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G. Rogel-Salazar
University of Rhode Island

H. Luna-Munguía
University of Rhode Island

See next page for additional authors

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Authors
G. Rogel-Salazar, H. Luna-Munguía, K. E. Stevens, and Walter G. Besio

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Transcranial focal electrical stimulation via tripolar concentric ring electrodes does not modify the short- and long-term memory formation in rats evaluated in the novel object recognition test

G Rogel-Salazar[1], **H Luna-Munguía**[1], **KE Stevens**[2,3], and **WG Besio**[1,*]

[1]Electrical, Computer, and Biomedical Engineering, University of Rhode Island, 4 East Alumni Ave., Kingston, Rhode Island 02881, USA

[2]Dept. of Psychiatry, University of Colorado, Denver, Anschutz Medical Campus, Aurora, Colorado 80045, USA

[3]Medical Research Service, Veterans Affairs Medical Center, Denver, Colorado, 80220;USA

Abstract

Noninvasive transcranial focal electrical stimulation (TFS) via tripolar concentric ring electrodes (TCREs) has been under development by Besio as an alternative/complementary therapy for seizure control. TFS has shown efficacy attenuating penicillin, pilocarpine, and pentylenetetrazole– induced acute seizures in rat models. This study evaluated the effects of TFS via TCREs on the memory formation of healthy rats as a safety test of TFS. The short and long-term memory formation was tested after the application of TFS using the novel object recognition (NOR) test. Independent groups were used: naïve, control (without TFS), and TFS (treated). Naïve, control, and stimulated groups spent more time investigating the new object than the familiar one during the test phase. TFS via TCREs given once does not modify the short- and long-term memory formation in rats in the NOR test. Results provide an important step towards a better understanding for the safe usage of TFS via TCREs.

Keywords

Transcranial focal electrical stimulation; TFS; Tripolar concentric ring electrodes; TCRE; short-term memory; long-term memory; novel object recognition test; NOR

1. Introduction

Brain stimulation is a promising new technology for the treatment of medically intractable epilepsy. However, most brain stimulation techniques involve invasive procedures to implant electrodes and electronic stimulators [for a review on various brain stimulation techniques for epilepsy see [1]. In contrast, noninvasive electrical stimulation does not

*Corresponding author to: Electrical, Computer, and Biomedical Engineering, University of Rhode Island, 4 East Alumni Ave., Kingston, Rhode Island 02881, USA. Tel. (401) 874-4738. besio@ele.uri.edu.

require the risks of implantation, and the electrodes can be moved easily as needed to determine where they may be the most effective in reducing seizure activity [2].

Besio has been developing noninvasive transcranial focal electrical stimulation (TFS) via tripolar concentric ring electrodes (TCREs) as an alternative/complementary therapy for seizure control. This innovative noninvasive stimulation technique has demonstrated excellent efficacy with penicillin, pilocarpine and pentylenetetrazole – induced rat seizure models [2–4]. Furthermore, when the scalp of the rat was analyzed results showed that TFS via TCREs did not damage it [5] or the underlying cortex [6].

The short and long-term side effects of TFS are not completely understood. It is possible to study the safety of the electrical stimulation in the brain through the analysis of their functional consequences on the memory formation [7, 8]. We are interested in demonstrating that TFS via TCREs has no undesirable effects on the memory formation and is safe *per se*. The aim of this study was to evaluate the effects of the TFS via TCREs on the memory process of healthy rats. To explore this issue, we addressed the following question: what are the functional consequences of applying noninvasive TFS via TCREs on the short- and long-term memory formation, tested in the novel object recognition (NOR) test in healthy rats.

