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Sub: Manuscript Submission

Dear JoVE Editor,

I am excited to submit the attached manuscript titled **'Deep Brain Stimulation of the Anterior Thalamic Nucleus in Rat'** for publication in the **Journal of Visualized Experiments.** Deep Brain Stimulation is emerging as one of the leading surgical techniques to treat patients with neuronal disorders which are not responsive to drug intervention. At this time, I am optimistic about the possibilities unfolded by further basic research on DBS. I am eager to share the experience of our group on experimental DBS surgery in rats with the scientific community, with the hope that more exploration into the technique will be encouraged.

I am thankful to JoVE and its editors for the opportunity to publish our manuscript and for your tremendous support. It has been a great pleasure to interact with JoVE, especially editor **Ms. Jane Hannon** during the course of the manuscript preparation and submission. Myself and the co-authors of this manuscript have taken every effort to make sure that the manuscript adheres to the standards and guidelines for publication in JoVE.

I would also like to declare that the contents of this manuscript were not published elsewhere and not under consideration for any publication elsewhere. I am happy to answer any further questions and looking forward to working with you in the future.

Sincerely,

Travis Tierney

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Analysis of gene expression changes in the rat hippocampus after deep brain stimulation of the anterior thalamic nucleus

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Keywords

anterior thalamic nucleus; deep brain stimulation; dentate gyrus; hippocampus; epilepsy; gene expression; high-frequency stimulation; quantitative RT-PCR

Short Abstract

The mechanism underlying the therapeutic effects of Deep Brain Stimulation (DBS) surgery needs investigation. The methods presented in this manuscript describe an experimental approach to examine the cellular events triggered by DBS by analyzing the gene expression profile of candidate genes that can facilitate neurogenesis post DBS surgery.

Long Abstract

Deep brain stimulation (DBS) surgery, targeting various regions of the brain such as the basal ganglia, thalamus, and subthalamic regions, is an effective treatment for several movement disorders that have failed to respond to medication. Recent progress in the field of DBS surgery has begun to extend the application of this surgical technique to other conditions as diverse as morbid obesity, depression and obsessive compulsive disorder. Despite these expanding indications, little is known about the underlying physiological mechanisms that facilitate the beneficial effects of DBS surgery. One approach to this question is to perform gene expression analysis in neurons that receive the electrical stimulation. Previous studies have shown that neurogenesis in the rat dentate gyrus is elicited in DBS targeting of the anterior nucleus of the thalamus [1]. DBS surgery targeting the ATN is used widely for treatment refractory epilepsy. It is thus of much interest for us to explore the transcriptional changes induced by electrically stimulating the ATN. In this manuscript, we describe our methodologies for stereotactically-guided DBS surgery targeting the ATN in adult male Wistar rats. We also discuss the subsequent steps for tissue dissection, RNA isolation, cDNA preparation and quantitative RT-PCR for measuring gene expression changes. This method could be applied and modified for stimulating the basal ganglia and other regions of the brain commonly clinically targeted. The gene expression study described here assumes a candidate target gene approach for discovering molecular players that could be directing the mechanism for DBS.

Introduction

The history behind the development of Deep Brain Stimulation as a neurosurgical technique dates back to the 1870s when the possibility of electrically stimulating the brain circuitry was explored [2]. The use of chronic high-frequency stimulation as treatment for neuronal disorders started in the 1960s [3]. Later in the 1990s with the advent of chronic implantation DBS electrodes [4-6], the number of neuronal disorders that were treated by DBS continued to increase. Deep Brain Stimulation was first used in the United States as a treatment for essential tremor [6]. Today the surgery is used widely to treat neuronal disorders that are currently untreatable by pharmacological intervention. DBS is currently used to treat movement disorders of Parkinson's disease and dystonia [7-9]. Alzheimer's type dementia, Huntington's disease, epilepsy, pain and neuropsychiatric diseases such depression, OCD, Tourette's syndrome and addiction are some of the conditions amenable to treatment by DBS [10-12]. While DBS surgery is FDA approved for treating Parkinson's disease, dystonia and essential tremor, the use of DBS for treating other conditions mentioned above are in various stages of lab and clinical studies offering much promise to patients [13,14].

Clinically, DBS surgery is performed in two stages. The first stage involves surgically positioning the DBS electrodes at the targeted anatomical location using a combination of radiological positioning, CT, MRI as well as microelectrode readings for enhanced precision. The second stage involves implanting a pulse generator in the patient's upper chest and installing extension leads from the scalp to the pulse generator. Based on the neurological condition, several programming schemes for the pulse generator have been standardized and will be used to deliver the desired voltage. The final voltage is reached in a stepwise fashion so as to receive the best clinical response with minimal voltage [15]. However, in our studies, unlike the chronic DBS implants used clinically, for the sake of simplicity, we have resorted to studying a one-time high frequency stimulation (for 1 hr) in our animal models.

Part of our group's research focuses on investigating the use of DBS surgery for treatment-refractory epilepsy. Stereotactic surgical approaches using high frequency stimulation has been explored by many others as an effective option to treat medically-refractory epilepsy which constitutes about 30% of all incidences of epilepsy[10,16,17]. Cerebellar stimulation targeting the cortical surface as well as the deep cerebellar nuclei have been used in the past as targets to treat epilepsy[10,18,19]. In addition, hippocampus stimulation has also been tried but with mixed results[20,21]. Some of the other investigated DBS targets for epilepsy include the cerebral cortex, thalamus, subthalamic nucleus and vagus nerve [8]. However, following results from several studies in the past few years, the anterior thalamic nucleus (ATN) has emerged as the most common DBS target for epilepsy treatment[10,22]. Based on knowledge about neuroanatomical circuitry and findings from animal models, several studies have focused on the therapeutic effect of deep brain stimulation of the ATN in treating epilepsy[23-26]. The ATN is part of the limbic circuit and is located in the region of the brain that affects seizure frequency. Studies by Hamani *et al.*, have tested the efficacy of ATN-DBS in a pilocarpine induced epilepsy model and found that bilateral ATN stimulation prolonged latencies for pilocarpine-induced seizures and status epilepticus[24]. Furthermore, high frequency stimulation of the ATN was found to reduce seizure frequency in a pentylenetetrazol (PTZ) model of epilepsy[25,27-29]. Lee *et al.*, have reported a mean reduction in seizure frequency by about 75% upon chronic deep brain stimulation of the ATN in treating refractory partial epilepsy[30].

