Deep brain stimulation of the medial forebrain bundle promotes the extinction of active avoidance and is associated with mossy fiber sprouting in the hippocampus

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Competing interests

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Abbreviations

DBS: deep brain stimulation; ICSS: intracranial self-stimulation; MFB: medial forebrain bundle; mPFC: medial prefrontal cortex; MF: mossy fiber; SO: stratum oriens; TWAA: two-way active avoidance

ABSTRACT

Background: Post-traumatic stress disorder (PTSD) causes intrusive symptoms and avoidance behaviors due to dysregulation in various brain regions, including the hippocampus. Deep brain stimulation (DBS) shows promise for refractory PTSD cases. In rodents, DBS improves fear extinction and reduces anxiety-like behaviors, but its effects on active-avoidance extinction remain unexplored. Medial forebrain bundle intracranial self-stimulation (MFB-ICSS) enhances two-way active avoidance (TWAA) conditioning by activating brain regions involved in reinforcement, learning, and memory, including the hippocampus.

Methods: This study investigates whether reinforcing DBS in the MFB enhances the extinction of conditioned active avoidance responses and examines its effects on hippocampal mossy fiber sprouting using Timm staining. We administered MFB-ICSS treatment following two 50-trial extinction sessions and assessed short-term (24 hours) and long-term (28 days) extinction in a TWAA task in rats.

Results: MFB-ICSS enhances short-term extinction and accelerates long-term reacquisition of extinction in a spontaneous recovery test. MFB-ICSS also promotes mossy fiber sprouting in the CA2 and CA3 regions of the hippocampus, with CA3 staining positively correlated with the level of extinction.

Conclusions: These findings suggest that MFB stimulation may enhance extinction and promote neural plasticity mechanisms, including mossy fiber sprouting. However, it does not fully prevent spontaneous recovery, highlighting the need for further optimization of treatment parameters. These results are relevant for PTSD as they suggest a potential enhancement in therapy for extinguishing avoidance responses in patients.

Keywords

Active avoidance extinction improvement; Deep brain stimulation; Intracranial Self-stimulation; Medial forebrain bundle; Hippocampus; Mossy fibers

HIGHLIGHTS:

Self-stimulation (ICSS) facilitates the short-term extinction of avoidance behavior

ICSS does not fully prevent the long-term spontaneous recovery of avoidance response

ICSS promotes mossy fiber sprouting in the stratum oriens of the hippocampus

Mossy fiber sprouting in CA3 correlates positively with extinction in stimulated rats

INTRODUCTION

Post-traumatic stress disorder (PTSD) results from trauma exposure and manifests through intrusive symptoms and avoidance behaviors [1]. These symptoms are linked to dysregulation of several brain regions, including alterations in the medial prefrontal cortex (mPFC), hippocampus, and amygdala [2,3]. Given the resistance to conventional psychopharmacological treatments and psychotherapy [3], novel treatment options such as neuromodulation are being explored. Deep brain stimulation (DBS) targeting regions within the neurocircuitry functionally altered in PTSD-type behavior has been widely studied in rodents and in some clinical trials [4,5]. Clinical trials in patients have shown that DBS aimed at reducing basolateral amygdala activity or enhancing mPFC function to restore a balanced response to trauma cues is yielding promising results, particularly in refractory PTSD cases [2,4,6,7].

In rodents, DBS has been applied to the amygdala, mPFC, hippocampus and ventral striatum during or after fear extinction, leading to improved fear extinction and reduced anxiety-like behaviors such as freezing [2,8–17]. Although these studies primarily focus on fear extinction, PTSD encompasses a wide range of symptoms, including intrusive and persistent re-experiencing of the traumatic event, increased arousal (manifesting as hypervigilance and high physiological reactivity), anhedonia, and persistent avoidance of trauma-associated stimuli. To our knowledge, no animal studies have examined the effects of DBS on active-avoidance extinction behavior. Active-avoidance models, unlike fear conditioning studies that predominantly focus on passive defensive responses, provide a unique opportunity to investigate active defensive responses, which are crucial in the manifestation of PTSD [1]. Thus, extinction of the two-way active-avoidance paradigm (TWAA) offers valuable insights into how DBS could influence active defensive behaviors.

In animal studies, intracranial self-stimulation (ICSS) is a specific form of DBS, where subjects perform an instrumental response to self-administer electrical stimulation to brain areas of the reward system via chronically implanted electrodes. ICSS of the medial forebrain bundle (MFB-ICSS) immediately after training significantly enhances learning and memory across various paradigms, including both explicit and implicit memory, such

as TWAA conditioning [18–21]. However, the effects of this treatment on the extinction process of the conditioned response in TWAA have not been evaluated. Considering that extinction may involve a new learning process where existing associations are modified or new ones are created, this study aims to investigate whether MFB-ICSS, administered after extinction, can facilitate this process.

