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Deep brain stimulation in a rat model of post-traumatic stress disorder modifies forebrain neuronal activity and serum corticosterone

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ABSTRACT

Objective(s): Post-traumatic stress disorder (PTSD), one of the most devastating kinds of anxiety disorders, is the consequence of a traumatic event followed by intense fear. In rats with contextual fear conditioning (CFC), a model of PTSD caused by CFC (electrical foot shock chamber), deep brain stimulation (DBS) alleviates CFC abnormalities.

Materials and Methods: Forty Male Wistar rats (220–250 g) were divided into 5 groups (n=8) and underwent stereotactic surgery to implant electrodes in the right basolateral nucleus of the amygdala (BLn). After 7 days, some animals received a foot shock, followed by another 7-day treatment schedule (DBS treatment). Next, freezing behavior was measured as a predicted response in the absence of the foot shock (re-exposure time). Blood serum corticosterone levels and amygdala c-Fos protein expression were assessed using Enzyme-linked immunosorbent assay (ELISA) and Western blot, respectively. Furthermore, freezing behaviors by re-exposure time test and general anxiety by elevated plus-maze (EPM) were evaluated.

Results: PTSD decreased serum corticosterone levels and increased both amygdala c-Fos expression and freezing behaviors. Therefore, DBS treatment significantly (P<0.001) enhanced serum corticosterone levels and could significantly (P<0.001) reduce both c-Fos protein expression and freezing behaviors' duration. However, DBS treatment has no effect on the general anxiety in PTSD rats.

Conclusion: We argue that these outcomes might demonstrate the mechanism of DBS treatment, a complete therapeutic strategy, in PTSD patients.

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Introduction

Post-traumatic stress disorder (PTSD) is considered one of the most devastating anxiety disorders and is a consequence of a seriously traumatic event, such as intense fear and shock (1). The neural mechanism underlying PTSD is approximately the same as fear conditioning, including fear acquisition and extinction; hence, contextual fear conditioning (CFC) makes more sense as an effective, appropriate model for PTSD (2-4).

According to functional magnetic resonance imaging (fMRI), PTSD patients exhibit hyperactivity of the amygdala, which shows higher performance than normal (5-7). On the other hand, malfunction of BLn creates a serious defect in fear extinction recall, which occurs in PTSD patients. (8-11). According to a clinician-administered PTSD scale, the severity of PTSD symptoms is directly related to the intensity of BLn amygdala activity, which is strong evidence for controlling PTSD symptoms by manipulation of the amygdala (3, 9, 11).

As regards, modifying the BLn of the amygdala by surgical therapies, such as deep brain stimulation (DBS), which transmits a chronic, high-frequency current by an electrode through subcortical structures, seems appropriate (10, 12). Moreover, there are striking reports regarding symptom alleviation in PTSD patients or animal models treated by DBS (1, 13).

In fact, current treatments for PTSD patients, such as selective serotonin reuptake inhibitor (SSRI) and psychological therapy, did not have a significant influence on PTSD symptom reduction (1, 14). During the past decades, DBS provided significant progress in modifying some psychiatric conditions, such as depression (15, 16) and obsessive-compulsive disorder, despite its unknown mechanism (1, 10, 17, 18). Some studies have focused on the DBS mechanism and have revealed different, non-deterministic reasons for the DBS effect (19).

Although the literature on the function of the Hypothalamic-Pituitary-Adrenal axis (HPA) system in PTSD is inconsistent, several recent papers reported a decrease in HPA axis activity and indicated low serum stress hormone in PTSD patients (20, 21). Furthermore, the cortisol hormone in humans and corticosterone in rodents serve as inducing factors and play a significant role in PTSD and fear conditioning (8, 22, 23). Moreover, another indicator of fear learning memory is the expression of c-Fos protein in the amygdala, which provides neural activity monitoring (24, 25).

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On this basis, we examined whether DBS affects the symptoms in PTSD models of rats. Determining concurrent changes in stress hormones, c-Fos and PTSD symptoms induced by DBS will improve our knowledge of the underlying mechanism of DBS for PTSD therapy.

Materials and Methods

Animals

Male Wistar rats (weighing 220–250 g, Pasteur Institute, Tehran, Iran) were obtained 1 week prior to the experiments and housed individually in the laboratory at 22±2˚C under at 12:12 hr light/dark cycle for environmental adaptation, with free access to water and food, except for the duration of the experiment. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the medical department of Tehran University. Notably, experiments were conducted during daylight hours and under standard conditions.