A recent clinical study on treatment-refractory epilepsy has shown promising results after DBS surgery targeting the anterior thalamic nucleus (ATN)[22]. A multicenter randomized clinical trial with 110 patients undergoing bilateral DBS of the ATN for treatment refractory epilepsy (SANTE trial) indicated a drop in seizure frequency by approximately 40%[31]. The results from this study also hinted on a delayed optimal anti-epileptic effect observed at 2-3 months post surgery. Further studies by Toda et al., corroborated with these findings where they demonstrated neurogenesis happening at a later time post DBS (days 3-5) in animal models[1]. In addition, Encinas *et al.,* have reported hippocampal neurogenesis in the adult mouse dentate gyrus after high frequency stimulation of the ATN[32]. Previous studies[33-35] have reported declining hippocampal neurogenesis in certain epileptic cases such as chronic temporal lobe epilepsy and an association with learning deficits, memory impairment and spontaneous recurrent motor seizures. Furthermore, there was a reduction in neural stem cell progenitor factors such as FGF2 and IGF-1 in the chronically epileptic hippocampus in animal models[33]. Considering this, interventional strategies such as DBS that show an augmentation of neurogenesis in the dentate gyrus are exciting avenues for research. These findings have encouraged us to explore further deeply into the mechanism underlying neurogenesis post-DBS treatment for epilepsy. We have targeted the ATN both unilaterally (data not reported) as well as bilaterally (in representative results) and seen elevated neurotrophin (BDNF) expression in the rat dentate gyrus. Our current hypothesis is that BDNF expression initiates a gene expression cascade that culminates in neurogenesis that translates to the anti-epileptic effect of DBS surgery. In this paper, we present our methods for DBS surgery targeting the ATN in rats followed by gene expression analysis as an attractive approach to study the mechanism underlying the benefits of DBS.

Protocol

Ethics Statement: All procedures discussed in this manuscript are in accordance with the NIH guidelines for Animal Research (Guide for the Care and Use of Laboratory Animals) and are approved by the Harvard Medical School IACUC Committee.

1. Pre-surgical Preparation:

1.1 Make sure that all surgical instruments are sterilized by either autoclaving or by cleansing with antiseptic solution and/or ethanol as necessary. Where possible, use sterile disposable equipment such as scalpels, needle and syringes.

1.2 Cover the workbench with surgical drapes and ensure that there is a biohazard waste disposal available.

1.3 Weigh the rat and calculate anesthesia dose. Use a Ketamine/Xylazine mix (Ketamine 75mg/kg and Xylazine 10 mg/kg) to anesthetize the rats. *Note: Between 200-250 g is the optimal weight for proper fixing of the animal in the stereotactic frame as well as for accurate targeting of the ATN. Isoflurane can also be used as the anesthetizing agent.*

1.4 Mount and secure two electrodes on the electrode holder (Figure 2) of a stereotactic surgical frame and with the help of a microscope (10X - 40X magnification), inspect the tips of the electrode for proper alignment. Secure the two electrodes 3.0 mm apart. *Note: Take care to avoid damaging the electrode tip by touching on hard surfaces.*

2. DBS Surgery:

2.1 Inject the Ketamine/Xylazine mix intraperitoneally and after confirming that the animal has reached a surgical plane of anesthesia (by checking for the toe-pinch reflex, respiratory rate, and depth and regularity of breathing) secure and position the animal on the stereotactic frame.

2.2 Apply eye lubricant to the animal's eyes to protect from over-drying. Remove hair from the region of the scalp that will be incised and disinfect with betadine by swabbing the reagent in a circular motion starting from the centre and then gradually moving to the outside.

2.3 Use circulating warm water pads or heating lamps to maintain the animal's body temperature at an optimal level.

2.4 Use the following stereotactic coordinates for targeting the ATN (Anterior thalamic nucleus): anterioposterior -1.6mm, mediolateral 1.5mm and dorsoventral 5.2mm [1]. *Note: The stereotactic coordinates are based on the Paxinos and Watson (6th edition) rat brain atlas [36].*

2.5 Make an incision in the scalp sagittally to reveal the skull. Using a pair of retractors, secure the incised scalp to expose the skull. Using sterile swabs dipped in ethanol, clean the incised region to expose the sutures clearly. Locate the bregma and mark with a black marker. To guide the position of the burr holes, make two more marks at approximately 1.5 mm mediolaterally on both sides from the sagittal suture and 1.6 mm posterior to the coronal suture.

2.6 Use a hand-held drill to make the burr holes. Make sure the tip of the burr hole is sterile by sterilizing it with ethanol. Hold the drill at about a 45 degree angle to the skull surface when drilling. Frequently switch between the two burr holes to avoid excessive heat at the location of any burr hole.

2.7 Continue drilling until the dura is exposed. Using a needle with its tip bent resembling the shape of an 'L', remove any broken pieces of bone that would obstruct the insertion of the electrode. Take care to avoid damaging the underlying dura and/or brain tissue while removing bone fragments using the bent needle. *Note: Using a blunted needle or fine blunt forceps is also an option.*

2.8 Fix the dual electrode assembly to the rotating handle of the stereotactic frame and fix the handle at a 90 degree angle. Using the adjustments in the stereotactic frame, position the left electrode exactly above the bregma.

2.9 Using the stereotactic adjustments for mediolateral positioning, precisely move the left electrode 1.5 mm to the left side of bregma such that now there are two electrodes perfectly aligned along the coronal suture but spaced apart at 1.5 mm mediolaterally from the bregma.

2.10 Using the anterioposterior stereotactic adjustments, move the electrodes 1.6 mm posterior to the coronal suture.

2.11 Use the dorsoventral adjustments to lower the electrodes to first check if the burr holes have been made at the right location such that the electrodes can be inserted with ease, without touching the rough edges of the burr holes. If so, insert the electrodes to a depth of 5.2mm from the surface of the skull.