At the neurophysiological level, studies of c-Fos, Nurr1, and Arc protein expression have revealed that MFB-ICSS, administered following TWAA acquisition, activates brain regions associated with reinforcement as well as other regions involved in various learning and memory systems, including the hippocampus [18–21]. Since the hippocampus is implicated in extinction and ICSS promotes behavior-related neural plasticity in this region [22], this study also aims to evaluate the effects of the treatment on hippocampal mossy fiber (MF) sprouting as a form of neural plasticity, using Timm staining. This sprouting in the stratum oriens (SO) of the hippocampus has been positively correlated with the level of learning training, as shown in a spatial task [23].

METHODS

Subjects

This study used a total of 16 male Wistar rats, aged between 3-4 months at the beginning of the experiment. The rats had an average weight of 431.46 g (SD = 76.56) and were obtained from the Psychobiology Laboratory breeding stock (Autonomous University of Barcelona, registry number B99-00029). Each rat was individually housed and maintained on a 12-hour light/dark cycle, and all training sessions were conducted during the light period. All procedures were carried out in compliance with the European Community Council Directive for care and use of laboratory animals and approved by the Ethics Committee at the UAB (order number 3942). Experimental timeline is summarized in Figure 1.

Stereotactic surgery

The rats underwent anesthesia with a solution of ketamine (110 mg/kg, Ketolar®, i.p.)

and xylazine (23 mg/kg, Rompun®, i.p.) for the surgical procedure. Each rat was implanted with a monopolar stainless-steel electrode (150 µm in diameter) targeting the lateral hypothalamus (LH), specifically within the fibers of the MFB. The coordinates from Bregma were as follows: AP = −2.3 mm; ML = 1.8 mm (right hemisphere) and DV = −8.8 mm, with the cranium surface as dorsal reference [24]. ICSS electrodes were anchored to the skull with jeweler's screws and dental cement. The rats were regularly monitored throughout the 7-day post-surgery recovery period.

ICSS behavior shaping and optimal intensity search

On day 14, 8 rats (ICSS group) were trained to self-stimulate their own brain by pressing a lever in a conventional Skinner box (25 × 20 × 20 cm). Electrical brain stimulation consisted of 0.3 s trains of 50 Hz sinusoidal waves at intensities ranging from 40 to 100 µA. The ICSS behavior was shaped for each subject to establish the range of current intensities that would support responding on a continuous reinforcement schedule. Afterwards, the rats were tested across a spectrum of intensities to establish the individual optimal intensity (OI), defined as the intensity that elicited the maximum response rate. The OI would subsequently be used to deliver the ICSS treatment following the extinction sessions. For more details, see [25].

Two-way Active Avoidance (TWAA) task

Apparatus. TWAA was conducted in a 50 x 24 x 23 cm automated two-way shuttle-box (AccuScan Instruments Inc. Columbus, Ohio, USA). The apparatus was housed within a sound-attenuating box, equipped with an extractor fan for ventilation, and controlled by Fusion software.

Habituation. On day 14, the rats underwent a 5-minute free ambulation session to acclimate to the conditioning cage.

Acquisition. From days 17 to 19, the rats underwent three conditioning sessions. one per day. Each session included 2 minutes of free ambulation in the shuttle box, followed by 50 conditioning trials. The conditioning followed a variable training schedule of 1 minute (±10 seconds) in a no-door configuration. The conditioned stimulus (CS) was a 1kHZ, 80dB tone with a maximum duration of 13 seconds. The unconditioned stimulus (US) was a 0.6 mA electrical foot shock presented 3 seconds after the onset of the CS

and lasting up to 10 seconds, following a trace procedure. Crossing before the shock (US) presentation was classified as an avoidance response, while crossing during the shock presentation was categorized as an escape response. The absence of any response throughout the 13-second duration of the trial was recorded as a non-response. The time delay (latency) between the onset of the CS presentation and the animal's response (avoidance or escape) was also recorded.

Extinction. On days 20-21, all animals underwent two extinction sessions (E1 and E2). The protocol mirrored the acquisition phase with 50 trials per session, but without the presentation of the US.

Spontaneous recovery. *On day 49,* 28 days after the last extinction session, rats underwent a single SR test of 50 trials, identical to the previous extinction sessions. This session allowed for the evaluation of long-term extinction maintenance of the conditioned response.

ICSS treatment

The ICSS treatment consisted of 2500 trains at each subject's OI. This treatment was administered immediately after each extinction session to the ICSS group (n=8). The Control group (n=8) received a sham treatment, which involved placing the rats in the Skinner box for 45 minutes, matching the average duration of the ICSS treatment, without any stimulation. Parameters such as treatment duration (in minutes) and the total number of lever presses during the treatment session (total responses) were recorded.

