased ipsilaterally in the DG [15]. Thus, present and previous data suggest that ICSS may have either bilateral or lateralized effects over pathways of signal transduction activating different plasticity events.

Although it could be argued that the increase in migration observed in subjects treated with non-contingent ICSS could be related to an increment in maturation ratio, the functional consequences of these anatomical differences are currently unclear and may not depend on the specific layering but instead on the integration into existing circuits [51]. In this sense, it has been reported that the functional integration into hippocampal circuits of the newly generated cells is not increased in mice subjected to entorhinal cortex DBS applied for memory facilitation, despite the evident surge of DG proliferation in the ipsilateral stimulated hemisphere [55]. Moreover, it has been reported that some altered patterns of brain activity, such as seizures, could induce changes in migration guidance of granule cells producing abnormal layering [56]. Therefore, further research is needed to unravel the functional consequences of increased migration of new-born granular cells due to MFB-ICSS.

In order to evaluate the potential effect of ICSS over cell survival, a population of new-born cells was labelled with BrdU just before the first session of T2 treatment or sham treatments. It is important to note that we do not report the total surviving cells generated on day 90, since a single BrdU injection only labels a portion of the population of new-born cells, and BrdU could also incorporate onto cells undergoing DNA repair or could induce side effects on cell proliferation and differentiation [57]; [58]. Despite these limitations, the dose of BrdU used in this study could offer an

advantage, especially considering that lower doses did not detect DNA repair [59] and that BrdU toxicity is dose-dependent [60]. In this sense, a short pulse of 200 mg/Kg BrdU did not appear to affect cell proliferation on the rat hippocampus [61]. No effective pro-survival effects of the ICSS treatment was observed on the developing DG cells in 7-months-old rats, since the final total number of surviving BrdU labelled cells remained unaltered. However, in our model, non-concurrent ICSS treatment seems to have affected the subjects differentially, since there is a high and significant variability among subjects of the T1+T2 group. This, together with the fact that we observed that ICSS induced gene expression of neuroprotective genes in a previous study [18], does not allow us to rule out that a chronic ICSS with different parameters could be neuroprotective. In this regard, clinical and preclinical studies indicate that different protocols regarding DBS targeting or age of administration may exhibit different neuroprotective properties [62],[63],[64],[65],[66]. Nevertheless, clinical evidence of neuroprotective long-term effect of DBS is controverted [67],[68], which denotes the importance on further elucidate these putative neuroprotective outcome.

4.3. Conclusions

Taken together, our findings allow us to confirm that ICSS administered immediately after the acquisition sessions of TWAA conditioning facilitates acquisition and long-term retention (10 days later) and provide the first evidence of maintenance of some facilitating effect of ICSS on a remote retention test. Furthermore, we can also conclude that the administration of some additional ICSS sessions in the rest period between retention tests clearly contributes to the strengthening of the conditioned response, as revealed by in its increase in the remote retention test as well as by a greater resistance to being extinguished. Finally, our results show that this additional ICSS treatment

administered non-concurrently to training is also able to increase neurogenesis and boost migration of newly generated cells over to the granular layer of the DG in 7-month-old rats.

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6. REFERENCES

[1] S. García-Brito, I. Morgado-Bernal, N. Biosca-Simon, P. Segura-Torres, Intracranial self-stimulation also facilitates learning in a visual discrimination task in the Morris water maze in rats., Behav. Brain Res. 317 (2017) 360–366. doi:10.1016/j.bbr.2016.09.069.

[2] J.P. Huston, C.C. Mueller, Enhanced passive avoidance learning and appetitive T-maze learning with post-trial rewarding hypothalamic stimulation, Brain Res. Bull. 3 (1978) 265–270.

[3] C. Soriano-Mas, D. Redolar-Ripoll, L. Aldavert-Vera, I. Morgado-Bernal, P. Segura-Torres, Post-training intracranial self-stimulation facilitates a hippocampus-dependent task, Behav. Brain Res. 160 (2005) 141–147. doi:S0166-4328(04)00448-6 [pii]; 10.1016/j.bbr.2004.11.025 [doi].

[4] P. Segura-Torres, I. Portell-Cortes, I. Morgado-Bernal, Improvement of shuttle-box avoidance with post-training intracranial self-stimulation, in rats: a parametric study, Behav. Brain Res. 42 (1991) 161–167.

[5] L. Aldavert-Vera, D. Costa-Miserachs, E. Massanés-Rotger, C. Soriano-Mas, P. Segura-Torres, I. Morgado-Bernal, Facilitation of a distributed shuttle-box conditioning with posttraining intracranial self-stimulation in old rats, Neurobiol. Learn. Mem. 67 (1997) 254–258. doi:10.1006/nlme.1997.3760.

[6] D. Coulombe, N. White, Posttraining self-stimulation and memory: a study of some parameters., Physiol. Psychol. 10 (1982) 343–349.

[7] E. Massanes-Rotger, L. Aldavert-Vera, P. Segura-Torres, M. Marti-Nicolovius, I.

Morgado-Bernal, Involvement of the parafascicular nucleus in the facilitative effect of intracranial self-stimulation on active avoidance in rats, Brain Res. 808 (1998) 220–231. doi:S0006-8993(98)00845-2 [pii].

[8] D. Redolar-Ripoll, L. Aldavert-Vera, C. Soriano-Mas, P. Segura-Torres, I. Morgado-Bernal, Intracranial self-stimulation facilitates memory consolidation, but not retrieval: its effects are more effective than increased training, Behav. Brain Res. 129 (2002) 65–75. doi:10.1016/S0166-4328(01)00325-4.

[9] J. Ruiz-Medina, D. Redolar-Ripoll, I. Morgado-Bernal, L. Aldavert-Vera, P. Segura-Torres, Intracranial self-stimulation improves memory consolidation in rats with little training, Neurobiol. Learn. Mem. 89 (2008) 574–581. doi:10.1016/j.nlm.2007.11.005.

[10] L. Aldavert-Vera, P. Segura-Torres, D. Costa-Miserachs, I. Morgado-Bernal, Shuttle-box memory facilitation by posttraining intracranial self-stimulation: Differential effects in rats with high and low basic conditioning levels, Behav. Neurosci. 110 (1996) 346–352.