2.12 Connect the electrodes via leads to a stimulator set at 130Hz, 2.5V and 90 µs pulsewidth [1].

2.13 Deliver high frequency stimulation for an hour (or for a desired period of time as per experimental setup). Perform unilateral or bilateral stimulation based on one's experimental needs. Include controls such as low frequency stimulation (for e.g., 10Hz) and unstimulated animals (inserting electrodes with no subsequent stimulation).

2.14 After stimulation is done, remove the electrodes carefully and suture the incision with 3-0 sutures or with sterile surgical staples.

2.15 Administer buprenorphine (0.05mg/kg) subcutaneously as analgesia. Monitor the animal until it returns to normal activity and then return it to the housing facility.

2.16 After a set period of time based on the experimental design (for e.g., 0, 3, 6 or 12 hours post DBS surgery), euthanize the animal with anesthesia overdose. After confirming the absence of vital signs, decapitate the animal.

2.17 Dissect out the brain by first removing the skin using scissors. Cut through the bone along the sagittal suture using dissection scissors. Make two more incisions (about an inch each) through the bone on both the lateral sides. Using forceps, lift the partially severed piece of bone from the top of the skull to expose the brain.

2.18 Using fine scissors or forceps, dislodge the brain from the skull and transfer to a petridish with cold PBS on ice. *Note: Take care to avoid damage to the brain while making the incisions through the bone.*

3. Hippocampus isolation:

Note: Perform all the subsequent steps in this section on ice.

3.1 Place the brain on a pre-cooled acrylic brain matrix on ice. Using a razor blade, cut the brain coronally at approximately 7-8 mm from the anterior-most edge of the brain. Make a second cut coronally and posterior to the first cut such that an approximately 5 mm thick brain slice could be removed.

3.2 Transfer the brain slice to a petridish with ice-cold PBS. Using razor blade, sever the two hemispherical sections and take care to note which hemisphere corresponds to the left and right sides respectively. This is especially important while performing unilateral stimulations.

3.3 Using fine forceps and scissors remove the hippocampus carefully.

3.4 Flash freeze the hippocampal tissue on dry ice and store in -20 °C freezer until ready for the subsequent RNA extraction steps. *Note: For long term storage, it is advisable to store tissue in a -80 °C freezer.*

4. RNA extraction and quantitative PCR:

4.1 Make sure the tissue stays frozen on dry ice until ready for homogenization.

4.2 Note: Perform this step in the hood.

4.2.1 Add 1 ml of Tri reagent to hippocampal tissue in a 1.5 ml centrifuge tube and homogenize it by first pipetting multiple times until the tissue is broken in smaller pieces. Further homogenize the tissue by passing through a syringe with a 25G needle until there is no unbroken tissue visible. Perform the homogenization steps on ice.

4.2.2 After homogenizing the tissue, allow the cell suspension to stand at room temperature for 5 minutes to allow cell lysis.

Note: After this point the cell suspension can be quick frozen and stored in -70 °C until further processing.

4.3 Add 0.2 ml chloroform and mix by vortexing for 20 secs and let the solution rest at room temperature for 15 mins.

4.4 Centrifuge the samples at 12000x g for 15 mins at 4 °C. After centrifugation, remove the tubes carefully without disturbing the three separate layers that will be visible. *Note: The bottom (red) phase contains proteins, middle cloudy phase contains DNA and the top clear phase contains RNA.*

4.5 Transfer the top clear phase (RNA) into a fresh 1.5 ml centrifuge tube (typically 500 µl of the RNA containing phase is obtained). To this add 0.5 ml isopropanol and mix by vortexing. To this add 2-3 µl glycogen (20 mg/ml) as the carrier for the RNA. Allow the sample to rest on the table at room temperature for 10 minutes.

4.6 Centrifuge the sample at 12000x g for 1 hour at 4 °C. Make sure that the RNA pellet is visible at the bottom of the tube.

4.7 Discard the supernatant and wash the RNA pellet by adding 1 ml of 75% ethanol (made with nuclease free water). Invert the sample multiple times until the pellet dislodges from the bottom of the tube and floats in the solution. Centrifuge the solution at 12000x g for 10 mins at 4 °C. *Note: The pellet in 75% ethanol can be stored at -20 ° C until further steps.*

4.8 Allow the RNA pellet to air dry by leaving the tubes open for 5 minutes at room temperature. Take precaution to avoid over drying the pellet as this might affect its dissolution in water in the subsequent step.

5. Removing DNA from the RNA preparation:

5.1 Add 9 µl of nuclease free water to the RNA pellet and make sure the pellet is dissolved before proceeding by gently vortexing the tube. To this add 1 µl of 10X Dnase I Buffer and 1 µl recombinant DNase (from DNase I kit), mix by gently flicking the tubes, briefly spin the tubes and incubate at 37 °C in a water bath for 30 mins.

5.2 Add 2 µl DNase inactivation reagent (from DNase I kit) to the sample and incubate at room temperature for 2 mins and mix often by gently flicking the tube. Centrifuge at 12000x g for 10 mins and transfer approximately 10 µl of the clear supernatant to a fresh 1.5 ml centrifuge tube.

5.3 Measure RNA concentration using a nanodrop or spectrophotometer.

6. Making cDNA from RNA:

6.1 Add requisite volume that contains 1 µg of RNA and bring the total volume to 8 µl by adding nuclease free water. To this add 1 µl 10 mM dNTP mix and 1 µl of random hexamers from the Superscript First Strand Synthesis Kit. Gently mix and incubate at 65 °C for 5 mins either in a water bath or on a pre-programmed thermocycler.

6.2 Make a premix containing the following reagents: 2 µl of 10X RT Buffer, 4 µl of 25 M $MgCl_2$, 2 µl of 0.1M DTT and 1 µl of RNAse OUT (40 U/µl). *Note: Volumes given are per reaction, user would need to scale up according to one's experimental needs.*

6.3 After removing the RNA containing sample from 65 °C, set the tube at room temperature for a minute. Add 9 µl premix solution and incubate at room temperature for 2 minutes. Add 1 µl of Superscript II reverse transcriptase enzyme (50U/µl) and then incubate at room temperature for 10 mins.