Tissue collection

After completing the SR test, the rats were given a lethal dose of pentobarbital (200 mg/kg body weight, i.p.) and transcardially perfused by with a 0.1M phosphate buffer saline (PBS) solution, pH 7.4, followed by a 4% paraformaldehyde solution in PBS. The brains were post-fixed in a 4% paraformaldehyde PBS solution for 2 hours, then immersed in 15% sucrose PBS solution for 3 days. Finally, they were transferred to a 30% sucrose PBS solution at 4 °C until they sank. The brains were then cut into 40 µm thick coronal sections using a cryostat. The sections were immediately mounted on chrome alum gelatine coated slides and stored at -80ºC until use.

Timm staining

Neo-Timm staining was performed according to the protocol described by [26] to stain glutamatergic synapses of MF (zinc-rich mossy fiber boutons). Briefly, air dried sections were pre-incubated in Neo-Timm's solution (0.1% sodium sulfide and 3% glutaraldehyde in a 0.1 M Sorensen phosphate buffer, pH 7.4) on a shaker at 4ºC for 72 hours. After pre-incubation, the slices were carefully rinsed twice in 0.1 M phosphate buffer for 10 min and air-dried for 10 minutes at 35ºC. Subsequently, the slides were stained with Timm's developing solution (30% gum arabic, 1.7% hydroquinone, and 0.11% silver lactate in 0.2 M citrate buffer) for 45 minutes in darkness at 35ºC. After removing the developer, the sections were rinsed in distilled water for 10 minutes, contrasted with a 5% sodium thiosulfate solution, and rinsed again in distilled water. Finally, the sections were dehydrated through a series of graded alcohol and cover-slipped using Histomount mounting media. Images of the hippocampal region between Bregma coordinates -2.5 to -4.5 were obtained using a digital camera (OlympusXC-50) coupled to OlympusVanox-T microscope with a 20X objective. The images were analyzed using the free software Image J v1.50i® (Wayne Rasband, National Institutes of Health, USA). The extent of MF sprouting was evaluated blindly by two observers using the Timm-stained sections in both ipsilateral and contralateral hemispheres. The Timm's stained area was manually delineated, and the inverse pixel density was recorded and normalized to the background in the SO-CA2 and SO-CA3 hippocampal regions as a measure of MF terminal staining. The boundaries of the CA regions were delineated for each Bregma according to [24] and [27]. Timm's labeling in each region was averaged from three to four sections per subject and combined data from the two observers.

Design and data analysis

Statistical analyses were performed using IBM SPSS Statistics 22.0. Analysis of the performance in the TWAA task (avoidances, latency, and non-responses) over acquisition sessions was conducted using mixed ANOVA 2×3 (GROUP×SESSION). The effects of the ICSS treatment on extinction (E2 and SR test) were studied by dividing sessions into 5 blocks of 10 trials (ANOVA 2×5, GROUP×BLOCK), allowing for a detailed study of the intrasession evolution. A survival Kaplan-Meyer analysis was also

performed for the SR test. The time event was defined as the trial in which rats showed a stable extinction response (5 consecutive non-responses to the CS). Pairwise comparisons between groups were computed with a Log rank (Mantel Cox) test, by weighting all time points equally.

Differences in Timm's intensity staining values have been assessed using a mixed ANOVA (GROUP × HEMISPHERE) in SO-CA2 and SO-CA3. The Spearman's correlation coefficient (rho) was used to analyze correlated patterns of Timm's staining and behavioral variables, along with simple linear regression. Results in figures are expressed as means (± SEM). Statistical significance was set at P= .05.

RESULTS

ICSS electrode placement and Treatment Parameters

All rats in the ICSS group had the tip of the electrode correctly located in the MFB, in the lateral hypothalamus between the coordinates -2.12 mm to -2.56 mm posterior to Bregma [24] (Figure S1). The ICSS rats took an average of 50,31 minutes (SE: ± 2.86) to complete the administration of the 2500 reinforcements in each treatment session, at an average intensity of 63.75 μA (± 4.97μA).

No Differences Between Groups in TWAA Acquisition and the First Extinction Session (E1)

The analysis of avoidances in the three acquisition sessions did not detect significant effects for either the GROUP factor ($F_{1,14}$= 0.558; P= .467) or the GROUP X SESSION interaction ($F_{2,28}$= 0.582; P= .565). As depicted in Figure 2A, both groups present an increase in avoidances between A1 and A2, reaching an asymptotic level in A3 (*linear function*: GROUP: $F_{1,14}$= 29.297; P= <.001; GROUP×SESSION: $F_{1,14}$= 0.009; P= .927; *quadratic function*: GROUP: $F_{1,14}$= 20.655; P= <.001; GROUP×SESSION: $F_{1,14}$= 1.795, P= .202). The analysis of latency (Figure 2B) and non-responses (Figure 2C) corroborated that, before ICSS treatment, both groups performed similarly levels during acquisition (GROUP: $F_{1,22}$= 0.324; P= .578; $F_{1,22}$= 0.324; P= .578, respectively; GROUP×SESSION: $F_{1,14}$ = 0.763; P= .397; $F_{1,14}$ = 0.763; P= .397, respectively).