[11] B.S. Shankaranarayana Rao, T.R. Raju, B.L. Meti, Long-lasting structural changes in CA3 hippocampal and layer V motor cortical pyramidal neurons associated with self-stimulation rewarding experience: a quantitative Golgi study, Brain Res. Bull. 47 (1998) 95–101. doi:S0361-9230(98)00056-2 [pii].

[12] K. Ramkumar, B.N. Srikumar, B.S. Shankaranarayana Rao, T.R. Raju, Self-stimulation rewarding experience restores stress-induced CA3 dendritic atrophy, spatial memory deficits and alterations in the levels of neurotransmitters in the hippocampus, Neurochem. Res. 33 (2008) 1651–1662. doi:10.1007/s11064-007-9511-x.

[13] D. Yoganarasimha, B.L. Meti, Amelioration of fornix lesion induced learning deficits by self-stimulation rewarding experience, Brain Res. 845 (1999) 246–251.

[14] H.-R. Berthoud, H. Münzberg, The lateral hypothalamus as integrator of metabolic and environmental needs: from electrical self-stimulation to opto-genetics., Physiol. Behav. 104 (2011) 29–39. doi:10.1016/j.physbeh.2011.04.051.

[15] L. Aldavert-Vera, G. Huguet, D. Costa-Miserachs, S.P. de Ortiz, E. Kádár, I. Morgado-Bernal, P. Segura-Torres, Intracranial self-stimulation facilitates active-avoidance retention and induces expression of c-Fos and Nurr1 in rat brain memory systems., Behav. Brain Res. 250 (2013) 46–57. doi:10.1016/j.bbr.2013.04.025.

[16] A. Arvanitogiannis, T.M. Tzschentke, L. Riscaldino, R.A. Wise, P. Shizgal, Fos expression following self-stimulation of the medial prefrontal cortex, Behav. Brain Res. 107 (2000) 123–132.

[17] E. Kádár, E. Vico-Varela, L. Aldavert-Vera, G. Huguet, I. Morgado-Bernal, P. Segura-Torres, Increase in c-Fos and Arc protein in retrosplenial cortex after memory-improving lateral hypothalamic electrical stimulation treatment, Neurobiol. Learn. Mem. 128 (2016) 117–124. doi:10.1016/j.nlm.2015.12.012.

[18] G. Huguet, L. Aldavert-Vera, E. Kádár, S. Peña de Ortiz, I. Morgado-Bernal, P. Segura-Torres, Intracranial self-stimulation to the lateral hypothalamus, a memory improving treatment, results in hippocampal changes in gene expression, Neuroscience. 162 (2009) 359–374. doi:10.1016/j.neuroscience.2009.04.074.

[19] E. Kadar, L. Aldavert-Vera, G. Huguet, D. Costa-Miserachs, I. Morgado-Bernal, P. Segura-Torres, Intracranial self-stimulation induces expression of learning and

memory-related genes in rat amygdala, Genes. Brain. Behav. 10 (2011) 69–77. doi:10.1111/j.1601-183X.2010.00609.x; 10.1111/j.1601-183X.2010.00609.x.

[20] J. Chamorro-López, M. Miguéns, I. Morgado-Bernal, A. Kastanauskaite, A. Selvas, A. Cabané-Cucurella, L. Aldavert-Vera, J. DeFelipe, P. Segura-Torres, Structural Plasticity in Hippocampal Cells Related to the Facilitative Effect of Intracranial Self-Stimulation on a Spatial Memory Task., Behav. Neurosci. (2015). doi:10.1037/bne0000098.

[21] B.S. Shankaranarayana Rao, T.R. Raju, B.L. Meti, Self-stimulation rewarding experience induced alterations in dendritic spine density in CA3 hippocampal and layer V motor cortical pyramidal neurons, Neuroscience. 89 (1999) 1067–1077. doi:S0306-4522(98)00394-7 [pii].

[22] T. Takahashi, Y. Zhu, T. Hata, C. Shimizu-Okabe, K. Suzuki, D. Nakahara, Intracranial self-stimulation enhances neurogenesis in hippocampus of adult mice and rats, Neuroscience. 158 (2009) 402–411. doi:10.1016/j.neuroscience.2008.10.048.

[23] F. Chamaa, W. Sweidan, Z. Nahas, N. Saade, W. Abou-Kheir, Thalamic Stimulation in Awake Rats Induces Neurogenesis in the Hippocampal Formation., Brain Stimul. 9 (2016) 101–8. doi:10.1016/j.brs.2015.09.006.

[24] A. Liu, N. Jain, A. Vyas, L.W. Lim, Ventromedial prefrontal cortex stimulation enhances memory and hippocampal neurogenesis in the middle-aged rats., Elife. 4 (2015). http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4381300&tool=pmcentrez&rendertype=abstract (accessed 26 May 2016).

[25] A. Ronaghi, M.I. Zibaii, S. Pandamooz, N. Nourzei, F. Motamedi, A. Ahmadiani,

L. Dargahi, Entorhinal cortex stimulation induces dentate gyrus neurogenesis through insulin receptor signaling, Brain Res. Bull. 144 (2019) 75–84. doi:10.1016/J.BRAINRESBULL.2018.11.011.

[26] S. Hao, B. Tang, Z. Wu, K. Ure, Y. Sun, H. Tao, Y. Gao, A.J. Patel, D.J. Curry, R.C. Samaco, H.Y. Zoghbi, J. Tang, Forniceal deep brain stimulation rescues hippocampal memory in Rett syndrome mice., Nature. 526 (2015) 430–4. doi:10.1038/nature15694.

[27] S. Hescham, Y. Temel, S. Schipper, M. Lagiere, L.-M. Schönfeld, A. Blokland, A. Jahanshahi, Fornix deep brain stimulation induced long-term spatial memory independent of hippocampal neurogenesis., Brain Struct. Funct. (2016). http://www.ncbi.nlm.nih.gov/pubmed/26832921 (accessed 27 April 2016).

[28] C. Winter, T. Bregman, M. Voget, R. Raymond, R. Hadar, J.N. Nobrega, C. Hamani, Acute high frequency stimulation of the prefrontal cortex or nucleus accumbens does not increase hippocampal neurogenesis in rats., J. Psychiatr. Res. 68 (2015) 27–9. http://www.sciencedirect.com/science/article/pii/S0022395615001533 (accessed 29 April 2016).

[29] C. Paxinos, G Watson, The rat brain in stereotaxic coordinates, Sixth edit, Elsevier Academic Press, 2007.