6.4 Incubate the samples at 42 °C for 50 mins for the reverse transcription to occur.

6.5 Incubate the samples at 70 °C for 15 mins to terminate the reaction.

6.6 Place the samples on ice briefly and add 1 µl RNAseH (2U/µl) and incubate at 37 °C for 20 mins.

6.7 Briefly centrifuge the tubes at 3000x g to spin down the condensate liquid. *Note: This is the cDNA that will be used for quantitative PCR. cDNA can be stored in -20 °C freezer until PCR setup. cDNA can also be diluted with nuclease free water before proceeding to PCR.*

7. Quantitative PCR:

7.1 Make a master mix containing the following reagents (volumes given are per reaction):6.3 µl of 2X Sybr Green Mix, 0.6 µl of Forward Primer (10 µM), 0.6 µl of Reverse Primer (10 µM) and 0.5 µl of Nuclease free water. *Note: Primer design was done using the 'Pick Primers' option in NCBI's nucleotide sequence page for the gene of interest.*

7.2 Add 10 µl mastermix to each well of a 96 well- PCR Plate. Add 2.5 µl of cDNA made earlier to the well containing the mastermix. Make sure to set up triplicate PCR reactions for each sample.

7.3 After completing the addition of mastermix and cDNA, cover the PCR plate using an optical adhesive sheet to seal the wells. Spin the plate briefly for 5 mins at 500x g in a tabletop centrifuge to settle all the liquid to the bottom of the well.

7.4 Load the PCR plate onto a pre-programmed RT-PCR machine. Use the PCR parameters provided in Table 1.

7.5 **Data analysis:** Use the C_t values from the qPCR output for further calculations. Along with the test gene, set up PCR reactions for internal control genes, 18S rRNA (to ensure equal input) and β-actin (negative control). Calculate fold changes based on ΔΔCt method [37].

Representative Results:

Figures 1A and 1B show the relative expression of BDNF and GABRD relative to the control gene β-actin. BDNF, a neurotrophin is often associated with neuroprotective effects in many neuronal diseases[38-41]. It is therefore interesting to analyze the expression profile of BDNF in response to stimulation of the ATN which yields therapeutic benefits to epileptic patients. In Figure 1A which shows the gene expression profile of BDNF across the indicated time-points post DBS stimulation, BDNF up-regulation is observed immediately (0 hr) after DBS surgery along with the peak expression (3 fold greater than unstimulated) at 3 hours post stimulation. This observation suggests that enhanced BDNF expression and the resulting neuroprotection could contribute to the therapeutic benefit of DBS. Another gene GABRD (Figure 1B) was also investigated using the qPCR method. GABRD is a GABA receptor which is one of the potential targets for designing anti-epilepsy drugs[42]. The expression profile of GABRD also shows enhanced expression in the stimulated animals compared to the unstimulated control animals at 3 hours post DBS. Considering that GABA agonists are used as effective seizure suppressors, it is interesting to observe enhanced GABRD expression post DBS, implicating a possible role for GABA in the anti-epileptic effect of DBS.

The RT-PCR protocol described here yields reproducible and quantitative results that reveal gene expression patterns and the relative fold differences compared to the control animals. The data analysis is performed in the following manner: The qPCR output gives the threshold C_t value for the test gene for each sample analyzed. C_t values are also obtained for the control gene β-actin and 18S rRNA (input control). The $\Delta\Delta C_t$ method will then be used to calculate the gene expression profile using these C_t values [37]. For example, to calculate the gene expression changes for BDNF, for a given sample, the difference between the C_t value for BDNF and 18S rRNA is calculated and is the first ΔC_t. For the same sample, the difference between the C_t value for β-actin and 18S rRNA is calculated to give the second ΔC_t. The difference between the two ΔC_t values is calculated to give $\Delta\Delta C_t$. This $\Delta\Delta C_t$ value is used to calculate 2^ ($-\Delta\Delta C_t$) which gives the relative template abundance for BDNF compared to β-actin. By plotting this value across the different time-points alongside the unstimulated control, the gene expression changes induced by DBS across time-points can be visualized. The above described method could be used effectively to investigate changes in expression for other genes which are potential candidates that are responsive to DBS stimulation and to investigate some of the downstream effects of modulating the expression of these genes.

[Place Figure 1 Here]

Figure 1: A. Time course analysis of BDNF expression in response to high frequency stimulation of the ATN. Tissue harvesting, RNA extraction, cDNA preparation and q-PCR were performed as explained in the protocol. Relative changes in gene expression are calculated after normalizing for input (by amplifying 18S rRNA) as well as a control gene (β-actin). C_t values obtained from the real-time PCR were used to calculate expression levels by the $\Delta\Delta$ C_t method [37]. The time-points analyzed are 0, 3, 6 and 12 hours post DBS stimulation. *Note: The timepoints selected here are with respect to a particular study and is subject to change according to the hypothesis and experimental plan.*
B. Time course analysis of GABA A receptor delta subunit (GABRD) levels in response to DBS at 130 Hz targeting the ATN. Methods and calculations were done as similar to the BDNF data.

Figure 2. DBS electrodes and stimulator set up.

Table 1. PCR parameters.

Discussion:

Following the landmark work by Benabid *et al.* in using deep brain stimulation to treat Parkinson's disease and essential tremor, the DBS surgical technique has been investigated with much interest over the past decade to treat many neurological disorders [6,10,43]. DBS studies targeting various neuro-anatomical regions of the brain circuitry are currently performed by many groups to address major neuronal diseases and are in various stages of clinical trials. Stimulation of the subthalamic nucleus (STN) or the internal segment of the globus pallidus (GPi) is FDA approved and used in treating movement disorders in Parkinson's disease [10]. The SANTE clinical trial has shown promising trends for epileptic patients receiving high frequency stimulation of the anterior thalamic nucleus [31]. Results from a phase I clinical trial of bilateral forniceal stimulation have shown a delay in the rate of cognitive decline and a reversal of the glucose hypometabolic uptake seen in Alzheimer's disease as well as activation of the memory circuitry [8,44]. Furthermore, in recent years neurosurgeons have conducted DBS trials for treating neuropsychiatric disorders such as OCD (Obsessive Compulsive Disorder), treatment-resistant depression, Tourette syndrome and addiction [10,45-55].