After the acquisition sessions, both groups underwent a single session of extinction (E1). In E1, both experimental groups exhibited a similarly low number of avoidances ($F_{1,14}$= 1.334; P= .267; Figure 2A), and a comparable increased response latency ($F_{1,14}$= 0.872; P= .366; Figure 2B) and a similar number of non-responses ($F_{1,14}$= 0.972; P= .341; Figure 2C). The results for escape responses are presented complementarily in Figure S2 (A), where it can be observed that there are also no differences between groups either in the acquisition phase or in E1, prior to the administration of the treatment. Thus, before treatment, both groups exhibited similar extinction patterns in E1.

ICSS Improves Short-term Extinction

Effects on E2 (24 hours). The ICSS group showed a better extinction of the conditioned response, with lower response latency ($F_{1,14}$= 5.466; P= .035) and a greater number of non-responses ($F_{1,14}$= 6.434; P= .024). No differences were observed in the number of avoidances (Figure 3A) or escape responses (Figure S2), since the number of responses from both groups in this extinction session was very low. In Figures 3B and 3C, latency and non-responses are represented in the 5 blocks of 10 trials into which E2 was divided to study the evolution of extinction throughout the session. The GROUP×BLOCK factor was not significant for any variable (latency: $F_{4,56}$= 1.457; P= .228; Figure 3B; non-responses: $F_{4,56}$= 0.746; P= .565; Figure 3C), indicating that a single session of ICSS (indicated as ICSS-1 in Figure 3) facilitated extinction from the first trials.

After E2, both groups underwent the SR test. Between last block E2/first block SR, increases in the number of avoidances ($F_{1,14}$= 15.385; P= .002) and decreases in latency ($F_{1,14}$= 33.421; P< .001) and number of non-responses ($F_{1,14}$= 56.041; P< .001) were observed, independently of the group (GROUP×BLOCK: avoidances $F_{1,14}$= 1.385; P= .259; latency $F_{1,14}$= 0.159; P= .696; non-responses $F_{1,14}$= 0.368; P= .554).

In the SR test, although no differences between groups were observed (avoidances: $F_{1,14}$= 2.358; P= .147, Figure 3A; latency: $F_{1,14}$= 0.236; P= .635, Figure 3B; non-responses: $F_{(1,14)}$= 0.044; P= .837, Figure 3C; escape responses: $F_{1,14}$= 3.373; P= .088, Figure S2), the ICSS group tended to display higher latency ($F_{1,14}$= 4.175; P= .060) and a greater number

of non-responses ($F_{1,14}$= 4.211; P= .059) in the last 3 blocks of 10 trials. This trend was confirmed by a survival analysis, which compared the number of trials required by subjects to display a stable extinction response, defined as 5 non-responses in consecutive trials (Figure 4). According to a Kaplan-Meier analysis, the ICSS group showed a greater and faster reacquisition of the extinction (χ^2= 5.067; P= .024). Specifically, by trial 21, 100% of the ICSS-treated subjects reached the extinction criterion, while only 50% of the Control group reached it.

ICSS Increases Mossy Fiber Sprouting in the Hippocampus

The large terminals formed by hippocampal MF, which are rich in zinc, were revealed using Timm's staining. In the stratum lucidum (SL), consistently intense staining was observed, likely because this region receives most of the projections of the MF of the dentate gyrus. In contrast, various levels of staining were observed in the SO-CA3 region, and little or no staining was observed in the SO-CA2 region (see Figures 5A and 5B). A hemispheric comparison showed greater staining intensity in the right hemisphere, ipsilateral to the electrode, in SO-CA3 ($F_{1,14}$= 8.856; P= .01), and a trend towards greater intensity in SO-CA2 ($F_{1,14}$= 4.082; P= .063). However, a significant correlation was observed between the ipsilateral and contralateral hemispheres in both SO-CA2 (rho= 0.785; P= .021) and SO-CA3 (rho= 0.810; P= .015) in the ICSS group. No correlation in Timm labeling levels was observed between SO-CA2 and SO-CA3 regions in any of the experimental groups.

In the SO-CA2 region, differences between groups were observed ($F_{1,14}$= 8.018; P= .013), regardless of hemisphere (GROUP×HEMISPHERE: $F_{1,14}$= 0.207; P= .656). The ICSS group exhibited a higher intensity of MF terminals staining in both the ipsilateral (P= .032) and contralateral (P= .009) hemispheres (Figures 5C and 5D). In the SO-CA3 region, the GROUP×HEMISPHERE interaction showed a trend towards significance ($F_{1,14}$= 3.976; P = .066). Given the very high intensity shown by both groups in the ipsilateral hemisphere, the effect of ICSS was analyzed for each hemisphere separately. The ICSS group showed greater intensity of MF terminal labeling in the contralateral hemisphere (P= .05) but not in the ipsilateral hemisphere (P= .844), where a ceiling effect may have occurred (Figures 5C and 5D).