Experimental design

Animals were randomly divided into 2 groups of non-PTSD (non-CFC rats) and PTSD (CFC rats), which were each divided into subgroups (n=8) (23). The non-PTSD (non-CFC rats) subgroups were as follows: (A) intact (no manipulation) and (B) sham-operation (surgery circumstance without electrode implantation). The PTSD (CFC rats) subgroups were as follows: (A) PTSD without surgery (positive control), (B) PTSD with surgery and no treatment (control), and (C) both PTSD and DBS treatment (Figure 1).

Surgical method

Once the habituation period was over, the groups that needed surgery underwent implantation of a single intracranial electrode in the right amygdala. The animals were anesthetized with intraperitoneal injections of ketamine (30 mg/kg, IP) and xylazine (15 mg/kg, IP). The rat's head was held by a stereotaxic frame, and the fur on top of the rat's head was removed and cleaned with iodine and ethanol; an incision was made based on the exact modeling of the cranium. The area of infusion was determined and marked following the coordinates given in Paxinos and Watson Atlas (1987). The certain point (lateral 4.8, anterior-posterior -2.5, ventral-dorsal 7.4) on the right amygdala was secured. A small hole was created by a dental drill at the exact point. The electrode, which was attached to the plastic connector of the stereotaxic arm, was gently inserted into the BLn. The connector and 4 screws were fixed on the surface of the skin using dental cement, and the arms of the stereotaxic device were released. Following the operation, the animals were given 7 days to rest. Buprenorphine (0.1- 0.5 mg/kg, IP) was administered daily for postoperative analgesia for 3 days.

Contextual fear conditioning: inescapable foot shock

CFC training and behavioral testing occurred in a rodent observation cage (30 × 37 × 25 cm) that was situated inside a soundproof chamber. The sidewalls of the cage were assembled with stainless steel, and the back walls and doors were assembled with clear Plexiglas. The floor contained 20 steel rods, which delivered electrical shocks generated by a science beamshock generator.

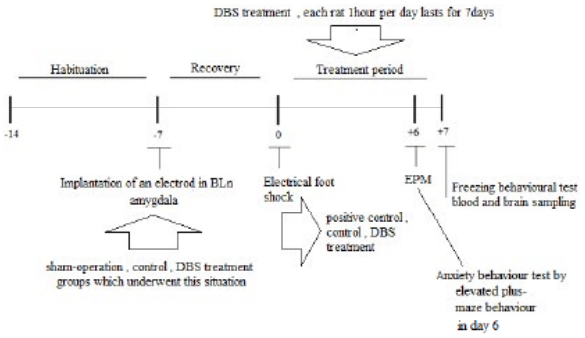

Figure 1. Animal treatment schedule. Evaluation of rats was performed after post-surgery recovery (7 days) of the electrode implantation in the right BLn amygdala. In the first day (day 0 cited in Figure 1) after recovery, electrical foot shock (CFC modeling) was performed for positive control, control, and DBS treatment groups. Consequently, for 7 more days, DBS treatment group was treated by induced stimulation while others got no treatment until the end of the process. EPM test was evaluated on day +6 as cited in Figure 1, for all the groups (without interrupting DBS treatment protocol). Next day (day +7 cited in Figure 1) those groups that had electrical foot shock undertook the freezing behavior test in the re-exposure time. Ultimately, blood sampling from orbital sinus as will be mentioned later was collected and all animals were sacrificed fast enough for avoiding any problem in brain sampling

Each observation cage was cleaned with an ethanol 90% solution before and after each test. On the conditioning day, the rats were individually transferred to the cage, and after 15 min of habituation, the rat received ten 1-sec foot shocks (unconditional stimuli) of 2 mA intensity for 5 min with 30-sec intervals (1, 27). The rats were removed from the cage after 10 min. For the conditioning test after 7 days (retention), the rats were placed back into the cage (CFC) in the absence of electrical foot shock and remained there for approximately 8 min for a context test. A camera recorded behavior during both training and testing. The time spent by each rat, whether freezing or active, was counted by an individual who was blinded to experimental conditions. Freezing as a behavioral marker is interpreted as an immobile posture except for respiration movement. Behavior was scored for the testing part, and the behavior evaluated during the 8-min re-exposure to the context and scores for each part were studied individually (23).