In addition to the clinical trials, over the past few years, animal surgery has offered us great opportunities to study the physiological changes induced by the surgical technique in a live animal, in a manner unparalleled by any *in vitro* technique. In this manuscript, we have discussed the methods involved in performing deep brain stimulation surgery in rodents. Stereotactic surgery in rodents as described here could also be used for potential DBS target searches and to test out the efficacy of the surgery using disease model animals. One of the challenges for the experimenter here is to be able to target the correct anatomical locus in a reproducible manner. There is especially a need for a skilled technician for the surgery because checking for correct targeting is possible only after the stimulation is done and the animal euthanized. Also occasionally, one might accidentally injure a key blood vessel which could lead to significant blood loss and sometimes even death of the animal. In addition, the need to dissect out the hippocampus for further biochemical analysis limits the possibility of immunohistological verification for proper electrode targeting in the same animal which requires an intact brain specimen for tissue sectioning. Proper electrode targeting could possibly be checked on a different animal stimulated in an identical manner. However, this does not provide evidence for proper targeting in the test animal and is a limitation of this approach. Recent publications have tried to circumvent this problem by conducting DBS surgery with simultaneous fMRI [56]. One possible improvement of the technique described here could be a study on the effects of chronic stimulation via an implanted stimulator in the animal. However, we have limited our analysis for a single dose (1 hr) of high-frequency stimulation as the first step to understanding the changes induced by DBS at a cellular level.

Considering the use of DBS surgery for a variety of neuronal disorders, it is essential that we know the mechanism underlying the beneficial effects of DBS. This information is critical for developing future improvements in the surgery and also to explore the utility of DBS as a treatment for other conditions which haven't been investigated by experts in DBS. In addition, modifications to the surgical technique can be implemented to avoid certain deleterious side-effects of the procedure and to effectively deal with recurrence of the disease condition.

An in-depth mechanistic analysis of DBS is possible by examining the gene expression changes induced by DBS. Either a candidate gene approach based on existing knowledge about neuronal pathways that respond to depolarizing stimuli such as high frequency stimulation, or a global transcriptome analysis can give important insights into the molecular events triggered by DBS. The gene expression analysis techniques (candidate gene approach as well as high-throughput method) are powerful tools that are key to exploring the molecular mechanisms and cellular changes associated with DBS surgery. Recent developments in this area have made it possible for us to get a wealth of information about several aspects of cellular physiology in a very short time, which was not possible a few years ago. With the advent of high throughput sequencing technology such as ChIPseq, it is possible to characterize the genome-wide location of important transcription factors which respond to DBS [57,58]. Recent discoveries linking noncoding RNA such as microRNA with neurodegenerative diseases, enable us to analyze possible changes in miRNA levels in neurons post-DBS using miRNA sequencing technology [59,60]. Possible changes in epigenetic signatures such DNA methylation and histone modifications in response to DBS could also be explored. However, despite the advantages, it is important to acknowledge some of the limitations of these techniques as well as potential problems that might arise during analyses. An important concern with gene expression analyses has been reproducibility and technical errors. It is important that the experimenter takes note of this and plans on having adequate number of repetitions to ensure reliability. A common problem with some of the high throughput screening studies has been the difficulty in interpretation of the tremendous amount of data that is generated. Sometimes it becomes important to determine whether the gene expression changes observed are due to the direct effect of the experimental treatment or is a downstream effect. This usually requires additional studies that are designed to address this issue.

In addition to the genomic studies, an extensive immunohistochemical analysis of the spatio-temporal localization of the key players that respond to DBS and a quantitation of the changes in their levels in various regions of the brain will be a great asset to future developments of the DBS surgical procedure. The cumulative findings from the gene expression analyses as well as the immunohistochemical studies can reveal novel interactions between key factors as well as key molecular events that regulate cellular processes such as neurogenesis or neurodegeneration. Identification of such critical molecular markers may also enable future drug discoveries. Such findings could shed light on the general functioning of the brain circuitry, which is valuable information from the perspective of scientists working to understand many neuronal diseases. Directing our future efforts on integrating latest technological advances made in the clinic as well as the laboratory is likely to offer us substantial advantages in our fight against disease.

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Disclosures:
The authors have no disclosures.

References:

1 Toda, H., Hamani, C., Fawcett, A. P., Hutchison, W. D. & Lozano, A. M. The regulation of adult rodent hippocampal neurogenesis by deep brain stimulation. *Journal of neurosurgery* **108**, 132-138, doi:10.3171/JNS/2008/108/01/0132 (2008).

2 Perlmutter, J. S. & Mink, J. W. Deep brain stimulation. *Annual review of neuroscience* **29**, 229-257, doi:10.1146/annurev.neuro.29.051605.112824 (2006).

3 Bergstrom, M. R., Johansson, G. G., Laitinen, L. V. & Sipponen, P. Electrical stimulation of the thalamic and subthalamic area in cerebral palsy. *Acta physiologica Scandinavica* **67**, 208-213, doi:10.1111/j.1748-1716.1966.tb03302.x (1966).

4 Benabid, A. L. *et al.* Chronic electrical stimulation of the ventralis intermedius nucleus of the thalamus as a treatment of movement disorders. *Journal of neurosurgery* **84**, 203-214, doi:10.3171/jns.1996.84.2.0203 (1996).

5 Benabid, A. L. *et al.* Long-term electrical inhibition of deep brain targets in movement disorders. *Movement disorders : official journal of the Movement Disorder Society* **13 Suppl 3**, 119-125 (1998).

6 Benabid, A. L. *et al.* Long-term suppression of tremor by chronic stimulation of the ventral intermediate thalamic nucleus. *Lancet* **337**, 403-406 (1991).