The NOR test has become the task of choice for assessing aspects of declarative memory in rodents [9–11]. It has been widely demonstrated that spontaneous exploratory activity in the rat can be used to provide a valid measure of memory function [10]. The NOR test exploits the natural tendency of rats to explore novel stimuli in preference to familiar stimuli [10, 12], and gives information on working, short-term or long-term memory depending on the elapsed testing phase [13]. For example, during the test phase, the memory formation could be tested for short-term (the first 90 min) and long-term (24–48h) memory [9]. Advantages of the NOR test includes no pre-training and involves no explicit reinforcement (such as food or electric shocks) [9, 10, 12].

2. Material and methods

2.1. Subjects

Male Sprague-Dawley rats (weighing 250–300 g) were ordered from Harlan Laboratories (Madison, WI) and housed in groups of 2–3 subjects in polycarbonate cages (48.2 × 26.6 × 20.3) with bedding material (7092 Corncob, Harlan Laboratories Inc., Madison, WI). They were kept under 12:12h cycle conditions and a room temperature of 24 ± °C. All behavioral tests were conducted between 1000 and 1400h. Subjects were provided with free access to water and rat chow (2020SX Teklad Global 18% soy protein-free extruded rodent diet sterilizable, Harlan Laboratories Inc., Madison, WI) throughout the experiments. At the end of the study, rats were euthanized by CO_2 inhalation. The experimental protocol was approved by the University of Rhode Island IACUC.

2.2. Novel Object Recognition (NOR) Test

2.2.1. Apparatus—The NOR test was performed in a blue acrylic opaque open field chamber (60 × 60 × 60 cm) (Clever System Inc.) with faint black-painted squares (15 × 15 cm). The open field chamber was placed on a table (80 cm from the floor) in a dark room illuminated only by a 60 W light bulb mounted 1 m above the area. White-noise source from one extraction hood provided constant background noise (72 dB). A video camera mounted directly above the box was used to record the testing session. Behaviour was videotaped for later manual scoring.

2.2.2. Objects—The familiar objects, and duplicates, were made of glass. The familiar object was a clean copy of the two identical objects used during the familiarization phase, thus ensuring that the familiar object had not been scent-marked during the familiarization phase. The novel objects varied in shape and color and were made of plastic. Preliminary observations showed that rats had no exploration preference between objects (plastic vs. glass). All the rats were tested with the same objects. The sizes of the objects were no smaller than the size of the rat and no larger than 2.5 times the size of the rat [12]. The objects were secured to the floor of the open field chamber using Velcro strips, which also served as marks that ensured that the objects were always placed in the same location within the open field [14].

2.2.3. Habituation—During the habituation phase, each rat was handled (rats were gently held by the experimenter from tail and body) for 5 min each day for 5 consecutive days. After 30 min of handling, rats were placed inside the acrylic opaque open field chamber (always facing to the opposite wall where objects were placed later) and allowed to explore and become familiar with the empty arena (context) for 5 min. No object was placed inside the box during habituation. The open field chamber was carefully cleaned with 60% alcohol prior to habituation of the next rat.

2.2.4. Testing—Testing consisted of four phases presented in the following order: (1) Re-habituation, (2) Familiarization, (3) Delay and (4) Test. The behavior was videotaped for later scoring. Between each phase the box and objects were cleaned with 60% alcohol to avoid odour trails.

1. **Re-habituation:** Each rat was placed in the empty open field and allowed to explore for 1 min. Afterwards, animals were removed from the box and placed in their home cage (for 1 min) meanwhile two equal objects were put in the arena.
2. **Familiarization:** One minute later after re-habituation, rats were returned to the open field and allowed to explore two identical objects for 3 min.
3. **Delay:** During the delay rats were removed from the open field chamber (and placed into their home cage) and the familiar object was paired with a novel object. Delay times were: 10 s, 1 min, 10 min, 90 min, 24 h and 48 h.
4. **Test:** After completion of the delay interval, the rats were placed back in the open field chamber and allowed to explore the two objects for 3 min. Exploration was defined as the animal directing the nose within 2 cm of the object while looking at or sniffing the object. Exploration was not scored when the rat climbed on top of the object or if another part of the rat’s body touched the object. The recognition index (RI) was used to evaluate cognitive function. RI was calculated by dividing the novel object exploration time by the total exploration time (novel/novel + familiar investigation) [15]. Values of RI close to 0.5 indicate that animals spent equal time exploring both objects (familiar and the novel), while RI values greater than 0.5 denote a preference to explore the novel object over the familiar one.