Deep brain stimulation (DBS)

High-frequency DBS treatment started the first day after CFC. All DBS groups were kept in individual cages during DBS. The external pulse generator was attached to the intracranial electrode with the plastic connector. There was enough wire to allow the animal to move freely in the cage. The DBS current flowed for 1 hr through the implanted electrodes constantly for 7 days in each rat. During DBS induction, a rat which was connected to stimulator was alone in the DBS cage until the end of the stimulation. The current setting involved monopolar, 120 ms pulse width, 160 Hz frequency, and 2.5 volts electrical potential (1). The control group rats were also linked to the pulse generator in the same setting, but without any pulse delivered.

Elevated plus-maze (EPM) test

The apparatus is a cross-shaped maze with 4 arms

made of wood. Each arm pathway is 90 cm away from the common center. The two opposite arms open to the environment, and the other opposite arms are surrounded by 40 cm walls, except for the path to the common center. This maze is normally used to evaluate anxiety in rats. Anxiety is calculated based on the time that animals spend in the open arms in 5 min. First, rats are placed in the center of the cross, facing one of the open arms. A blind examiner recorded movement factors. All important factors, such as open arm entries (%OAE), which is a percentage of the open arm entry number divided by the total number of open and closed arms entries were defined. Additionally for open arm time (%OAT) criterion, the same calculation process was evaluated by considering the amount of time spent in open arm divided by total time spent in both arms (1, 11).

Corticosterone measurement

To analyze corticosterone level in the serum of PTSD and non-PTSD rats, approximately 2 ml blood was obtained from the orbital sinus of the rat's eye (28). Some samples were collected before training, and some after the testing. Consequently, the blood samples were centrifuged under the following settings: 3500 rpm for 5 min at room temperature. The serum was stored at -70 °C. Corticosterone was quantified using an Enzyme-linked immunosorbent assay (ELISA) DRG Corticosterone kit (Cat. No. EIA4164).

c-Fos Western blots

Expression of c-Fos protein was evaluated by Western blot as previously described. All groups were sacrificed at the end of the experiment, and the whole brain tissue was removed quickly and carefully (less than 2 min, transferred from nitrogen tank to the storage place -80 °C). The BLn was neatly dissected from the brain (almost 1 g) and placed in 1 ml or 400 µl of ice-cold SDS lysis buffer (2% SDS, 0.3% DTT, 10% glycerol in 40 mM Tris-cl, pH 6.8). Consequently, the samples were homogenized by ultrasonic disruption, and the obtained solution heated to 85 °C for 8 min and centrifuged (12000 rpm for 12 min). Protein samples were separated from the pelleted debris. Total protein segments were separated by SDS-PAGE gel on a Bio-Rad system and immune-blotted. Subsequently, semidry electron-transfer to nitrocellulose membranes was performed (0.2 µm pore size) and blots were placed in blocking solution (5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBS-T)) overnight at 4 °C with a gentle shaking. Following the overnight incubation, the blots were incubated in primary antibody solution (anti-c-Fos antibody - ChIP Grade, rabbit polyclonal to c-Fos - ChIP Grade, Abcam ab7963. 1:200), which was dissolved in TBS-T for 2 hr at 4 °C with gentle shaking. The blots were then washed three times for 15 min with TBS-T and incubated for 1 hr at 1:10000 dilution of secondary antibody (Dako REAL EnVision, Rabbit/Mouse, HRP conjugated), which was dissolved in TBS-T. Next, the membranes were washed three times with TBS-T for 5 min each. Finally, to visualize the blots, we used diaminobenzidine (DAB), the common substrate for horseradish peroxidase (HRP), which was conjugated to the second antibody. For quantification, the c-Fos band (as the examined protein) compared with the bate-actin band (as the housekeeping protein) after normalizing by the optical density of each band was evaluated, and densitometry values for each experimental group were calculated by using the Image J software and reported as mean ±SEM.

Statistical analyses

The quantitative findings were presented as the mean ± SEM. One-way analysis of variance and complementary Tukey's test were used to compare intergroup means. A *P*-value <0.05 was considered significant.