[30] Y. Martínez, S. Díaz-Cintra, U. León-Jacinto, A. Aguilar-Vázquez, A.C. Medina, G.L. Quirarte, R.A. Prado-Alcalá, Effects of postnatal malnutrition and senescence on learning, long-term memory, and extinction in the rat, Behav. Brain Res. 203 (2009) 48–53. doi:10.1016/j.bbr.2009.04.016.

[31] E. Kádár, M. Ramoneda, L. Aldavert-Vera, G. Huguet, I. Morgado-Bernal, P.

Segura-Torres, Rewarding brain stimulation reverses the disruptive effect of amygdala damage on emotional learning., Behav. Brain Res. 274 (2014) 43–52. doi:10.1016/j.bbr.2014.07.050.

[32] P. Segura-Torres, L. Aldavert-Vera, A. Gatell-Segura, D. Redolar-Ripoll, I. Morgado-Bernal, Intracranial self-stimulation recovers learning and memory capacity in basolateral amygdala-damaged rats, Neurobiol. Learn. Mem. 93 (2010) 117–126. doi:10.1016/j.nlm.2009.09.001.

[33] P. Segura-Torres, L. Capdevila-Ortís, M. Martí-Nicolovius, I. Morgado-Bernal, Improvement of shuttle-box learning with pre- and post-trial intracranial self-stimulation in rats, Behav. Brain Res. 29 (1988) 111–117. doi:10.1016/0166-4328(88)90058-7.

[34] D. Yoganarasimha, B.S. Shankaranarayana Rao, T.R. Raju, B.L. Meti, Facilitation of acquisition and performance of operant and spatial learning tasks in self-stimulation experienced rats, Behav. Neurosci. 112 (1998) 725–729.

[35] L. Velley, J.M. Chassaing, B. Cardo, Learning improvement of appetitively or aversively reinforced light-dark discrimination and reversal four weeks after electrical stimulation of the lateral hypothalamus of the rat, Brain Res. Bull. 6 (1981) 377–383.

[36] M.E. Bouton, S. Trask, R. Carranza-Jasso, Learning to inhibit the response during instrumental (operant) extinction., J. Exp. Psychol. Anim. Learn. Cogn. 42 (2016) 246–258. doi:10.1037/xan0000102.

[37] A.R. Delamater, R.F. Westbrook, Psychological and neural mechanisms of experimental extinction: A selective review, Neurobiol. Learn. Mem. 108 (2014) 38–51. doi:10.1016/j.nlm.2013.09.016.

[38] J.E. Dunsmoor, Y. Niv, N. Daw, E.A. Phelps, Rethinking Extinction, Neuron. 88 (2015) 47–63. doi:10.1016/j.neuron.2015.09.028.

[39] B. Monti, C. Berteotti, A. Contestabile, Subchronic Rolipram Delivery Activates Hippocampal CREB and Arc, Enhances Retention and Slows Down Extinction of Conditioned Fear, Neuropsychopharmacology. 31 (2006) 278–286. doi:10.1038/sj.npp.1300813.

[40] E. Kádár, G. Huguet, L. Aldavert-Vera, I. Morgado-Bernal, P. Segura-Torres, Intracranial self stimulation upregulates the expression of synaptic plasticity related genes and Arc protein expression in rat hippocampus, Genes, Brain Behav. 12 (2013) 771–779. doi:10.1111/gbb.12065.

[41] E. Kádár, E.V. Varela, L. Aldavert-Vera, G. Huguet, I. Morgado-Bernal, P. Segura-Torres, Arc protein expression after unilateral intracranial self-stimulation of the medial forebrain bundle is upregulated in specific nuclei of memory-related areas, BMC Neurosci. 19 (2018) 48. doi:10.1186/s12868-018-0449-5.

[42] D.U. Jeong, J.E. Lee, S.E. Lee, W.S. Chang, S.J. Kim, J.W. Chang, Improvements in memory after medial septum stimulation are associated with changes in hippocampal cholinergic activity and neurogenesis., Biomed Res. Int. 2014 (2014) 568587. doi:10.1155/2014/568587.

[43] G.W. Kirschen, S. Ge, Young at heart: Insights into hippocampal neurogenesis in the aged brain, Behav. Brain Res. 369 (2019) 111934. doi:10.1016/J.BBR.2019.111934.

[44] S. Jinno, Aging affects new cell production in the adult hippocampus: A quantitative anatomic review., J. Chem. Neuroanat. (2015). http://www.sciencedirect.com/science/article/pii/S0891061815000940 (accessed

27 April 2016).

[45] O. Lazarov, M.P. Mattson, D.A. Peterson, S.W. Pimplikar, H. van Praag, When neurogenesis encounters aging and disease., Trends Neurosci. 33 (2010) 569–79. http://www.sciencedirect.com/science/article/pii/S0166223610001347 (accessed 16 September 2015).

[46] V. Vedam-Mai, M. Baradaran-Shoraka, B.A. Reynolds, M.S. Okun, Tissue Response to Deep Brain Stimulation and Microlesion: A Comparative Study, Neuromodulation Technol. Neural Interface. 19 (2016) 451–458. doi:10.1111/ner.12406.

[47] T. Sankar, M.M. Chakravarty, A. Bescos, M. Lara, T. Obuchi, A.W. Laxton, M.P. McAndrews, D.F. Tang-Wai, C.I. Workman, G.S. Smith, A.M. Lozano, Deep Brain Stimulation Influences Brain Structure in Alzheimer's Disease., Brain Stimul. 8 (2015) 645–54. doi:10.1016/j.brs.2014.11.020.

[48] A.W. Laxton, D.F. Tang-Wai, M.P. McAndrews, D. Zumsteg, R. Wennberg, R. Keren, J. Wherrett, G. Naglie, C. Hamani, G.S. Smith, A.M. Lozano, A phase I trial of deep brain stimulation of memory circuits in Alzheimer's disease., Ann. Neurol. 68 (2010) 521–34. doi:10.1002/ana.22089.

[49] G.S. Smith, A.W. Laxton, D.F. Tang-Wai, M.P. McAndrews, A.O. Diaconescu, C.I. Workman, A.M. Lozano, Increased cerebral metabolism after 1 year of deep brain stimulation in Alzheimer disease., Arch. Neurol. 69 (2012) 1141–8. doi:10.1001/archneurol.2012.590.