2.3. Application of noninvasive TFS via TCREs

On the day prior to the NOR test the rat scalp was shaved. The day of the experiment, subjects were held by one researcher while another used conductive paste to apply the TCREs on the scalp. Rats were randomly assigned to the control and treatment groups. Only the treatment group received TFS via TCREs. The TFS was applied immediately after the familiarization phase.

The parameters and methods for the TFS via TCREs used in this experiment were based on our previous studies that have shown efficacy attenuating penicillin, pilocarpine, and pentylenetetrazole– induced acute seizures in rat models [2–4]. One TCRE was placed at the top center of the head. Flexible cables connected the TCREs to the stimulator. The TFS via TCRE was given once according to these specifications: 2 min, 300 Hz, 200 μs equal biphasic pulses at 50 mA. The control group was fully instrumented like the treatment group, but did not receive TFS.

2.4. Stimulation System

The stimulator was custom designed and built by our group with frequency, phase, and time duration of the TFS output signals programmable. The magnitude of the stimulation is adjusted manually. The stimulation controller, a Basic Stamp 2P (Parallax, Inc), was preprogrammed to apply TFS automatically when triggered. The TFS was programmed for charge balance to improve safety.

2.5. Locomotor Activity Test

The locomotor activity was evaluated during the evaluation of memory, the number of times the subject crossed with all paws from one square to another (crossings) was counted during 3-min periods. The open field chamber was carefully cleaned between tests with 60% alcohol [16].

2.6. Experimental groups

For evaluating the effects of TFS on memory, three groups were needed: Naïve, Control (without TFS), and TFS (treated). The naïve group (n=12) received habituation for handling, familiarization in the empty open field, and evaluation with the NOR test. Animals for control and TFS groups (n=12 and 13, respectively) received habituation for handling, familiarization with the empty open field, and also habituation for the TFS procedure. The control group received faked TFS, and only the TFS group was administered TFS immediately after the familiarization phase. The delay intervals were chosen to assess the specific memory types: short-term memory (10 sec, 1, 10 and 90 minutes) and long-term memory (24 and 48 hours) [12, 13].

2.7. Statistical analyses

The results are expressed as the mean ± standard error of the mean (S.E.M.). A two-way repeated analysis of variance (ANOVA) followed by the Holm-Sidak test was performed to analyze differences between delays (or groups) and objects in the NOR test. Here groups are: Naïve vs. Control vs. TFS groups. The locomotor activity tested differences within the naïve, control, and treated groups and were analyzed using the one-way analysis of variance (ANOVA) followed by the Holm-Sidak test. A *P* value of less than 0.05 was considered significant. Sigma Plot with Sigma Stat Integration (version 9.0, Systat Software, Inc., San Jose, California, USA) was used for all statistical analyses.

3. Results

3.1. Familiarization Phase

Figure 1 shows that during the familiarization phase, animals exhibited a comparable amount of time exploring the two identical objects in the naïve, control and TFS. There was no main effect of group ($F_{(2,22)}$=0.39, P=0.68) or object ($F_{(1,22)}$=0.61, P=0.45) nor was there a group x object interaction ($F_{(2,22)}$=1.02, P=0.37).

3.2. Test Phase

Figure 2 shows the recognition index (RI) during the test phase for the naïve, control and stimulated groups in the object recognition test. The naïve, control, and treated groups showed more preference for exploring the novel object than the familiar one at all the delay times (10 s, 1 min, 10 min, 90 min, 24h and 48h). The two-way repeated analysis of variance (ANOVA) did not find significant differences for the factor group ($F_{(2,110)}$=1.37, P=0.275) and the interaction between factors (Group-Time; $F_{(10,110)}$=1.49, P=0.152) but showed differences for the factor time ($F_{(5,110)}$=3.01, P=0.018).

