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Temporal Interference Stimulation Regulates Eye Movements and Neural Activity in the Mice Superior Colliculus*

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Abstract — **Temporal interference (TI) stimulation is a novel electrical stimulation technique which offers noninvasive deep brain stimulation (NDBS) in mice. The purpose of this study is to investigate the effect of TI stimulation on deep layers superior colliculus (SC) nerve activity and eye movements in mice. Six male C57BL / 6J mice were used in this study. Different parameters of TI stimulation were applied to the deep layers of mice SC. Each TI stimulation lasted for 20 seconds and were repeated five times. We analyzed the synchronous recording of Ca^{2+} signals in deep layers mice SC and the eye movement amplitudes. Our results show that TI stimulation can evoke eye movements and the neural activity in deep layers of mice SC. Changing the difference frequency of TI stimulation can regulate the frequency of the nerve activity and eye movements. Granger causality analysis indicates that the neural activity in deep layers of mice SC may cause the eye movements during TI stimulation.**

I. Introduction

Electrical brain stimulation is a form of electrotherapy, which can regulate the activities and functions of neurons and neural networks in the brain with high time resolution [1, 2]. Electrical brain stimulation is widely used in the clinical treatment of Alzheimer's disease (AD), Parkinson's disease (PD), epilepsy, depression and other neurological and mental diseases [3-6]. Traditional electrical brain stimulation methods include non-invasive transcranial electrical stimulation (tES) and invasive deep brain stimulation (DBS). TES has low spatial accuracy thus cannot target deep brain regions selectively [7]. Being an invasive electrical stimulation technique, DBS is controversial too. For example, implanted electrodes in the brain may lead to cerebral hemorrhage and infection, and the implanted parts also have the risk of damage [8]. Recently, researchers have developed a novel temporal interference (TI) stimulation, which offers noninvasive deep brain stimulation (NDBS) in mice with the "beat frequency" coming from two similar transcranial high frequency current stimulations [9]. TI stimulation is based on the assumption that neurons do not respond to high-frequency current stimulation, while neurons are responsive to the envelope of interference current, which causes low-frequency stimulation in the deep brain areas [10]. Some studies have shown that TI stimulation is able to selectively activate the hippocampus and stimulate motor cortex to evoke whiskers and forepaws movements [9].

The superior colliculus (SC) is a midbrain structure important for sensorimotor transformation in mammals [11]. Some studies have shown that there is a topographic map for eye movement amplitudes in the deep layers of the mice SC. Eye movements can be evoked by stimulating the invasive electrode in the deep layers using micro current [12]. However, the activity of neurons in the deep layers during the electrical-evoked eye movements is still unclear. Furthermore, the invasive electrodes may lead to cerebral hemorrhage.

In this paper, we aim to explore the relationship between eye movements and neural activity in the deep brain areas in mice SC during TI stimulation. Specifically, we recorded Ca^{2+} signals and eye movement amplitudes synchronously, demonstrated that TI stimulation can evoke the neural activity in the deep layers of SC and the eye movements. The Granger causality (GC) analysis indicated that when the current exceeds 1 mA, the neural activity in deep layers of mice SC may cause the eye movements during TI stimulation. Changing the difference frequency of TI stimulation can also regulate the frequency of the deep layers neural activity and eye movements.

II. Methods

A. The design of TI stimulator

TI stimulator consists of two constant current sources. Each constant current source includes a direct digital synthesis signal generator, a group of constant current feedback circuits, an inverse reference circuit and other control circuits.

B. Animals

All procedures had been approved by Animal Care and Use Committees in the Shenzhen Institute of Advanced Technology (SIAT), Chinese Academy of Sciences (CAS). Six adult (4-5 month old) male C57BL / 6J mice were used in this study. All mice were maintained on a 12/12-h light/dark cycle at 25 °C. Food and water were available ad libitum.