7 Tierney, T. S. & Lozano, A. M. Surgical treatment for secondary dystonia. *Movement disorders : official journal of the Movement Disorder Society* **27**, 1598-1605, doi:10.1002/mds.25204 (2012).

8 Tierney, T. S., Sankar, T. & Lozano, A. M. Some recent trends and further promising directions in functional neurosurgery. *Acta neurochirurgica. Supplement* **117**, 87-92, doi:10.1007/978-3-7091-1482-7_14 (2013).

9 Tierney, T. S., Sankar, T. & Lozano, A. M. Deep brain stimulation emerging indications. *Progress in brain research* **194**, 83-95, doi:10.1016/B978-0-444-53815-4.00015-7 (2011).

10 Sankar, T., Tierney, T. S. & Hamani, C. Novel applications of deep brain stimulation. *Surgical neurology international* **3**, S26-33, doi:10.4103/2152-7806.91607 (2012).

11 Tierney, T. S., Vasudeva, V. S., Weir, S. & Hayes, M. T. Neuromodulation for neurodegenerative conditions. *Frontiers in bioscience* **5**, 490-499 (2013).

12 Mayberg, H. S. *et al.* Deep brain stimulation for treatment-resistant depression. *Neuron* **45**, 651-660, doi:10.1016/j.neuron.2005.02.014 (2005).

13 Lyons, M. K. Deep brain stimulation: current and future clinical applications. *Mayo Clinic proceedings* **86**, 662-672, doi:10.4065/mcp.2011.0045 (2011).

14 T, T. & A, L. in *Rehabilitation in movement disorders* eds Iansek R & Morris ME) Ch. 4, 36-43 (Cambridge University Press, 2013).

15 Goodwin, R., Tierney, T., Lenz, F. & Anderson, W. in *Single Neuron Studies of the Human Brain: Probing Cognition* eds Itzhak Fried, Ueli Rutishauser, Moran Cerf, & Gabriel Kreiman) Ch. 15, 275-293 (MIT Press, 2014).

16 Hauser, W. A. Epidemiology of epilepsy in children. *Neurosurgery clinics of North America* **6**, 419-429 (1995).

17 Hauser, W. A. Recent developments in the epidemiology of epilepsy. *Acta neurologica Scandinavica. Supplementum* **162**, 17-21 (1995).

18 Davis, R. & Emmonds, S. E. Cerebellar stimulation for seizure control: 17-year study. *Stereotactic and functional neurosurgery* **58**, 200-208 (1992).

19 Levy, L. F. & Auchterlonie, W. C. Chronic cerebellar stimulation in the treatment of epilepsy. *Epilepsia* **20**, 235-245 (1979).

20 Velasco, A. L. *et al.* Electrical stimulation of the hippocampal epileptic foci for seizure control: a double-blind, long-term follow-up study. *Epilepsia* **48**, 1895-1903, doi:10.1111/j.1528-1167.2007.01181.x (2007).

21 Tellez-Zenteno, J. F., McLachlan, R. S., Parrent, A., Kubu, C. S. & Wiebe, S. Hippocampal electrical stimulation in mesial temporal lobe epilepsy. *Neurology* **66**, 1490-1494, doi:10.1212/01.wnl.0000209300.49308.8f (2006).

22 Kerrigan, J. F. *et al.* Electrical stimulation of the anterior nucleus of the thalamus for the treatment of intractable epilepsy. *Epilepsia* **45**, 346-354, doi:10.1111/j.0013-9580.2004.01304.x (2004).

23 Hamani, C. *et al.* Deep brain stimulation of the anterior nucleus of the thalamus: effects of electrical stimulation on pilocarpine-induced seizures and status epilepticus. *Epilepsy research* **78**, 117-123, doi:10.1016/j.eplepsyres.2007.09.010 (2008).

24 Hamani, C. *et al.* Bilateral anterior thalamic nucleus lesions and high-frequency stimulation are protective against pilocarpine-induced seizures and status epilepticus. *Neurosurgery* **54**, 191-195; discussion 195-197 (2004).

25 Mirski, M. A., Rossell, L. A., Terry, J. B. & Fisher, R. S. Anticonvulsant effect of anterior thalamic high frequency electrical stimulation in the rat. *Epilepsy research* **28**, 89-100 (1997).

26 Mirski, M. A. & Ferrendelli, J. A. Interruption of the mammillothalamic tract prevents seizures in guinea pigs. *Science* **226**, 72-74 (1984).

27 Mirski, M. A. & Ferrendelli, J. A. Anterior thalamic mediation of generalized pentylenetetrazol seizures. *Brain research* **399**, 212-223 (1986).

28 Mirski, M. A. & Ferrendelli, J. A. Interruption of the connections of the mammillary bodies protects against generalized pentylenetetrazol seizures in guinea pigs. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **7**, 662-670 (1987).

29 Mirski, M. A., McKeon, A. C. & Ferrendelli, J. A. Anterior thalamus and substantia nigra: two distinct structures mediating experimental generalized seizures. *Brain research* **397**, 377-380 (1986).

30 Lee, K. J., Jang, K. S. & Shon, Y. M. Chronic deep brain stimulation of subthalamic and anterior thalamic nuclei for controlling refractory partial epilepsy. *Acta neurochirurgica. Supplement* **99**, 87-91 (2006).

31 Fisher, R. *et al.* Electrical stimulation of the anterior nucleus of thalamus for treatment of refractory epilepsy. *Epilepsia* **51**, 899-908, doi:10.1111/j.1528-1167.2010.02536.x (2010).

32 Encinas, J. M., Hamani, C., Lozano, A. M. & Enikolopov, G. Neurogenic hippocampal targets of deep brain stimulation. *The Journal of comparative neurology* **519**, 6-20, doi:10.1002/cne.22503 (2011).

33 Hattiangady, B., Rao, M. S. & Shetty, A. K. Chronic temporal lobe epilepsy is associated with severely declined dentate neurogenesis in the adult hippocampus. *Neurobiology of disease* **17**, 473-490, doi:10.1016/j.nbd.2004.08.008 (2004).