Moreover, in the ICSS treated group, contralateral SO-CA3 MF staining intensity correlated with a better extinction of the conditioned response in E2. As depicted in Figure 5E, the greater the MF staining intensity, the greater the latency (rho= 0.810; P= .015) and number of non-responses (rho= 0.855; P= .007), and the lower the number of avoidances (rho= -0.848; P= .008). Additionally, the contralateral SO-CA3 MF staining intensity also positively correlated with the level of extinction in the SR test, measured by response latencies, in the ICSS group (rho= 0.738; P= .037), while a similar trend is observed in the Control group (rho= 0.690; P= .058, Figure 5F).

DISCUSSION

This study is the first to investigate the use of reinforcing brain stimulation in the medial forebrain bundle (MFB-ICSS) as a novel approach to enhance the extinction of a conditioned active avoidance response. We examined the impact of MFB-ICSS administered after each of two 50-trial extinction sessions on short-term extinction retention (24 hours) and long-term maintenance (28 days) in a two-way active avoidance (TWAA) paradigm. The results indicate that ICSS enhances short-term extinction of the conditioned response and accelerates long-term extinction reacquisition in the TWAA task. Additionally, the facilitative effect of MFB-ICSS on extinction was associated with increased mossy fiber (MF) terminal sprouting in the stratum oriens (SO) of the CA2 and CA3 hippocampal regions.

Our findings revealed a significant effect of the MFB-ICSS treatment administered after the first extinction session (E1), leading to increased latency and a higher number of non-responses in the second extinction session (E2) conducted 24 hours later. However, no significant differences were detected in the number of avoidances during the E2 session. This may be because this variable is not sensitive enough to assess the extinction of the conditioned response, given the reduced number of avoidance responses the animals make after undergoing a high number of extinction trials, as in E2. These findings align with compelling evidence supporting the ability of ICSS —with its dual components of reinforcement and neuromodulatory activation—to enhance learning and memory. Moreover, they underscore the potential of MFB-ICSS as a

promising therapeutic approach for improving short-term extinction. This outcome is consistent with reductions in conditioned fear observed following DBS of brain regions within the extinction circuit, including the ventral striatum, prefrontal cortex, amygdala, or hippocampus [2,6].

In fact, stimulation of the MFB produces a generalized cerebral arousal that modulates the activation not only of regions related to the extinction circuit but also those involved in different memory systems, as revealed by c-Fos expression studies [18,21,28–30]. In this context, MFB-ICSS might enhance extinction by influencing circuits connecting the prefrontal cortex, hippocampus, and subcortical brain regions involved in suppressing conditioned responses. Additionally, it may facilitate the formation of new learning, particularly the association between the CS and the absence of the US, through activation of regions such as the hippocampus or amygdala. Supporting this latter notion, in previous studies, we have demonstrated that a treatment similar to that in the present work, but administered after the learning acquisition sessions, can facilitate the acquisition and retention not only of active avoidance conditioning [18,31], but also of other non-emotional implicit tasks [32], as well as explicit memory tasks [22,33]. It should be noted that in the present experiment, massed training was used, which seems to pose a greater challenge for the extinction of the conditioned response compared to distributed training [34]. Thus, MFB-ICSS treatment could be particularly effective for extinction under more unfavorable conditions, as has been demonstrated in the acquisition and retention of active avoidance conditioning [35–37].

The absence of differences between the experimental groups in the initial blocks of the SR test suggests that while MFB-ICSS facilitates short-term extinction of the active avoidance response, it does not prevent animals from showing spontaneous recovery after 28 days. However, in the SR test, the MFB-ICSS group presented a greater and faster reacquisition of the extinction of the response to the conditioned stimulus and/or the context than the Control group. MFB-ICSS treatment enabled all animals to reach an extinction criterion in just 21 trials. In contrast, untreated animals require a greater number of extinction trials to achieve this response, and not all succeeded. These results suggest that MFB-ICSS may accelerate long-term reacquisition of extinction learning and

underscore the importance of considering not only overall group differences but also the temporal dynamics of behavior during extinction trials. Notably, the existing literature has primarily focused on short-term extinction effects of DBS without investigating long-term spontaneous recovery [2]. Our findings indicate that merely two sessions of 2500 trains of reinforcing stimulation are sufficient to positively affect short- and long-term extinction, but they may be insufficient to prevent spontaneous recovery. Given that the facilitating effect of ICSS on active avoidance conditioning is maintained in the long term when a greater number of treatment sessions is administered [19,37], it is plausible to assume that a greater number of ICSS sessions may help to strengthen long-term extinction and prevent spontaneous recovery.