C. Viral injections

For fiber photometry experiments, AAV-syn-GCaMP6s virus was used. C57BL / 6J mice were anesthetized with pentobarbital (1% m / v, 10 ml /kg) and fixed on stereotaxic apparatus (RWD, Shenzhen, China). During the surgery, mice were kept anesthetized with isoflurane (1.5%) and placed on a heating pad to keep the body temperature at 35 °C. A 10 μL microsyringe with a 33-Ga needle was connected to a microliter syringe pump (UMP3/Micro4; WPI, USA) and used

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for virus injection into SC (coordinates: AP, -4 mm; ML, 0.8 mm; DV, -1.6 mm).

D. Implantation of optical fibers

A 200 μm optical fiber (NA: 0.37; NEWDOON, China) was chronically implanted in the SC (coordinates: AP. -4 mm; ML, 0.8 mm; DV, -1.4 mm) of C57BL / 6J mice 2–3 weeks following virus expression for fiber photometry experiments. Then a nylon head plate was implanted into the skull to fix the head. After the surgery, all mice were allowed to recover for at least 1 weeks.

E. Experimental preparation

After virus injection and optical fiber implantation, we performed a third operation on C57BL / 6J mice (n = 3). In addition, C57BL / 6J mice (n = 3) without virus injection and optical fiber implantation were subjected to the same operation. The mice were anesthetized with 4% isoflurane and maintained at 1.5% concentration. Erythromycin eye ointment was applied to the eyes of the mice. The scalp and face of the mice were shaved and disinfected with 70% ethanol. We used two stainless steel cranial nails (0.8 * 4 mm) as electrodes, which were fixed on the mouse skull with dental acrylic acid. The skull electrodes were located at stereotactic coordinates (relative to bregma) of anteroposterior - 4 mm, mediolateral + 2.5 mm and - 1.7 mm. 2 days later, head-fixed awake mice were then habituated to restraint for three consecutive days for 30 min respectively. During this time, the animals were fixed on the top of the polystyrene foam ball. They could stay still or run and try to turn the ball forward / backward. In order to prevent mice from being fatigue, each experiment was completed within 2 hours.

F. Monitoring eye position.

We used a 200Hz macro lens (Point Grey FL3-U3-13E4M) to shoot the eye position of head-fixed mice. The shooting distance was 30 cm, the magnification was 1.6, the depth of field was more than 2mm, and the resolution was 1280 * 1024. We fixed 940 nm high-power infrared LED below the front of the mouse eye to illuminate the mouse eye (Fig. 1A). We used a high transmittance infrared filter to filter the reflection of the infrared lamp on the mouse's eyeball to extract the mouse's pupil.

G. TI stimulation and data recording

The head-fixed mice were stimulated by two skull electrodes. During TI stimulation, two electrically isolated currents I_1 and I_2 were applied transcranially via electrodes that were connected to the stimulator via thin silver wires (Fig. 1B). Current I_1 was applied via the skull electrode that was located at coordinates relative to bregma AP - 4 mm, ML + 2.5 mm. Current I_2 was applied via the skull electrode that was located 4.2 mm laterally to the I_1 electrode (distance between centers of electrodes). Each skull electrode was paired with a 8 mm diameter cloth electrode, that was attached to the ipsilateral cheek. The stimulation time was 20 s, repeated 5 times, and there was at least 30 s rest time between each trial. During transcranial stimulation, two cranial electrodes were paired. Transcranial alternating current stimulation (tACS) was applied to the head-fixed mice at 1 mA and 1 Hz. In the time window of 60s before and after stimulation, we recorded Ca^{2+} signals in deep neurons of SC with optical fiber recording system (Inper, Hangzhou, China), and simultaneously photographed the pupil position of mice with macro lens. We recorded the pupil and Ca^{2+} signals of three mice with optical fiber implantation, and the pupil data of three mice without optical fiber.