3.3. Locomotor activity

Table 1 shows the locomotor activity evaluation during the NOR test. During the familiarization phase naïve, control, and TFS groups had similar levels of locomotor activity ($F_{(2, 34)}$= 0.018, P=0.981). During the test phase in the delay of 10 s and 1 min all groups (naïve, control, and TFS) significantly decreased their locomotor activity relative to their familiarization phase (Holm-Sidak test P<0.05). The control and TFS groups, at 10 s delay ($F_{(2,34)}$=12.27, P<0.001) and the TFS group at 1 min delay ($F_{(2,34)}$= 3.61, P=0.038) significantly reduced their locomotor activity in comparison to the naïve group. The locomotor activity in all groups (naïve, control and TFS) for the delay of 48 h significantly increased relative to their familiarization phase (Holm-Sidak test P<0.05).

4. Discussion

In this study we found that the TFS via unique TCREs does not modify the short- and long-term memory formation in healthy rats evaluated with the NOR test. These results suggest that short- and long-term memory formation are not affected by the TFS via TCREs which provide a promising step towards a better understanding of its safe usage.

4.1. Effect of applying noninvasive TFS via TCREs on the memory formation

When a subject is familiar with an object, the subject will recognize the familiar object when exposed to it again; this is called recognition memory [13]. The recognition memory of naïve rats was assed in order to establish basal conditions for our experiment. Our results demonstrated that naïve rats showed more preference for novel objects than familiar objects. This observation is in agreement with the literature; the NOR paradigm is based on the natural tendency of rodents to explore new objects more -preference of novelty- in comparison to familiar objects [9–11]. These results verify that naïve animals displayed good memory performance under our experimental conditions.

The control group (similar to the naive group) showed higher exploration towards the novel object than the familiar object. The control group received placebo TFS via TCREs. This result suggests that the habituation to the procedure of TFS via TCREs does not affect the memory performance of the animals.

The main goal of this experiment was to establish the functional consequences of applying noninvasive TFS via TCREs on the memory formation. The present data showed that the TFS via TCREs does not modify the short- and long-term memory formation in healthy rats evaluated with the NOR test. This idea is supported by the fact that animals that received TFS via TCREs spent more time exploring the novel object than the familiar one (as also was exhibited in the naïve and control groups). These results constitute the first report that TFS via TCREs do not produce adverse effects in the memory formation.

4.2. Brain stimulation and memory formation

It is difficult to make comparisons of the effects of our TFS via TCREs on the memory formation to invasive electrical stimulation or even with other techniques of noninvasive brain stimulation. In general, some reports mention that invasive and noninvasive electrical stimulation induce augmentation of the memory formation while others indicate no apparent undesirable effects [17–25].

The deep brain stimulation (DBS, invasive technique) has been demonstrated to improve or at least not show apparent undesirable effects on the memory formation. For example, using the autoshaping task, the high frequency electrical (HFS) stimulation applied in the hippocampus produced an augmentation in the short-term but not in the long-term memory formation in healthy rats [17]. Also, the effects of DBS applied in the hippocampus of patients with temporal lobe epilepsy have shown no modifications in the short-term memory formation [18].

Similar to the invasive brain stimulation, transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS) (both noninvasive techniques) have been shown to enhance the memory process while not exhibiting adverse memory modifications [7, 19, 20]. For example, in healthy rats, the evaluation of the visuospatial working memory after applying tDCS in the frontal cortex demonstrated that the stimulation had no effect on the short-term memory but showed a long-term benefit (animals exhibited significantly more efficient place avoidance and skill retention in comparison to the controls) [21]. In healthy humans after applying anodal tDCS in the prefrontal cortex, results demonstrated that this stimulation enhanced the working memory performance while cathodal tDCS interfered with it [22].