[50] A.W. Laxton, N. Lipsman, A.M. Lozano, Deep brain stimulation for cognitive disorders., Handb. Clin. Neurol. 116 (2013) 307–11. doi:10.1016/B978-0-444-53497-2.00025-5.

[51] E.A. Mathews, N.A. Morgenstern, V.C. Piatti, C. Zhao, S. Jessberger, A.F. Schinder, F.H. Gage, A distinctive layering pattern of mouse dentate granule cells is generated by developmental and adult neurogenesis., J. Comp. Neurol. 518 (2010) 4479–90. doi:10.1002/cne.22489.

[52] K. Van der Borght, P. Meerlo, P.G. M. Luiten, B.J.L. Eggen, E.A. Van der Zee, Effects of active shock avoidance learning on hippocampal neurogenesis and plasma levels of corticosterone, Behav. Brain Res. 157 (2005) 23–30. doi:10.1016/J.BBR.2004.06.004.

[53] T.J. Shors, G. Miesegaes, A. Beylin, M. Zhao, T. Rydel, E. Gould, Neurogenesis in the adult is involved in the formation of trace memories, Nature. 410 (2001) 372–376. doi:10.1038/35066584.

[54] J. Van Reempts, M. Dikova, L. Werbrouck, G. Clincke, M. Borgers, Synaptic plasticity in rat hippocampus associated with learning, Behav. Brain Res. 51 (1992) 179–183. doi:10.1016/S0166-4328(05)80211-6.

[55] S.S.D. Stone, C.M. Teixeira, L.M. Devito, K. Zaslavsky, S.A. Josselyn, A.M. Lozano, P.W. Frankland, Stimulation of entorhinal cortex promotes adult neurogenesis and facilitates spatial memory., J. Neurosci. 31 (2011) 13469–84. doi:10.1523/JNEUROSCI.3100-11.2011.

[56] C. Gong, T.-W. Wang, H.S. Huang, J.M. Parent, Reelin Regulates Neuronal Progenitor Migration in Intact and Epileptic Hippocampus, J. Neurosci. 27 (2007) 1803–1811. doi:10.1523/JNEUROSCI.3111-06.2007.

[57] R.S. Nowakowski, N.L. Hayes, New neurons: extraordinary evidence or extraordinary conclusion?, Science. 288 (2000) 771. doi:10.1126/science.288.5467.771a.

[58] B. Lehner, B. Sandner, J. Marschallinger, C. Lehner, T. Furtner, S. Couillard-Despres, F.J. Rivera, G. Brockhoff, H.-C. Bauer, N. Weidner, L. Aigner, The dark side of BrdU in neural stem cell biology: detrimental effects on cell cycle, differentiation and survival., Cell Tissue Res. 345 (2011) 313–28. doi:10.1007/s00441-011-1213-7.

[59] P. Taupin, BrdU immunohistochemistry for studying adult neurogenesis: Paradigms, pitfalls, limitations, and validation, Brain Res. Rev. 53 (2007) 198–214. doi:10.1016/J.BRAINRESREV.2006.08.002.

[60] A. Duque, P. Rakic, Different effects of bromodeoxyuridine and [3H]thymidine incorporation into DNA on cell proliferation, position, and fate., J. Neurosci. 31 (2011) 15205–17. doi:10.1523/JNEUROSCI.3092-11.2011.

[61] B. Lehner, B. Sandner, J. Marschallinger, C. Lehner, T. Furtner, S. Couillard-Despres, F.J. Rivera, G. Brockhoff, H.-C. Bauer, N. Weidner, L. Aigner, The dark side of BrdU in neural stem cell biology: detrimental effects on cell cycle, differentiation and survival, Cell Tissue Res. 345 (2011) 313–328. doi:10.1007/s00441-011-1213-7.

[62] V. Visser-Vandewalle, C. van der Linden, Y. Temel, H. Celik, L. Ackermans, G. Spincemaille, J. Caemaert, Long-term effects of bilateral subthalamic nucleus stimulation in advanced Parkinson disease: a four year follow-up study, Parkinsonism Relat. Disord. 11 (2005) 157–165. doi:10.1016/j.parkreldis.2004.10.011.

[63] P.D. Charles, C.E. Gill, T.L. Davis, P.E. Konrad, A.-L. Benabid, Is deep brain stimulation neuroprotective if applied early in the course of PD?, Nat. Clin. Pract. Neurol. 4 (2008) 424–426. doi:10.1038/ncpneuro0848.

[64] S.S.D. Stone, C.M. Teixeira, K. Zaslavsky, A.L. Wheeler, A. Martinez-Canabal, A.H. Wang, M. Sakaguchi, A.M. Lozano, P.W. Frankland, Functional convergence of developmentally and adult-generated granule cells in dentate gyrus circuits supporting hippocampus-dependent memory, Hippocampus. 21 (2011) 1348–1362. doi:10.1002/hipo.20845.

[65] N. Chen, Y. Gao, N. Yan, C. Liu, J.-G. Zhang, W.-M. Xing, D.-M. Kong, F.-G. Meng, High-frequency stimulation of the hippocampus protects against seizure activity and hippocampal neuronal apoptosis induced by kainic acid administration in macaques, Neuroscience. 256 (2014) 370–378. doi:10.1016/j.neuroscience.2013.10.059.

[66] D.L. Fischer, T.J. Collier, A. Cole-Strauss, S.L. Wohlgenant, J.W. Lipton, K. Steece-Collier, F.P. Manfredsson, C.J. Kemp, C.E. Sortwell, High-Frequency Stimulation of the Rat Entopeduncular Nucleus Does Not Provide Functional or Morphological Neuroprotection from 6-Hydroxydopamine., PLoS One. 10 (2015) e0133957. doi:10.1371/journal.pone.0133957.

[67] D. Harnack, A. Kupsch, The Impact of Subthalamic Deep Brain Stimulation on Nigral Neuroprotection-Myth or Reality?, Neuromodulation Technol. Neural Interface. 13 (2010) 160–167. doi:10.1111/j.1525-1403.2010.00282.x.

[68] T.M. Herrington, J.J. Cheng, E.N. Eskandar, Mechanisms of deep brain stimulation., J. Neurophysiol. 115 (2016) 19–38. doi:10.1152/jn.00281.2015.