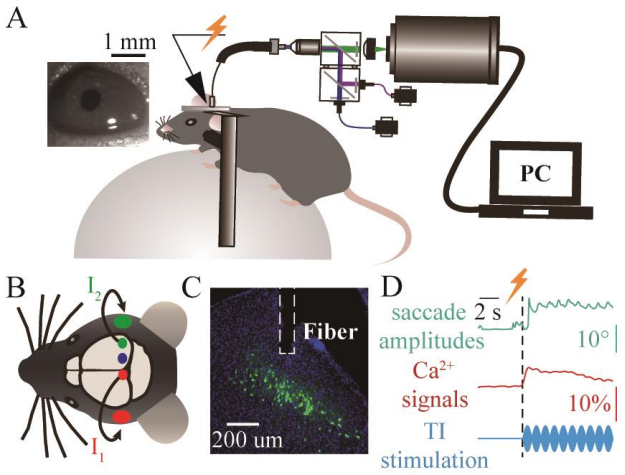

Figure 1. TI stimulation evokes eye movements and neural activity in deep layers of mice SC. (A) Schematic diagram of the experimental setup. (B) Two pairs of electrodes were used to stimulate the conscious head-fixed mice with I_1 and I_2. (C) Representative image of GCaMP6s virus expression in deep layers of SC (Green, GCaMP6s). (D) Mean values of eye movement amplitudes (top green curve) and Ca^{2+} signals in deep layer of SC (middle red curve) in three mice during TI stimulation (carrier frequency: 2000 Hz, difference frequency: 1 Hz, current: 1 mA; bottom blue curve).

H. Photometry data analysis

Firstly, InperDataProcessV0.2.3 (Inper, Hangzhou) was used to correct the baseline of the original data and reduce the photobleaching caused by long-time recording. We then subtracted the scaled 405 nm trace from the 470 nm trace to generate the corrected 470 nm signal [14]. Custom MATLAB (The MathWorks Inc. ©) scripts were developed for further analysis using R2017a. Signals were analyzed as $\frac{\triangle F}{F} = \frac{F - F_{baseline}}{F_{baseline}}$, where $F_{baseline}$ was defined as the baseline fluorescence within 5 seconds before stimulation. Then Gaussian function was used to smooth the data.

I. Eye movement data analysis

We used the Deeplabcut Toolbox to identify the center of the pupil in the videos [15]. The pupil displacement in the 2D image was converted to a rotation angle based on the estimated eyeball radius (1.67 mm) for adult C57BL/6 mice [16]. We also used Gaussian function to smooth the eye movement data. In order to analyze with the synchronous Ca^{2+}signals, all eye movement data were downsampled to 100Hz. To quantify eye movement amplitudes, the initial eye position was determined as the average of the eye position in the 5 s time window before TI Stimulation, and the position was set to 0 ° angle.

J. Statistics

All values were presented as mean ± SEM. Prism 8.0 (GraphPad Software) and MATLAB 2017a were used for the statistical analyses and graph plotting. Pairwise conditional

GC was computed using the state-of-the-art Multivariate Granger Causality (MVGC) Matlab Toolbox [17].

Before hypothesis testing, data were first tested for normality using the Shapiro-Wilk normality test and then for homoscedasticity by the F test. If the null hypothesis that the data had normal distribution and homogeneity of variances could not be rejected, parametric tests were used (for example, the paired Student's t-test for two groups). If the null hypothesis that the data had normal distribution and homogeneity of variances could not be rejected, nonparametric tests were used (for example, Wilcoxon signed rank test for two groups). * indicated $p < 0.05$, ** indicated $p < 0.01$, *** indicated $p < 0.001$.

III. Results

A. TI stimulation evokes neural activity in deep layers of SC and eye movements in awake mice

We explored whether TI stimulation could activate neural activity in deep layers of SC and eye movements in awake mice. We implanted the optical fiber into the deep layers of in head-fixed mice, photographed the eye movements of mice with macro lens. Fiber position was carefully verified in brain slices for each individual mouse at the end of experiments (Fig. 1C). The electrode on the left side of the skull above the SC area and the electrode on the left cheek formed a circuit, and the current was I_1; The electrode on the right side of the skull above the SC area and the electrode on the right cheek formed a circuit, and the current was I_2 (Fig. 1B). TI stimulation (I_1: 2001Hz, 1mA, I_2: 2000Hz, 1mA) induced eye movements in mice, and also enhanced the values of Ca^{2+}signals (Fig. 1D). These results indicate that TI stimulation can evoke the eye movements and neural activity in deep layers of SC noninvasively in mice.