Tissue confirmation

To confirm that the electrode was placed correctly in the BLn amygdala, a cannula implanted in BLn position and 1 µl of Trypan blue solution (10 µg/rat) was injected through the cannula with a Hamilton syringe.

Results

Proof of certain point of electrode in the BLn amygdala

To evaluate exact point of the electrode in the BLn amygdala, the animal was sacrificed and the brain slides confirmed the correct location of the cannula in the BLn area (Figure 2).

Significant reduction of the freezing behavior following 7 days of both treatment schedules in CFC rats (PTSD model)

Freezing behavior was observed in the context re-exposure time. Furthermore, the recorded mean time of the freezing behavior in the treatment group (DBS) was lower than that of the freezing behavior in non-DBS treatment groups, indicating a notable decrease in the freezing time (P<0.001). Additionally, there were remarkable differences between the treatment group (DBS treatment (2.00±0.20), P<0.001) and non-DBS g[illegible] (

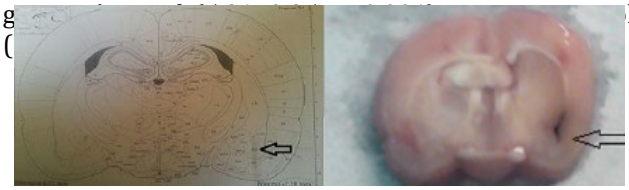

Figure 2. This image shows the position of the tip of the electrode (arrow) in the BLn of the right amygdala

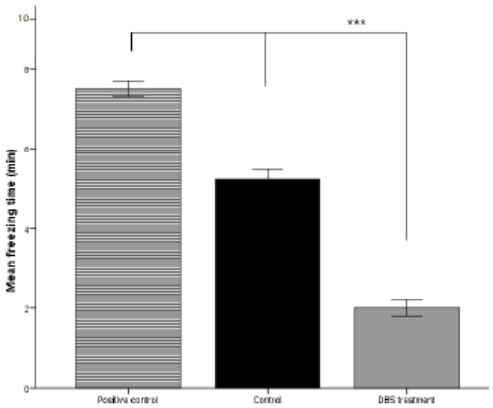

Figure 3. Changes in the freezing time. The exploratory behavior was assessed 7 days post-shock in the groups at the re-exposure time in the context. ***P<0.001, indicates a significant difference between the groups

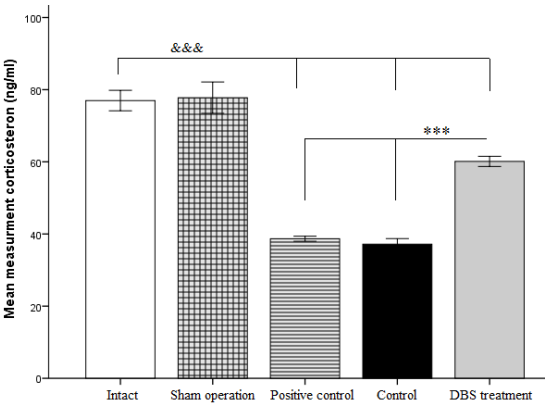

Figure 4. Serum corticosterone level presented after the re-exposure time in the groups. ***P<0.001, &&& P<0.001, indicate a significant difference between the groups

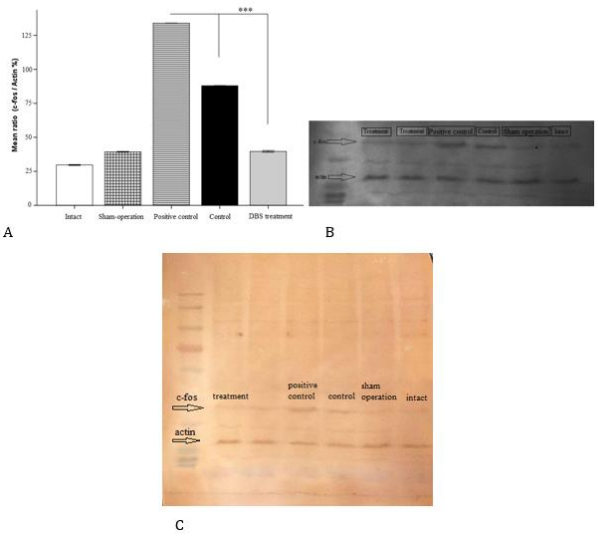

Figure 5A. (left), the amygdala c-Fos expression presented after the re-exposure time in the groups. ***P<0.001, significantly different from the treatment groups, **5B.** (right), Western-blot image. A c-Fos band and actin as the loading protein are marked in the image. There is a remarkable difference between the bands of each group, indicating the effect of DBS treatment (The treatment group was assessed twice), **5C.** Western-blot image (better resolution same picture)