Part of the behavioral effects of MFB-ICSS on extinction may be linked to its capacity to promote structural plasticity and neurogenesis in the hippocampus, as observed in studies that assess the facilitative effect of ICSS on the acquisition and retention of different types of tasks [19,22,38]. It has been shown that the TWAA training trials increased phosphorylation of CREB in the dorsal hippocampus, amygdala, amygdalo-hippocampal junction, and hypothalamus [39]. Studies using permanent or temporary lesions demonstrate that the hippocampus contributes to memory processing in extinction tasks [40], and that inactivation of the dorsal regions disrupts context-specific extinction of fear [41]. Furthermore, optogenetic studies have shown that the hippocampus plays a key role in generating representations of fear extinction, which could be involved in control of processes like fear suppression and long-term spontaneous recovery following extinction [42]. In this regard, a large-scale study showed a robust reduction of the hippocampal volume in individuals with PTSD [43], pointing to a hippocampal involvement in the modulation of fear extinction. Likewise, an increase in hippocampal volume in PTSD patients has been associated with better treatment outcomes [44].

In this study, we demonstrate that MFB-ICSS facilitates extinction in association with an enhancement in the sprouting of the mossy fiber in the oriens layer of hippocampal CA2 and CA3 regions. Despite the MF project mainly to the CA3 region, projections to pyramidal neurons of CA2 have also been described [45], and MF staining has been observed in the SO-CA2 region proximal to CA3 in rodents [46]. In the SO-CA3 region,

differences between groups are only observed in the hemisphere contralateral to the electrode location. Nevertheless, the correlation between hemispheres for the MFB-ICSS group suggests that the stimulation affected both hemispheres similarly. The greater intensity of Timm staining in the ipsilateral CA3 region may have caused a ceiling effect, preventing the detection of group differences. The increased ipsilateral labeling, observed in both groups, might be attributed to the metallic composition of the electrode, which crosses the hippocampal region to reach the MFB, potentially interfering with the labeling method used. However, this explanation is unlikely, as the electrode is fully insulated while passing through the hippocampus, except for the terminal tip, which is the only non-insulated part. Since all animals had an implanted electrode, any effect of the electrode –no matter how small– would not explain the differences observed between the stimulated and non-stimulated animals.

It is important to highlight that, in the ICSS group, the levels of SO-CA3 Timm staining are positively correlated with higher extinction in both the E2 session and the SR test. Despite synaptic plasticity in this area related to active avoidance conditioning not being previously described, other authors have related MF sprouting into the SO-CA3 region to spatial learning. It has been observed that MF sprout into the SO-CA3 region in trained rats with enhanced spatial learning and memory [23,47], indicating that physiological activity resulting from experience can induce structural synaptic formation and plasticity in this hippocampal region. Additionally, the involvement of the CA3 oriens layer in active place avoidance has been reported in a study using functional magnetic resonance in mice [48]. Thus, similar to observations in other tasks in rodents, in the extinction learning of TWAA, the MFB-ICSS treatment may contribute to the consolidation and preservation of neural plasticity changes in key areas such as SO-CA3. The MFB-ICSS treatment also induced MT bilateral sprouting into the SO-CA2, although this was not related to the improvement in extinction. To our knowledge, this is the first time that this form of structural plasticity in the SO-CA2 region has been associated with DBS. Previous evidence of this phenomenon exists only in patients with temporal lobe epilepsy and in a mouse epilepsy model [49]. However, future studies that directly manipulate the hippocampus and its connections will be required to establish a causal relationship between these neural changes and the behavioral outcomes observed.

Here, MFB-ICSS following extinction enhances avoidance extinction processes and promotes synaptic plasticity in the hippocampus. However, it does not fully prevent spontaneous recovery, highlighting the need for further optimization of treatment parameters. Further research into the specific molecular and cellular mechanisms involved in these synaptic changes may provide valuable insights into the broader implications of MFB-ICSS in the context of extinction. Collectively, our findings suggest that reinforcing DBS targeting the MFB could be a promising strategy for mitigating avoidance behavior, a key symptom of PTSD.

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AUTHOR CONTRIBUTIONS

C T-E*: conceptualization, investigation, formal analysis, writing - original draft; S G-B: investigation, formal analysis, writing - reviewing and editing; L V-S: investigation, formal analysis, writing - reviewing and editing; G H: investigation, formal analysis, writing – original draft, reviewing and editing; E K: investigation, formal analysis, writing - reviewing and editing; L A-V: investigation, formal analysis, writing - reviewing and editing; P S-T*: conceptualization, investigation, formal analysis, writing - original draft, reviewing and editing; G C-B*: investigation, formal analysis, writing – original draft, reviewing and editing.