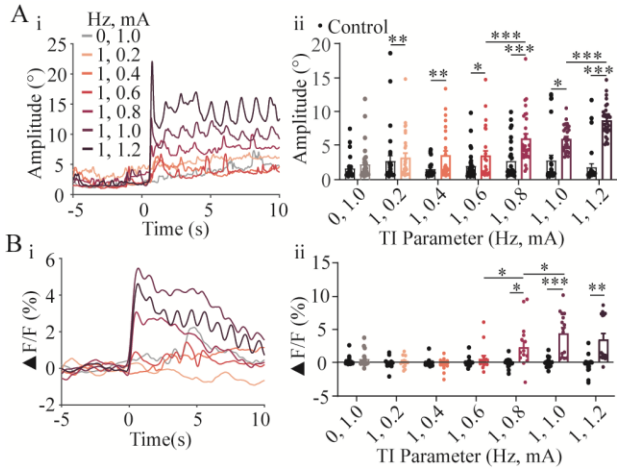

Figure 2. (A) Eye movement amplitudes response to TI stimulation (carrier frequency: 2000 Hz) with different intensities. (i) Mean values of eye movement amplitudes, n = 6 mice, 30 trials. (ii) Comparison of eye movement amplitudes before and during TI stimulation (control group: average values of eye movement amplitudes in 3 s time window before stimulation; TI group: average values of eye movement amplitudes in 3 s time window at the beginning of stimulation.). Each data point is from an individual trial. N = 6 mice. 30 trials. (B) Averaged Ca^{2+} signals in deep layers of SC response to TI stimulation with different intensities. N = 3 mice. 15 trials.

B. Effect of TI stimulation intensity on eye movements and neural activity in deep layers of SC

We next sought to test whether eye movements and neural activity in deep layers of SC can be affected through changing the TI stimulation intensity. We changed the TI stimulation's current intensity and measured the eye movement amplitudes and the values of Ca^{2+} signals in deep layers of SC during TI stimulation. We found that if the difference frequency was 0 Hz, TI stimulation whose current intensity was 1mA could not evoke eye movements (Fig. 2A) and neural activity in deep layers of SC (Fig. 2B). If the difference frequency was 1 Hz, TI stimulation whose current intensity was 0.2 mA, 0.4 mA, 0.6 mA, 0.8 mA, 1.0 mA and 1.2 mA could significantly increase the eye movement amplitudes. Only if the current intensity was greater than 0.8 mA, would the values of Ca^{2+} signals increase significantly.

In order to elucidate the relationship between the neural activity in deep layers of SC and eye movements in mice, we performed GC analysis. We found that when the current exceeded 1 mA, the neural activity in deep layers of SC might cause the eye movements during TI stimulation (Table Ⅰ).

These results show that the eye movement amplitudes and the values of Ca^{2+} signals do not increase linearly with the increase of TI stimulation's current intensity, but increase significantly when the current reach the thresholds (eye movement amplitudes: 0.8 mA and 1.2 mA; the values of Ca^{2+} signals: 0.8 mA). The neural activity in deep layers of SC displays in an all or none fashion when TI stimulation's current intensity is near threshold. In addition, GC analysis indicates that there may be a causal relationship between the neural activity in deep layers of SC and eye movements in mice during TI stimulation. The neural activity in deep layers of SC causes eye movements, but conversely, eye movements cannot cause the neural activity in deep layers of SC.

TABLE I. P-VALUES OF THE NULL HYPOTHESIS OF NO CAUSALITY

Parameters of TI stimulation	Neural activity causes eye movements	Eye movements cause neural activity
2000×2000Hz, 1.0mA	0.2817	0.2985
2000×2001Hz, 0.2mA	0.2865	0.5721
2000×2001Hz, 0.4mA	0.2331	0.2390
2000×2001Hz, 0.6mA	0.4436	0.4541
2000×2001Hz, 0.8mA	0.2537	0.6033
2000×2001Hz, 1.0mA	0.0380*	0.5375
2000×2001Hz, 1.2mA	0.0369*	0.5189