Serum corticosterone level increased markedly following 7-day DBS treatment schedule in CFC rats (PTSD model)

Consequently, statistical data showed that compared with both the intact (76.96±2.83, P<0.001) and sham-operated groups (77.78±4.33, P<0.001), the sham-PTSD group had a significant reduction in serum corticosterone levels (38.68±0.70, P<0.001). Compared with the control (37.18±1.5, P<0.001) and control positive groups (38.68±0.7, P<0.001), the treatment group (DBS treatment (60.12±1.41, P<0.001)) showed significant changes. However, there was a significant gap between the intact group (76.96±2.8, P<0.001), the DBS treatment group (60.12±1.41, P<0.001), and also control (37.18±1.5, P<0.001) and control positive groups (38.68±0.7, P<0.001) (Figure 4).

The c-Fos protein expression in the amygdala was decreased by treatment schedule in CFC rats (PTSD model)

Expression of c-Fos protein was evaluated by Western blotting after the end of the experiment. Statistical analysis indicated that compared with the intact (0.29± 0.005 P<0.001) and sham-operated groups (0.39±0.005, P<0.001), the positive control group (1.34±0.00, P<0.001) and control group (0.88±0.00, P<0.001) exhibited a significant increase in amygdala c-Fos protein expression. However, there were no significant differences between the DBS treatment group (0.39±0.11, P<0.001) and the sham-operated group, although compared with the treatment group, the control group and positive control group showed a significant increase (P<0.001) (Figures 5A, B, and C).

DBS treatment has no effect on anxiety-like behavior measured by the elevated plus maze in CFC rats (PTSD model)

General anxiety-like behavior was evaluated by the EPM test on day 6 post shock. There was no significant (P<0.001) difference between the DBS treatment group and other groups in open arm time (OAT %) and open arm entry (OAE %) ratio (Figures 6A, B).

Discussion

The current experiment might be an innovative method that explores treatment with high-frequency DBS that might conceivably alleviate PTSD symptoms under CFC. We also modified the DBS protocol by changing the stimulation period. In fact, DBS treatment was able to noticeably increase the levels of corticosterone in serum in PTSD rats. Moreover, overexpression of brain c-Fos protein was profoundly reduced in the treatment group. According to the outcome of this research, improvement in rat PTSD symptoms might be the result of the mentioned modifications, which indicated a significant decrease in freezing behavior. Moreover, studies have shown that DBS facilitates the extinction of the fear conditioning induced by the electrical foot shock chamber (27, 29). Additionally, recent findings indicated that DBS stimulation-induced modulation of pathological network activity might be a mechanism of DBS treatment (19). In this regard, DBS treatment might lead to decreased activity of the amygdala and the mesocorticolimbic system, followed by a reduction in cerebral dopamine and consequently, dopamine-dependent behaviors, such as freezing behavior in this study (30, 31). Consistent with our study, the results confirmed the obvious influence of the treatment module on avoidance and fear behaviors known as cue-related anxieties, indicated by a reduction in freezing behavior duration. However, no significant changes were observed in general trauma independent of anxiety and measured by the EPM test in the DBS treatment group in our experiment. Additionally, no significant differences were seen between the DBS treatment group and other groups in open arm time and open arm entry ratio, which indicates neutral effect of DBS on anxious behavior. In fact, animals with less anxiety spend more time in the open arms. Therefore it seems that DBS by modulating the mesocorticolimbic system via the amygdala connection might be more effective on avoidance behavior than anxiety behavior in PTSD rats.