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ETHICAL STATEMENT

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

DATA AVAILABILITY

Datasets generated during the current study are available from the corresponding author(s) upon reasonable request.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used IA-tool ChatGPT, only used as AI-assisted improvements to human-generated texts in order to improve readability and style of the manuscript, and to ensure that the texts are free of errors in grammar, spelling, punctuation and tone. These AI-assisted improvements do not include generative editorial work or autonomous content creation. After using this IA-tool, the author(s) carefully reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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FIGURE LEGENDS

Figure 1. Experimental overview. Sixteen rats were acclimated to experimenters over three days through handling (Days 1-3). On Day 4, all rats underwent electrode implantation surgery, followed by a recovery period (Days 5-11) and additional handling (Days 12-13). Starting on Day 14, following random group assignment, eight rats underwent ICSS shaping (ICSS group, n = 8), while the other eight rats were placed in the same apparatus without stimulation (Control group, n = 8). On the same day, all animals were habituated to the Two-Way Active Avoidance (TWAA) apparatus. From Days 17 to 19, rats underwent three TWAA acquisition sessions (A1 to A3), with 50 trials per session. TWAA extinction sessions (E1 and E2) were conducted on Days 20 and 21. Following E1 and E2, the ICSS group started ICSS treatment, while the Control group underwent sham treatment (placed in the apparatus without stimulation). On Day 49, long-term extinction was assessed in a Spontaneous Recovery (SR) test consisting of 50 extinction trials. After the SR test, rats were euthanized, and their brains were processed for Timm staining to study hippocampal synaptogenesis.

Figure 2. Performance during the Acquisition (A1, A2 and A3) and Extinction 1 (E1) sessions of the Two-Way Active Avoidance did not differ between the ICSS and Control groups prior to ICSS treatment. The variables studied, including Avoidances (A), Response Latency (in seconds) (B), and number of non-responses (C), showed no significant differences between the groups. Results are presented as mean ± SEM.

Figure 3. Effect of Intracranial self-stimulation (ICSS) on short-term (E2, 24h) and long-term maintenance (SR test, 28 days) of extinction. ICSS treatment started immediately after Extinction 1 (E1) and E2. The ICSS group exhibits enhanced extinction compared to the Control group in E2, reflected by increased non-responses (B) and latency in seconds (C). In the SR test, overall group differences are not statistically significant, but during the final 30 trials, ICSS animals show a trend toward greater extinction. No differences were observed in avoidance responses (A). Data are presented as mean ± SEM. *P < .05.

Figure 4. Survival function for the Spontaneous Recovery test session. This plot shows the cumulative percentage of rats from each experimental group that reached the extinction criterion in each extinction trial. Notably, all subjects in the ICSS group reached the extinction criterion by trial 21, whereas only 50% of the Control group reached it by this point (indicated by thin dotted lines). *P < .05

Figure 5: **Hippocampal mossy fiber (MF) synaptogenesis assessment using the Neo-Timm method.** Microphotographs of the CA2 and CA3 hippocampal regions representative of each group (Control and ICSS) corresponding to (A) the contralateral hemisphere and (B) the ipsilateral hemisphere to the electrode placement. Scale bar: 400 µm. *SL*: stratum lucidum; *SO: stratum oriens.* Arrowheads show MF projections in the SO layer. Timm's staining intensity in the SO-CA2 and SO-CA3 regions in the contralateral (C) and the ipsilateral hemispheres (D). * P < 0.05, ** P < 0.01 ICSS *versus* Control. (E) Correlation between the intensity of Timm labeling in the contralateral SO-CA3 and the response latencies, as well as the number of non-responses and avoidances observed for each experimental group in extinction session E2. (F) Correlation between Timm labeling in the contralateral SO-CA3 and response latencies in the spontaneous recovery (SR) test. Linear r^2, Spearman's rho and P values are shown for each group.