34 Kuruba, R., Hattiangady, B. & Shetty, A. K. Hippocampal neurogenesis and neural stem cells in temporal lobe epilepsy. *Epilepsy & behavior : E&B* **14 Suppl 1**, 65-73, doi:10.1016/j.yebeh.2008.08.020 (2009).

35 Hattiangady, B. & Shetty, A. K. Implications of decreased hippocampal neurogenesis in chronic temporal lobe epilepsy. *Epilepsia* **49 Suppl 5**, 26-41, doi:10.1111/j.1528-1167.2008.01635.x (2008).

36 Paxinos, G., Watson, C. *The Rat Brain in Stereotaxic Coordinates*. (Elsevier Inc., 2007).

37 Livak, K. J. & Schmittgen, T. D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 22DDCT Method. *METHODS* **25**, 402-408, doi:10.1006/meth.2001.1262 (2001).

38 Kells, A. P. *et al.* AAV-mediated gene delivery of BDNF or GDNF is neuroprotective in a model of Huntington disease. *Molecular therapy : the journal of the American Society of Gene Therapy* **9**, 682-688, doi:10.1016/j.ymthe.2004.02.016 (2004).

39 Han, B. H. & Holtzman, D. M. BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **20**, 5775-5781 (2000).

40 Hetman, M., Kanning, K., Cavanaugh, J. E. & Xia, Z. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *The Journal of biological chemistry* **274**, 22569-22580 (1999).

41 Stadelmann, C. *et al.* BDNF and gp145trkB in multiple sclerosis brain lesions: neuroprotective interactions between immune and neuronal cells? *Brain : a journal of neurology* **125**, 75-85 (2002).

42 Treiman, D. M. GABAergic mechanisms in epilepsy. *Epilepsia* **42 Suppl 3**, 8-12 (2001).

43 Benabid, A. L., Pollak, P., Louveau, A., Henry, S. & de Rougemont, J. Combined (thalamotomy and stimulation) stereotactic surgery of the VIM thalamic nucleus for bilateral Parkinson disease. *Applied neurophysiology* **50**, 344-346 (1987).

44 Laxton, A. W. *et al.* A phase I trial of deep brain stimulation of memory circuits in Alzheimer's disease. *Annals of neurology* **68**, 521-534, doi:10.1002/ana.22089 (2010).

45 Tierney, T. S., Abd-El-Barr, M. M., Stanford, A. D., Foote, K. D. & Okun, M. S. Deep brain stimulation and ablation for obsessive compulsive disorder: evolution of contemporary indications, targets and techniques. *The International journal of neuroscience* **124**, 394-402, doi:10.3109/00207454.2013.852086 (2014).

46 Kennedy, S. H. *et al.* Deep brain stimulation for treatment-resistant depression: follow-up after 3 to 6 years. *The American journal of psychiatry* **168**, 502-510, doi:10.1176/appi.ajp.2010.10081187 (2011).

47 Lozano, A. M. *et al.* Subcallosal cingulate gyrus deep brain stimulation for treatment-resistant depression. *Biological psychiatry* **64**, 461-467, doi:10.1016/j.biopsych.2008.05.034 (2008).

48 Ackermans, L. *et al.* Double-blind clinical trial of thalamic stimulation in patients with Tourette syndrome. *Brain : a journal of neurology* **134**, 832-844, doi:10.1093/brain/awq380 (2011).

49 Houeto, J. L. *et al.* Tourette's syndrome and deep brain stimulation. *Journal of neurology, neurosurgery, and psychiatry* **76**, 992-995, doi:10.1136/jnnp.2004.043273 (2005).

50 Maciunas, R. J. *et al.* Prospective randomized double-blind trial of bilateral thalamic deep brain stimulation in adults with Tourette syndrome. *Journal of neurosurgery* **107**, 1004-1014, doi:10.3171/JNS-07/11/1004 (2007).

51 de Koning, P. P., Figee, M., van den Munckhof, P., Schuurman, P. R. & Denys, D. Current status of deep brain stimulation for obsessive-compulsive disorder: a clinical review of different targets. *Current psychiatry reports* **13**, 274-282, doi:10.1007/s11920-011-0200-8 (2011).

52 Greenberg, B. D. *et al.* Deep brain stimulation of the ventral internal capsule/ventral striatum for obsessive-compulsive disorder: worldwide experience. *Molecular psychiatry* **15**, 64-79, doi:10.1038/mp.2008.55 (2010).

53 Muller, U. J. *et al.* Successful treatment of chronic resistant alcoholism by deep brain stimulation of nucleus accumbens: first experience with three cases. *Pharmacopsychiatry* **42**, 288-291, doi:10.1055/s-0029-1233489 (2009).

54 Zhou, H., Xu, J. & Jiang, J. Deep brain stimulation of nucleus accumbens on heroin-seeking behaviors: a case report. *Biological psychiatry* **69**, e41-42, doi:10.1016/j.biopsych.2011.02.012 (2011).

55 Porta, M. *et al.* Thalamic deep brain stimulation for treatment-refractory Tourette syndrome: two-year outcome. *Neurology* **73**, 1375-1380, doi:10.1212/WNL.0b013e3181bd809b (2009).

56 Younce, J. R., Albaugh, D. L. & Shih, Y. Y. Deep brain stimulation with simultaneous FMRI in rodents. *Journal of visualized experiments : JoVE*, e51271, doi:10.3791/51271 (2014).

57 Park, P. J. ChIP-seq: advantages and challenges of a maturing technology. *Nature reviews. Genetics* **10**, 669-680, doi:10.1038/nrg2641 (2009).

58 Valouev, A. *et al.* Genome-wide analysis of transcription factor binding sites based on ChIP-Seq data. *Nature methods* **5**, 829-834, doi:10.1038/nmeth.1246 (2008).

59 Fiore, R., Siegel, G. & Schratt, G. MicroRNA function in neuronal development, plasticity and disease. *Biochimica et biophysica acta* **1779**, 471-478, doi:10.1016/j.bbagrm.2007.12.006 (2008).

60 Hebert, S. S. & De Strooper, B. Alterations of the microRNA network cause neurodegenerative disease. *Trends in neurosciences* **32**, 199-206, doi:10.1016/j.tins.2008.12.003 (2009).