The use of repetitive transcranial magnetic stimulation (rTMS) has been shown to improve the animal's performance in the NOR test at high frequency (15Hz) and to impair the memory formation at lower frequency (1 and 8 Hz) in healthy mice [23]. Also studies evaluating the effect of TMS on the cognitive functions in humans are still controversial; results are not sufficiently conclusive to assert that the TMS enhances the memory process [24, 25].

When comparing stimulation techniques, several factors should be considered to evaluate the effects that invasive/noninvasive brain stimulation has on the memory formation: a) structure stimulated [hippocampus, prefrontal cortex, thalamus, etc]; b) characteristics of the electrical stimulation; c) evaluation of short- or long-term memory formation; d) which tests are used for evaluating the memory process; e) studies in humans or animal models; f) healthy or pathological subjects, etc.

4.3. Effect of applying noninvasive TFS via TCREs on the locomotor activity

One procedure that helps to evaluate the levels of anxiety-like behaviors in rodents is through the quantification of the locomotor activity in the open field chamber [16]. The NOR test gives the opportunity to evaluate the memory formation and at the same time the locomotor activity. Taking advantage of this possibility, we assessed the locomotor activity of the animals. Decrease/increase of the total locomotion activity is interpreted as an anxiolytic/anxiogenic-like effect, respectively [16].

All groups of animals that were submitted to the NOR test exhibited an increase in their anxiety levels during the first minute. One explanation for observing this anxiogenic-like effect is that the first minute of exposing the animals to a novel environment with objects is a very high stressful situation. In contrast, all groups displayed an anxiolytic-like effect in the 48h delay. This result could reflect that the animal's levels of anxiety-like behavior diminished due to the repetition of submitting them to the open field chamber. Despite the modification in the locomotor activity all the subjects showed an increased exploration of the novel object over the familiar one.

4.4. Final considerations

It is important to be critical about the precision with which TFS via TCREs can target specific parts of the brain. Presently, we can not assert that the electrical field was focally concentrated in a specific part of the rats' brain or if the rats received a generalized electrical stimulation. One preliminary report of our group indicates that the extra-cranial TFS current would be sufficient to cause the activation of neurons in the hippocampus [26]. Moreover future experiments should be carried out to determine what structures are being stimulated.

One limitation of this study is that prior to testing memory, the TFS via TCREs was applied on the scalp for two minutes only once. Previously, we proposed TFS via TCREs as a novel alternative/complementary therapy for seizure control where the TFS was triggered once or twice to stop PTZ-induced electrographic activity [27, 28]. In clinical practice, the application of the TFS via TCREs may need to be given more than once per day. More experiments are necessary to evaluate the consequence of repetitive application of TFS via TCREs in the memory formation under normal and pathological conditions.

In conclusion, TFS via TCREs given once does not modify the short and long-term memory formation in healthy rats tested in the NOR test. Considering that one dose of TFS on rat scalp [5] and multiple applications on cortex [6] caused no significant damage, along with these current findings on eloquent brain formation in behaving rats, the application of TFS seems to be safe. However, further research should be executed to understand the effect of applying TFS via TCREs on memory formation.

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Highlights

- The effect of focal stimulation was evaluated with a novel object recognition test
- Noninvasive focal electrical stimulation does not modify memory formation
- Noninvasive electrical stimulation via concentric ring electrodes is safe on rats