Figure 1

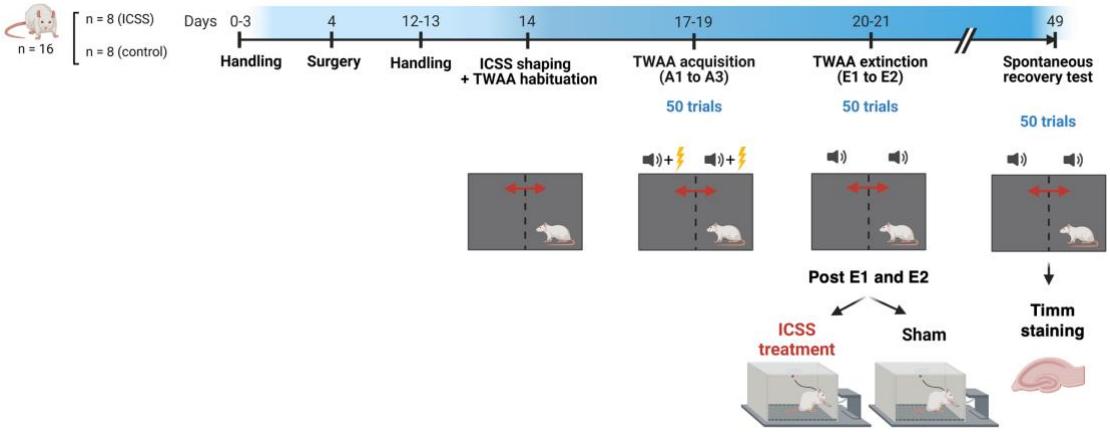

Figure 2

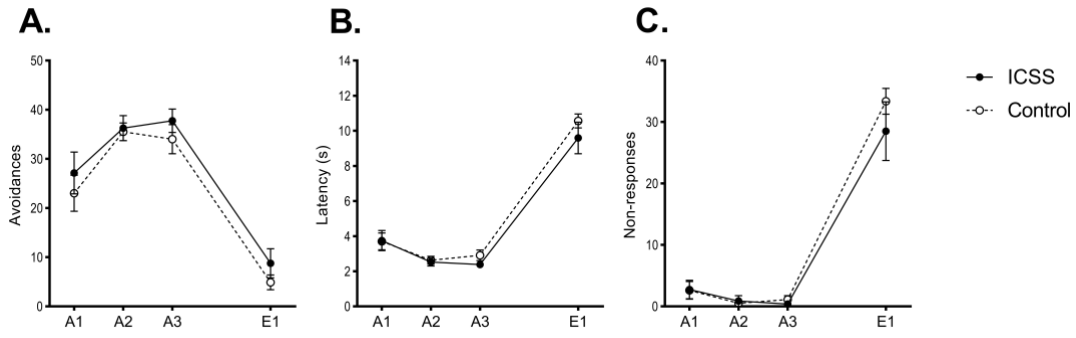

Figure 3

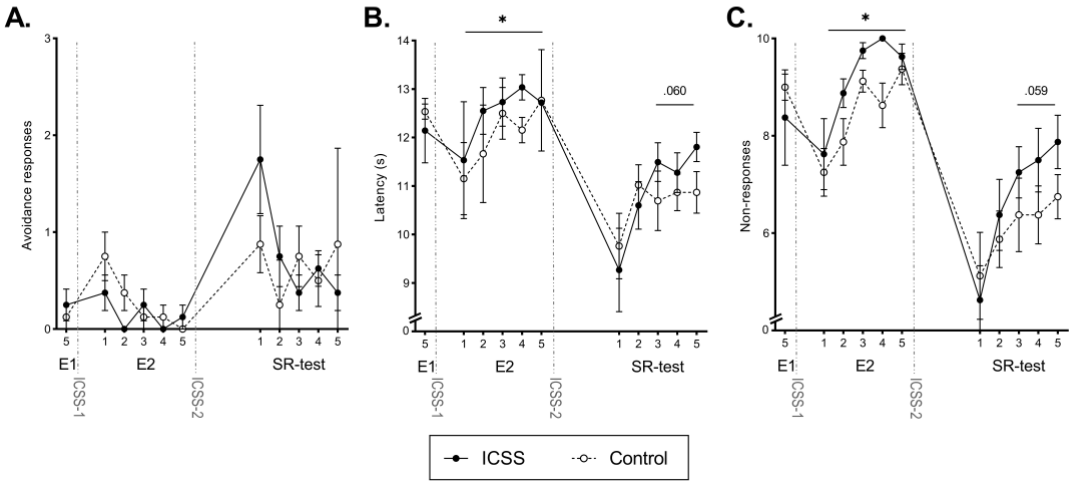

Figure 4

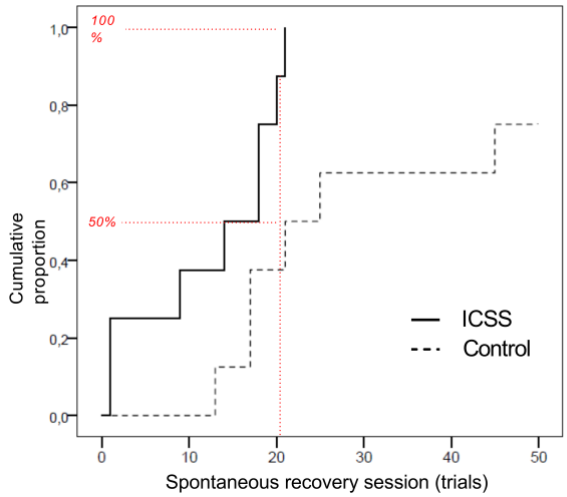

Figure 5