Even if we disregard all of the behavioral results, the elevated corticosterone levels in serum emphasize the

effect of DBS. However, there was a significant gap between the amount of blood serum hormone in the DBS treatment group and the intact group. It is worth noting that negative feedback is responsible for the secretion of glucocorticoid, which inhibits glutamate receptors (GluR) in the pituitary, hypothalamus, and amygdala, may not occur in some situations based on the severity of stress; consequently, the level of serum glucocorticoid remains high (8). It seems that this mechanism is not available in patients with PTSD; therefore, the patients demonstrate a decreased level of cortisol in serum and urine (4, 8). The results indicate that DBS might modify glutamate receptors (GluR) and change the mechanism of response to corticosterone by decreasing the expression of the GluR gene (8).

Furthermore, the HPA axis is highly involved in the neuroendocrine response to stress, which involves the corticotropin-releasing hormone (CRH) and vasopressin (8). In fact, corticosterone as a stress hormone plays a key role in the establishment and long-term expression of CFC and PTSD (34). Studies have demonstrated a decrease in glucocorticoid levels in PTSD conditions, probably due to the changes in the negative feedback action of the associated gene receptors (4, 8). Previous studies agree that DBS could directly or indirectly modulate through neural connections between the anatomical areas stimulated by DBS (32). According to this hypothesis, there is a neuro-connection between amygdala and catecholaminergic neurons in the raphe nucleus, which displays a catecholamine connection to the corticotropin-releasing hormone (CRH) neurons in the paraventricular nucleus (PVN) (33). In addition, a connection between the CRH neurons in the PVN and the amygdala has been revealed as an amygdala-hypothalamic axis for adrenocortical responses (33). Therefore, DBS might modify this neuro-connection and strongly influence the cortisol/corticosterone level in the blood. Although DBS treatment might increase the corticosterone hormone in PTSD rats, it still has a meaningful distance with the normal level. On the other hand, this obvious difference does not mean that the treatment is not successful because improving the condition of the PTSD rats by increasing the level of the hormone, although at the level indicated, has been able to well reduce the symptoms of the disease.

It has been previously demonstrated that the acquisition of an extinction memory is associated with c-Fos expression in prefrontal-amygdala circuits (34, 35). On the other hand, rats that show fear to an extinguished conditioned stimulus (CS) in the extinction re-exposure time, exhibited elevated c-Fos expression in different parts of the brain such as BL amygdala (34, 35). It has also been shown that pairing CSs with electrical stimulation of the BL speeds up extinction and reduces conditioned freezing, which might be related to the reduction of the c-Fos expression (34). Furthermore, correlational analysis showed that anxiety and stress were positively correlated with c-Fos neuronal activity of the certain part of the brain that was involved in the disorder (34, 36). Also it was shown that stressed animals had increased number of c-Fos expression cells in the other areas such as cerebellar dentate and fastigial nuclei (36). On the other hand, studies indicated that the electrical stimulation of specific brain targets like DBS has antidepressant effects (36). Other reviews on the effect of stimulation found that vmPFC stimulation significantly decreased the c-Fos activity within the cerebellar fastigial nucleus as compared to the non-treated group, which might be the same procedure as in the amygdala (36).

Besides, c-Fos protein expression in the amygdala increases during stressful situations and induces CRH release. Additionally, CRH has a substantial role in memory conformation in acute stress and trauma in the amygdala and cortico-limbic area (37). On the other hand, abnormal activity of the amygdala in PTSD patients markedly increases CRH release that consequently desensitizes CRH receptors and causes cortisol reduction in PTSD patients (34, 37). Our results indicated that following the effect of DBS on the amygdala, c-Fos protein expression decreased and CRH was released normally; consequently, receptors responded normally, and cortisol hormone levels rose. These results can address the mechanism of DBS response to PTSD.

Conclusion

In this study, we present the potential application of DBS to the treatment of PTSD symptoms through the stimulation of the BLn of the amygdala in CFC rats (a PTSD model). Furthermore, the present study aimed to reveal some aspects of the DBS mechanism in the amygdala in PTSD. The HPA axis as the main key in PTSD disorder conformation, which had a significant influence on our PTSD model and treatment, was considered. We demonstrate an outstanding therapeutic response, such as modified freezing behavior, by influencing c-Fos protein expression and corticosterone levels in rats following DBS treatment. It seems that the results of the current study, specifically the increased level of corticosterone and reduction in c-Fos expression, play an important role in PTSD mechanisms. This targeted therapy could lead to better therapeutic efficacy and provide an option for PTSD patients who are non-responders to current therapy.

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Conflicts of Intrest

The authors have no conflicts of interest to disclose.

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