FIGURE and TABLE CAPTIONS

Figure 1. Timeline of experimental design. T1: post-training ICSS treatment (5 sessions of 2500 trains each). T2: non-concurrent ICSS treatment (10 sessions of 60 minutes each). The days not specified in the figure correspond to rest periods of the rats under normal housing conditions.

Figure 2. Effects of post-training (T1) and non-concurrent (T2) ICSS treatments on TWAA task. Mean number of avoidance responses in **(A)** acquisition, **(B)** 10-day long-term retention (RT10), and **(C)** 90-day remote retention (RT90) of two-way active avoidance (* $p < 0.05$).

Figure 3. Effects of post-training (T1) and non-concurrent (T2) ICSS treatments on extinction of conditioned response. Mean number of avoidance responses **(A)**, escape **(B)** and no-responses **(C)** in the first 10 trials and total extinction session. **(D)** Cumulative representation of the above results for the whole extinction session. It can be observed how the group T1+T2 shows a high proportion of conditioned responses (escape and avoidance) in comparison with the two other groups. **(E)** Survival function represents, for each experimental group, the cumulative number of rats that reached the established extinction criterion in each extinction trial. It is noteworthy that, by the end of the session, 37.5% of the subjects in group T1+T2, but none in T1 and only 10% in the Sham group, had failed to show any signs of extinction (* $p < 0.05$).

Figure 4. Effects of ICSS on DCX positive cells in DG. (A) Representative photomicrographs of DCX immunopositive-cells for ipsilateral hemisphere *(coronal coordinates between -3,15 and -4,30 to bregma)* from one subject in each experimental

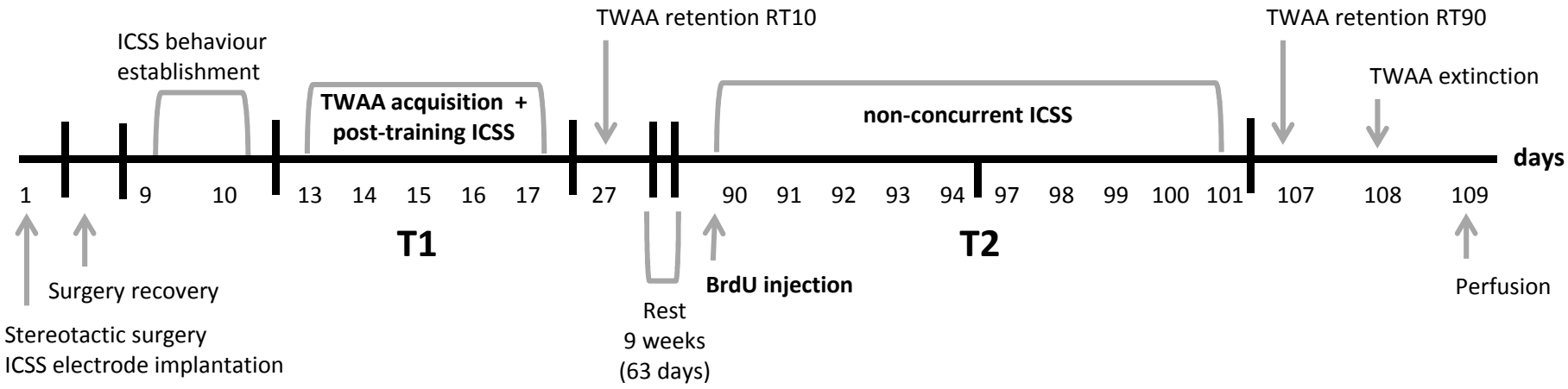
group: Sham (a, d and g), T1 (b, e and h) and T1+T2 (c, f and i). SP detail in d, e and f; and IP detail in g, h and i. Scale barr, 50µm (a-c) or 20 µm (d-i). SP: suprapyramidal DG blade, IP: infrapyramidal DG blade. **(B)** Quantification of DCX labelled cells in DG. Box plots show the effect of ICSS treatments on the number of DCX positive cells/mm (± SE) in the crest, SP blade or IP blade of the DG, on the ipsilateral or contralateral hemisphere respect to the electrode implantation. The number of DCX positive cells in the ipsilateral hemisphere is higher for group T1+T2 than group T1, in both the SP and IP blades; no changes were observed in the contralateral hemisphere (* $p< 0.05$).