C. TI stimulation can regulate the frequency of eye movements and neural activity in deep layers of SC

Next, we sought to test whether eye movements and neural activity in deep layers of mice SC were affected by changing the difference frequency of TI stimulation. We found that if TI stimulation was applied with the current of 1 mA, a carrier frequency of 2 kHz and a difference frequency of 1 Hz, 2 Hz or 5 Hz, the stimulation evoked periodic eye movements and neural activity in deep layers of SC, whose activity frequency was equal to the difference frequency (Fig. 3). When the tACS with frequency of 1 Hz, 2 Hz or 5 Hz was applied to the skull electrodes of mice, we did not observe periodic eye

movements or neural activity in deep layers of SC (Fig. 3, A-C). These results indicate that the frequency of neural activity in deep layers of SC and eye movements can be regulated by changing the difference frequency of TI stimulation.

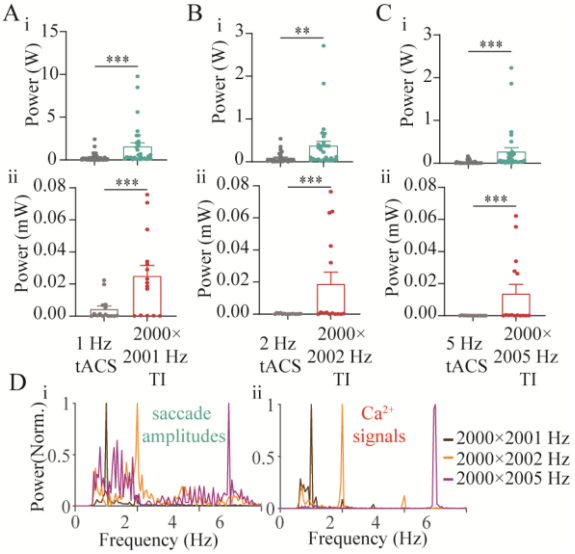

Figure 3. (A-C) (i) Power spectrum of eye movement amplitudes in 1 Hz, 2Hz, 5Hz band during stimulations. N = 6 mice. 30 trials. (ii) Power spectrum of Ca^{2+} signals in deep layers of SC in 1 Hz, 2Hz, 5Hz band during stimulations. N = 3 mice. 15 trials. (D) Normalized power spectrum of eye movement amplitudes (n = 6 mice. 30 trials) and Ca^{2+} signals in deep layers of SC (n = 3 mice. 15 trials) during TI stimulation.

IV. Discussion

We have known that there is a topographic map for eye movement amplitudes in the deep layers of the mice SC, and the micro stimulation of implanted electrode in SC can cause the eye movements in mice. We designed a TI stimulator and found that TI stimulator can also cause the neural activity in deep layers of SC, which causes the eye movements of mice. Moreover, the amplitudes and frequency of eye movements and the neural activity can be adjusted through altering the current amplitudes and difference frequency of TI stimulation.

When stimulating the implanted electrode in deep layers SC using micro current, the eye movements in mice present a "all or none" characteristic, in which the eye movement amplitudes would increase only after the current intensity reaches the threshold [12]. We found that the neural activity in deep layers of SC also presents "all or none". In addition, when TI stimulation intensity increases from 0.6 mA to 0.8 mA, eye movement amplitudes and the values of Ca^{2+} signals in deep layers of SC increase significantly. This suggests that the eye movements and the neural activity in deep layers of SC may have similar activation thresholds in mice.

When TI stimulation currents are less than 1 mA, GC values of neural activity causing eye movements are smaller than those of eye movements causing neural activity (Table Ⅰ). However, because p-values of the null hypothesis of no causality are greater than 0.05, our results cannot be interpreted as that the neural activity causes the eye movements when TI stimulation currents are small.

TI stimulation is a non-invasive method to stimulate deep neurons in the brain. It does not require chemical or genetic manipulation of the brain. We used TI stimulation to study the relationship between the neural activity in deep layers of SC and eye movements in mice, which provided more evidence for the application of TI stimulation in animal experiments and guidance for the study of sensory motor transformation in the superior colliculus.

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