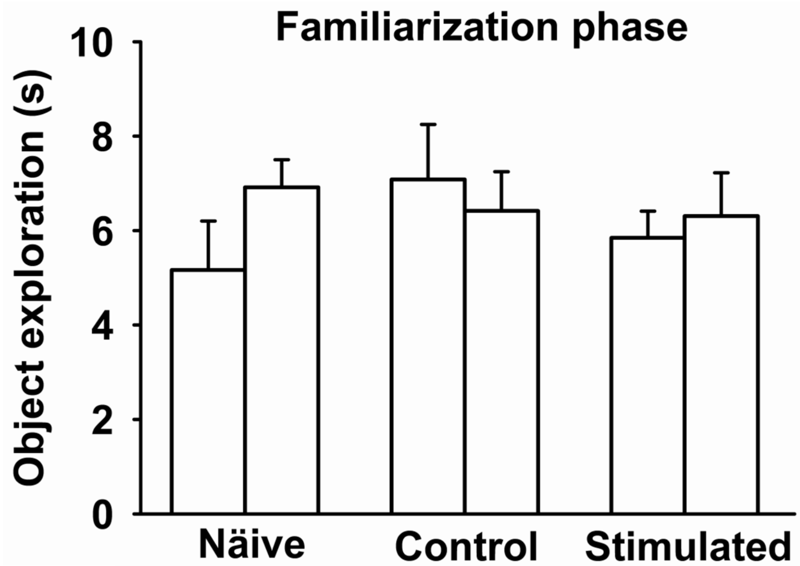

Fig. 1.

During the familiarization phase all groups of rats (naïve, control and stimulated) showed a comparable amount of time exploring two equal objects evaluated in the novel object recognition test. Data are presented as the mean ± the S.E.M. (*n*=12–13).

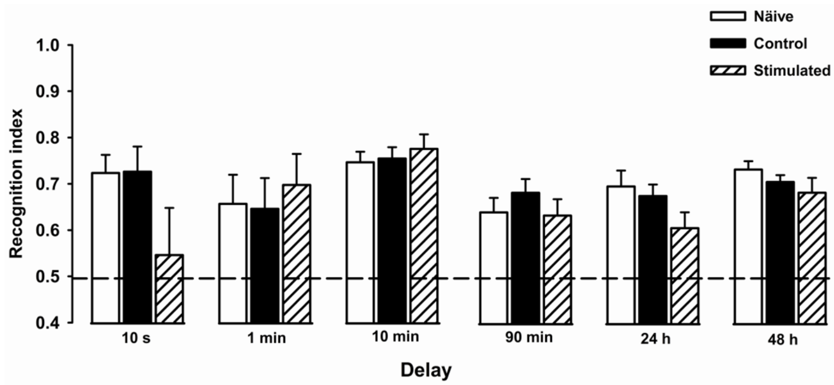

Fig. 2.

Effect of transcranial focal stimulation via tripolar concentric ring electrodes on memory performance (expressed as recognition index) of rats tested in the novel object recognition test. Animals were stimulated immediately after the familiarization phase and tested later according to the delay intervals for evaluating short-term memory (10 sec, 1, 10 and 90 minutes) and long-term memory (24 and 48 hours). Data are presented as the mean ± the S.E.M. (n=12–13).

Table 1

Locomotor activity of the rats evaluated in the Spontaneous Object Recognition Test.

	Familiar Phase	Test Phase					
		10 s	1 min	10 min	90 min	24 h	48 h
Naïve (n = 12)	63.00±6.72	38.75±5.00 *	34.50±4.55 **	53.58±4.90	64.08±5.42	76.58±4.90	91.50±5.46 **
Control (n = 12)	61.75±6.77	21.00±3.40 **/††	33.33±4.31 **	54.91±5.46	71.75±5.34	73.50±8.51	78.50±6.80 *
Stimulated (n = 13)	61.30±5.69	14.69±1.74 **/††	19.00±4.84 **/†	43.23±5.48	55.00±8.20	67.76±8.61	86.00±4.99 **

Data are expressed as mean values ± SEM (*n*=12–13). Number of counts per 3 min

* $P<0.05$

** $P<0.01$ vs. their proper familiarization phase,

† $P<0.05$ vs. naïve group