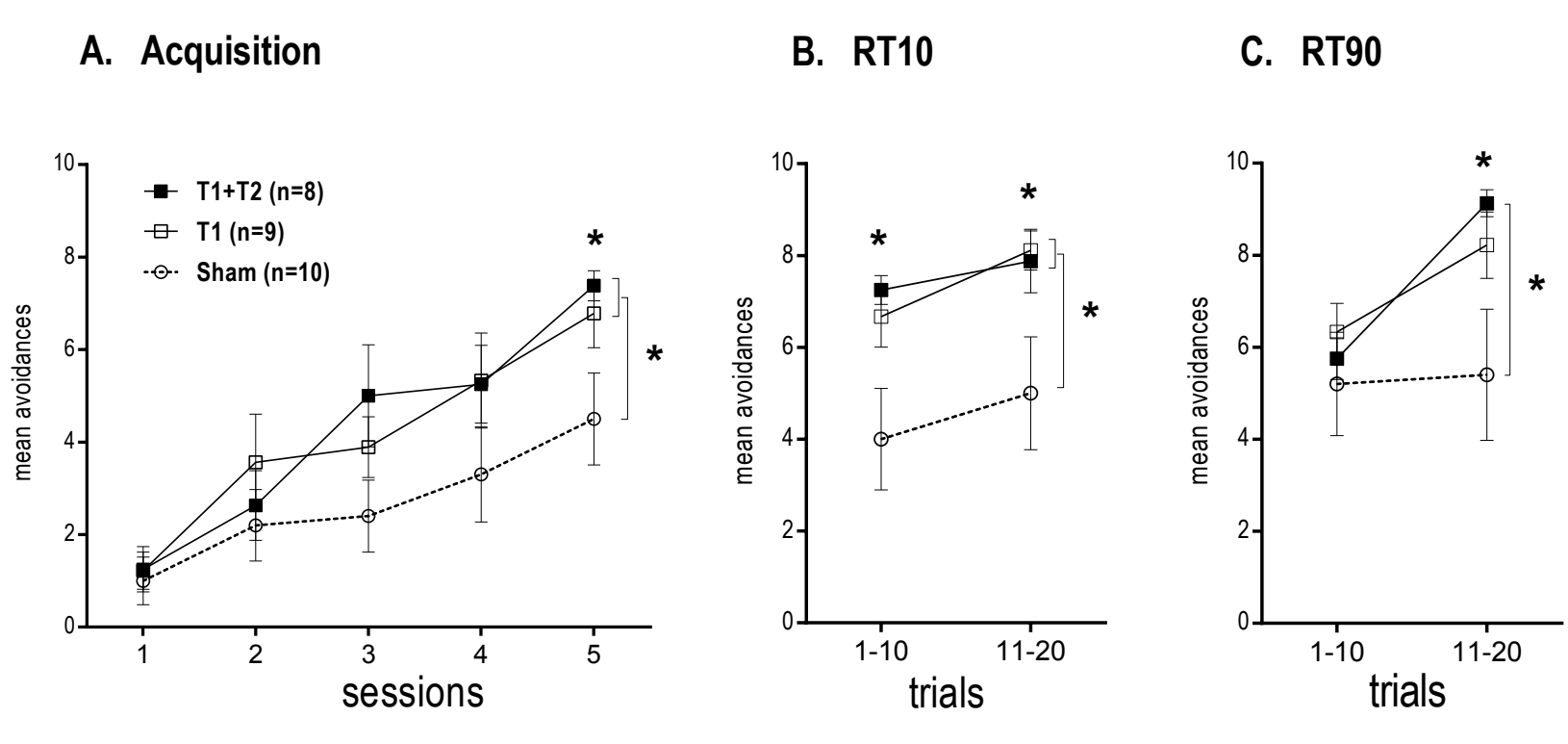
Figure 5. Effects of ICSS on BrdU positive cells in DG. (A) Representative photomicrographs of BrdU immunopositive-cells for ipsilateral hemisphere from one subject in each experimental group: a) Sham, b) T1 and c) T1+T2. Scale barr, 50µm. In the expanded top box, arrow indicates a granular cell, while arrowhead indicates a subgranular cell. **(B)** Quantification of BrdU positive granular and subgranular cells in DG. Box plots show the effect of ICSS treatments on the number of BrdU granular and subgranular cells (± SE) in the suprapyramidal or infrapyramidal DG blades of total (both hemispheres, SP and IP), ipsilateral (SP ipsi, IP ipsi) or contralateral (SP contra, IP contra) hemispheres respect the electrode placement. Comparison of total SP and IP showed that the number of BrdU positive cells is higher in the granular zone of subjects in T1+T2 group compared to the two other groups; no changes were observed in the subgranular zone (* $p< 0.05$).

Table 1: Mean values (and SD) of ICSS parameters in the T1 and T1+T2 treated groups. Intensity, responses and reward (number of trains received) in T1 and T2 sessions correspond to the average value of the 5 sessions (T1) or the 10 sessions (T2)

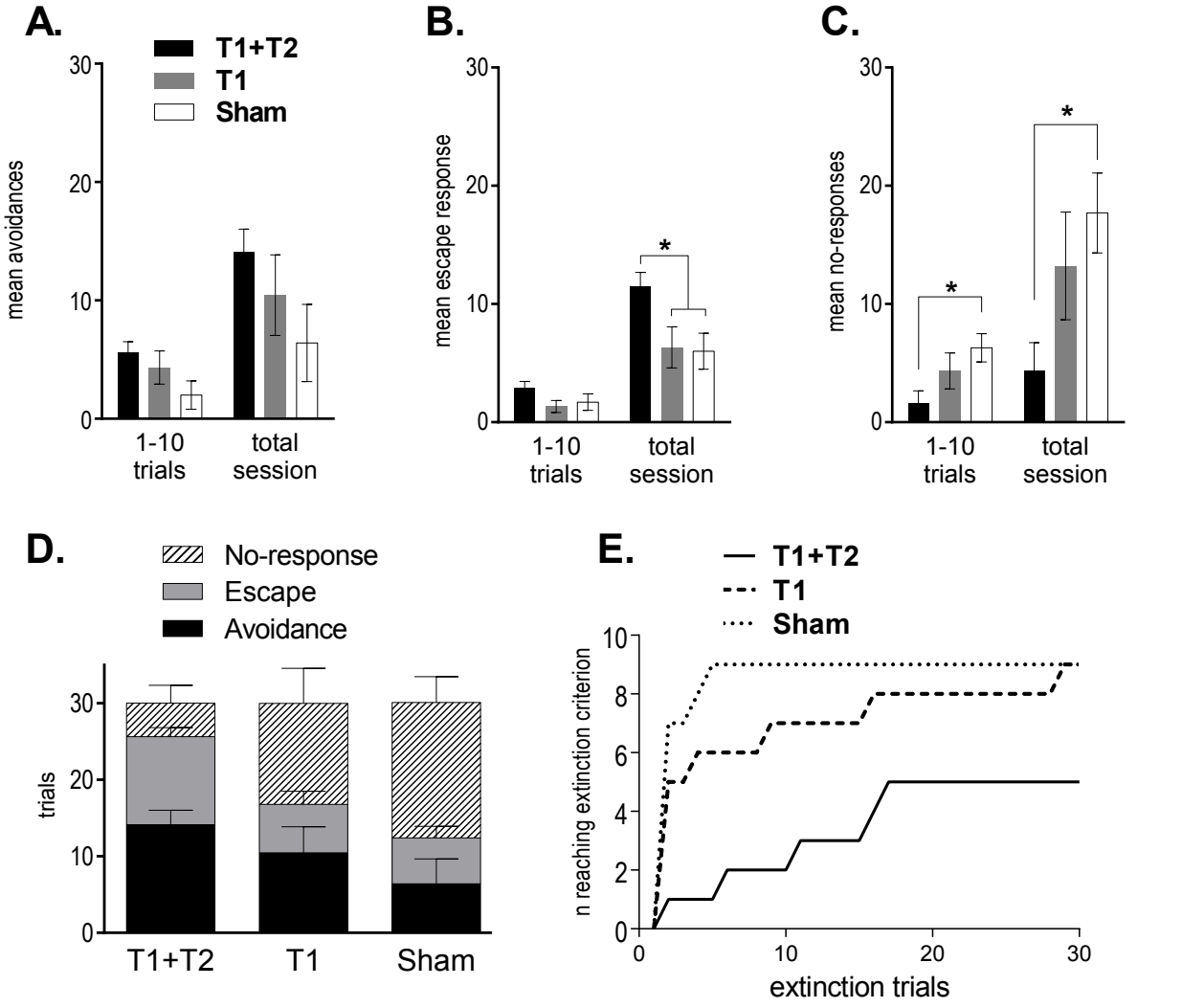
of each treatment. Intensity in the ICSS training session corresponds to the mean optimum intensity of ICSS individually determined to apply the ICSS treatments; Time: mean duration of the T1 treatment session. The p values corresponding to the T-test comparison are also indicated in the bottom row.

Table S1: Mean values (and SD) of the ICSS parameters in all the ICSS training, T1 treatment and T2 treatment sessions, in the T1 and T1+T2 groups. **A**. Current intensity. **B**. Number of responses (lever presses). **C**. Reward: number of stimulation trains received. **D**. Time: mean duration of the T1 treatment session. The p values corresponding to the T-test comparison are also indicated in the bottom row (p underlined values indicate when variance homogeneity is not assumed).

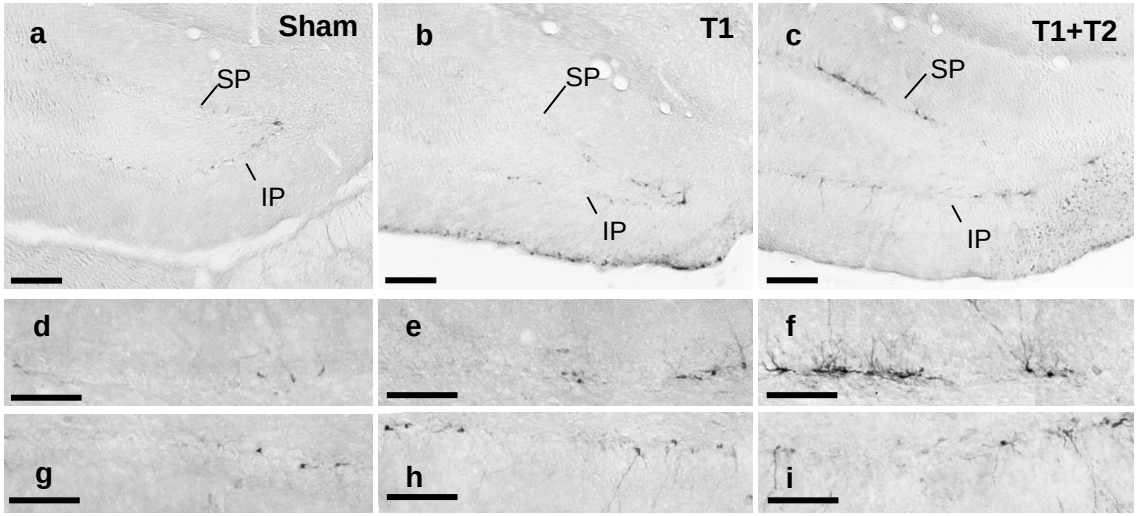

TWAA retention RT10
TWAA retention RT90
ICSS behaviour establishment
TWAA extinction
TWAA acquisition + post-training ICSS
non-concurrent ICSS
days
1
9
10
13
14
15
16
17
27
90
91
92
93
94
97
98
99
100
101
107
108
109
T1
T2
Surgery recovery
BrdU injection
Perfusion
Rest
9 weeks
(63 days)
Stereotactic surgery
ICSS electrode implantation


A. Acquisition
B. RT10
C. RT90
T1+T2 (n=8)
T1 (n=9)
Sham (n=10)
mean avoidances
sessions
trials
1-10
11-20
*

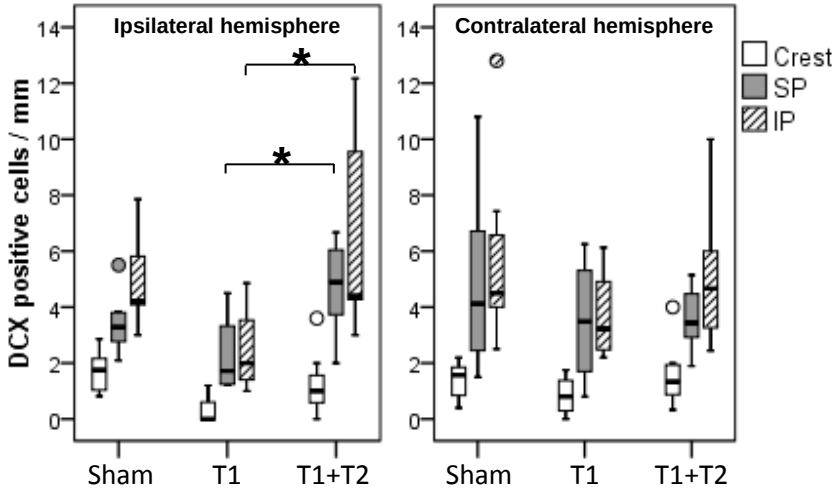
Extinction


A.
T1+T2
T1
Sham
mean avoidances
30
20
10
0
1-10 trials
total session
B.
mean escape response
*
C.
mean no-responses
*
*
D.
No-response
Escape
Avoidance
trials
T1+T2
T1
Sham
E.
T1+T2
T1
Sham
n reaching extinction criterion
extinction trials

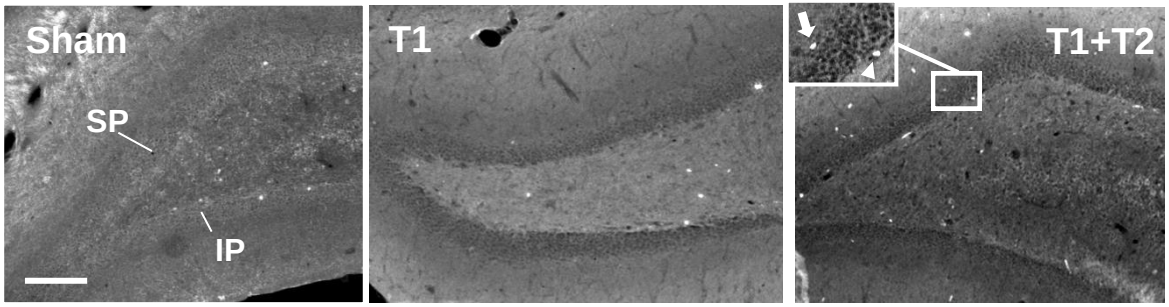
A.


a
Sham
SP
IP
b
T1
SP
IP
c
T1+T2
SP
IP
d
e
f
g
h
i

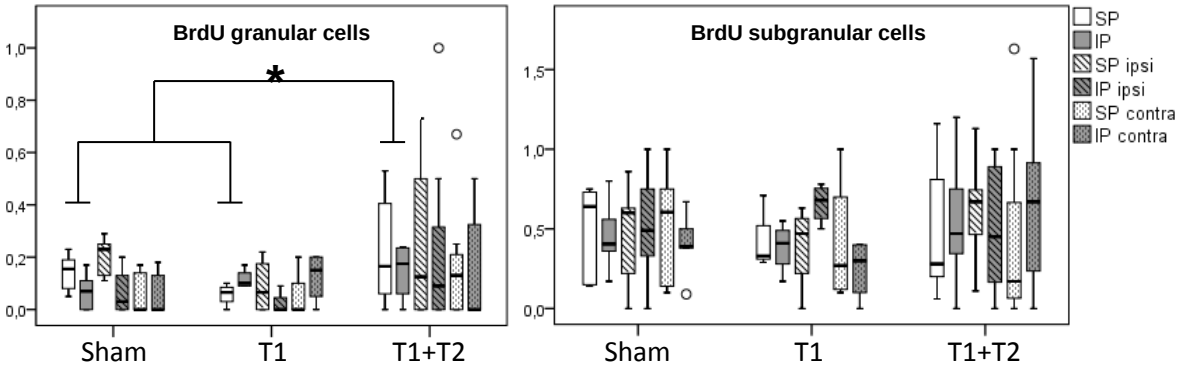
B.


Ipsilateral hemisphere
Contralateral hemisphere
DCX positive cells / mm
0
2
4
6
8
10
12
14
*
*
Sham
T1
T1+T2
Crest
SP
IP

A.


Sham
SP
IP
T1
T1+T2

B.


BrdU granular cells
*
1,0
0,8
0,6
0,4
0,2
0,0
Sham
T1
T1+T2
BrdU subgranular cells
1,5
1,0
0,5
0,0
Sham
T1
T1+T2
SP
IP
SP ipsi
IP ipsi
SP contra
IP contra

		ICSS parameters								
		Intensity (µA)			Responses			Reward (Trains)		Time (min)
		ICSS training	*T1 sessions*	*T2 sessions*	*Highest rate (RR/min)*	*T1 sessions*	*T2 sessions*	*ICSS training*	*T2 sessions*	*T1 sessions*
GROUPS	**T1**	78.3 (7.5)	71.7 (8.1)	-	67.3 (9.9)	2906.8 (151.7)	2500 fixed	430.0 (136.9)	-	50.9 (18.2)
	T1+T2	76.2 (8.3)	66.1 (7.9)	79.01 (19.2)	69.3 (11.1)	2923.6 (136.8)	3679.9 (763,1)	430.2 (110.7)	3236.1 (529.6)	45.1 (8.6)
Sig (T-Test)		0.596	0.169	-	0.703	0.815	-	0.997		0.431

	A. ICSS current intensity (μA)															
GROUPS	ICSS training		T1 sessions					T2 sessions								
	1	2	1	2	3	4	5	1	2	3	4	5	6	7	8	9	10
T1	84.4 (11.3)	72.2 (6.6)	75.5 (7.6)	70.0 (8.2)	71.6 (8.6)	71.1 (9.2)	70.5 (8.8)	-	-	-	-	-	-	-	-	-	-
T1+T2	85.0 (11.9)	67.5 (10.3)	71.2 (9.5)	66.2 (10.6)	64.3 (7.2)	63.7 (7.4)	65.0 (7.5)	62.0 (8.3)	66.0 (9.6)	71.0 (14.3)	74.0 (14.7)	78.0 (19.2)	82.0 (22.8)	88.0 (30.3)	88.0 (30.3)	90.0 (32.4)	91.0 (33.2)
Sig (T-Test)	0.923	0.275	0.319	0.427	0.082	0.094	0.186	-	-	-	-	-	-	-	-	-	-

	B. ICSS responses (lever presses)															
GROUPS	highest rate (responses/min) ICSS training	T1 sessions					T2 sessions									
		1	2	3	4	5	1	2	3	4	5	6	7	8	9	10
T1	67.3 (9.9)	3018.7 (258.3)	2900.4 (221.8)	2926.3 (180.3)	2814.3 (166.2)	2874.6 (139.7)	-	-	-	-	-	-	-	-	-	-
T1+T2	69.3 (11.1)	3018.6 (242.3)	2818.9 (125.2)	2902.8 (144.2)	2965.9 (261.8)	2912.1 (271.4)	4105.0 (877.4)	3522.6 (1209)	3821.6 (723.2)	3442.0 (842.0)	3822.4 (766.5)	3595.6 (772.9)	3911.6 (785.6)	3544.4 (820.3)	3676.6 (776.5)	3358.0 (845.4)
Sig (T-Test)	0.703	1.0	0.374	0.772	0.186	0.732		-	-	-	-	-	-	-	-	-

	C. Reward (number of trains of stimulation)												
GROUPS	ICSS training		T1 sessions (fixed)	T2 sessions									
	1	2	1 to 5	1	2	3	4	5	6	7	8	9	10
T1	399.5 (176.4)	460.4 (145.9)	2500	-	-	-	-	-	-	-	-	-	-
T1+T2	469.8 (83.3)	390.6 (180.6)	2500	3413.8 (465.4)	3009.4 (949.1)	3315.4 (427.7)	3081.0 (687.4)	3394.0 (502.2)	3248.2 (578.6)	3375.2 (470.4)	3174.4 (630.7)	3327.0 (615.1)	3023.2 (666.3)
Sig (T-Test)	0.307	0.392	-	-	-	-	-	-	-	-	-	-	-

	D. Time (duration of the ICSS treatment in min)					
GROUPS	T1 sessions					T2 sessions (fixed)
	1	2	3	4	5	1 to 10
T1	59.1 (28.9)	52.8 (19.5)	47.6 (18.0)	49.9 (17.9)	45.2 (11.7)	-
T1+T2	47.9 (7.3)	46.8 (10.5)	48.6 (12.8)	41.9 (10.1)	40.8 (9.0)	60
Sig (T-Test)	0.290	0.449	0.891	0.283	0.399	-