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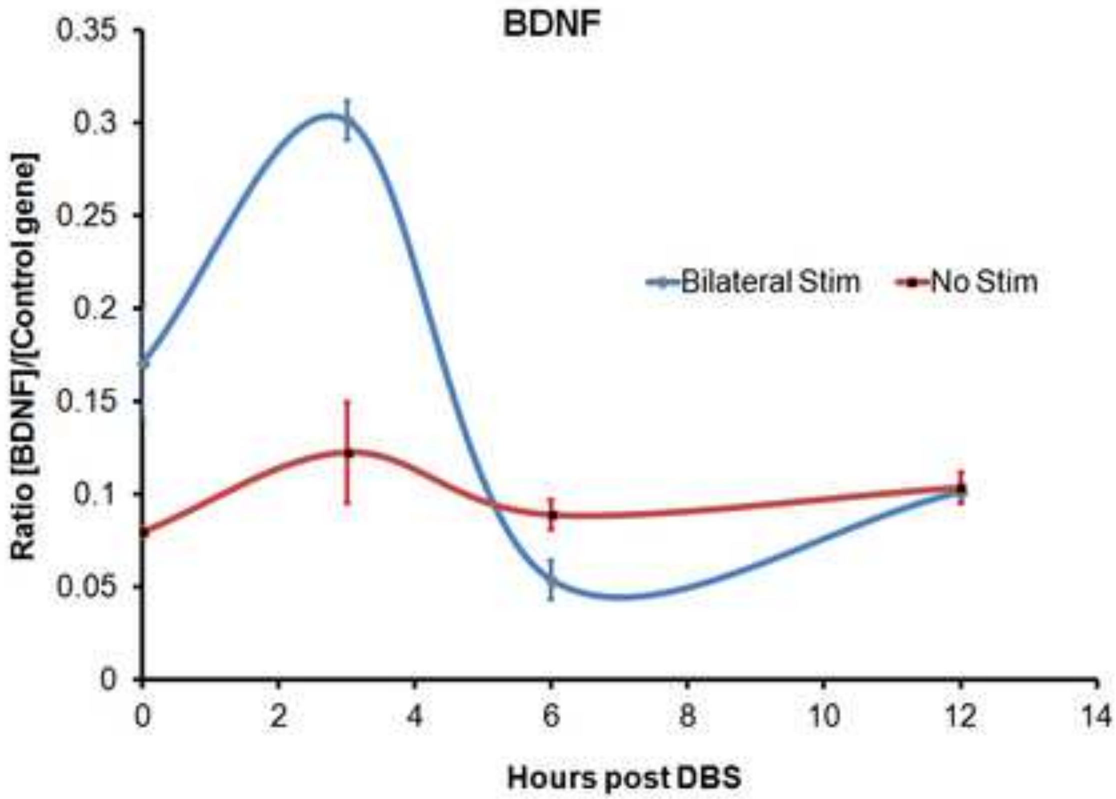

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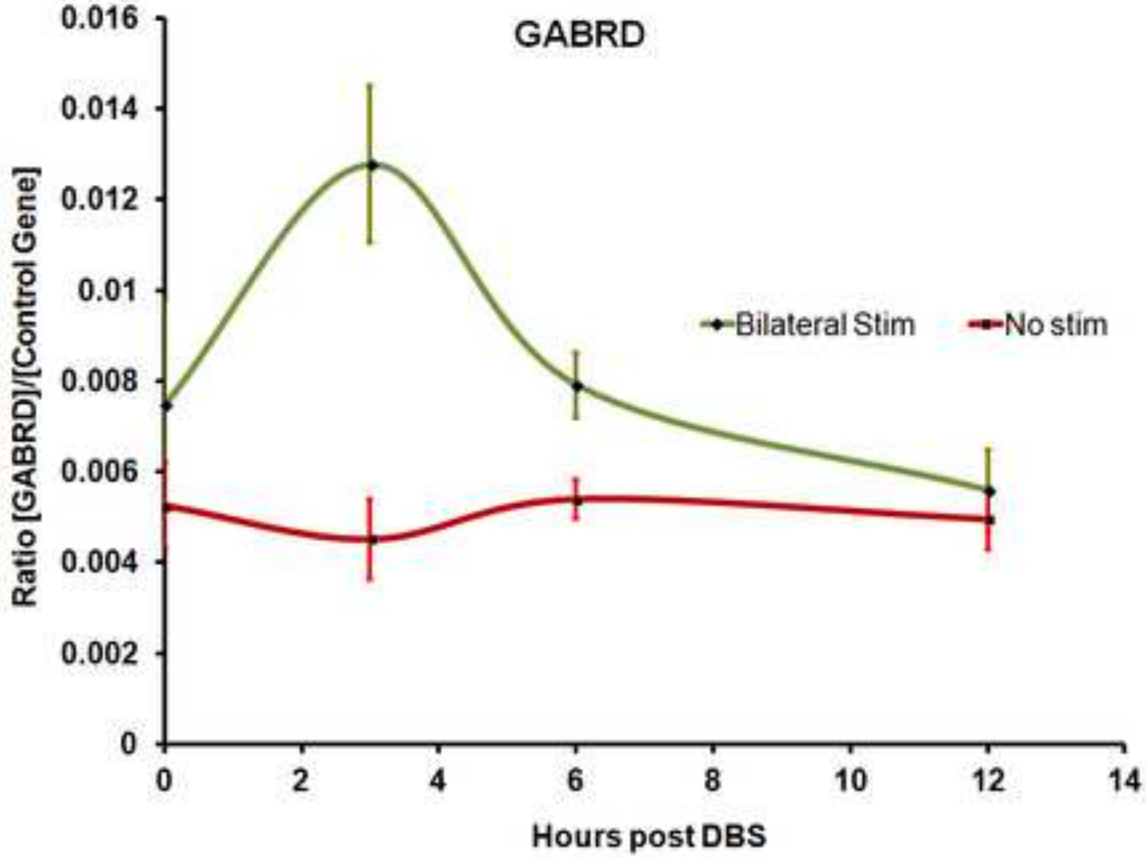

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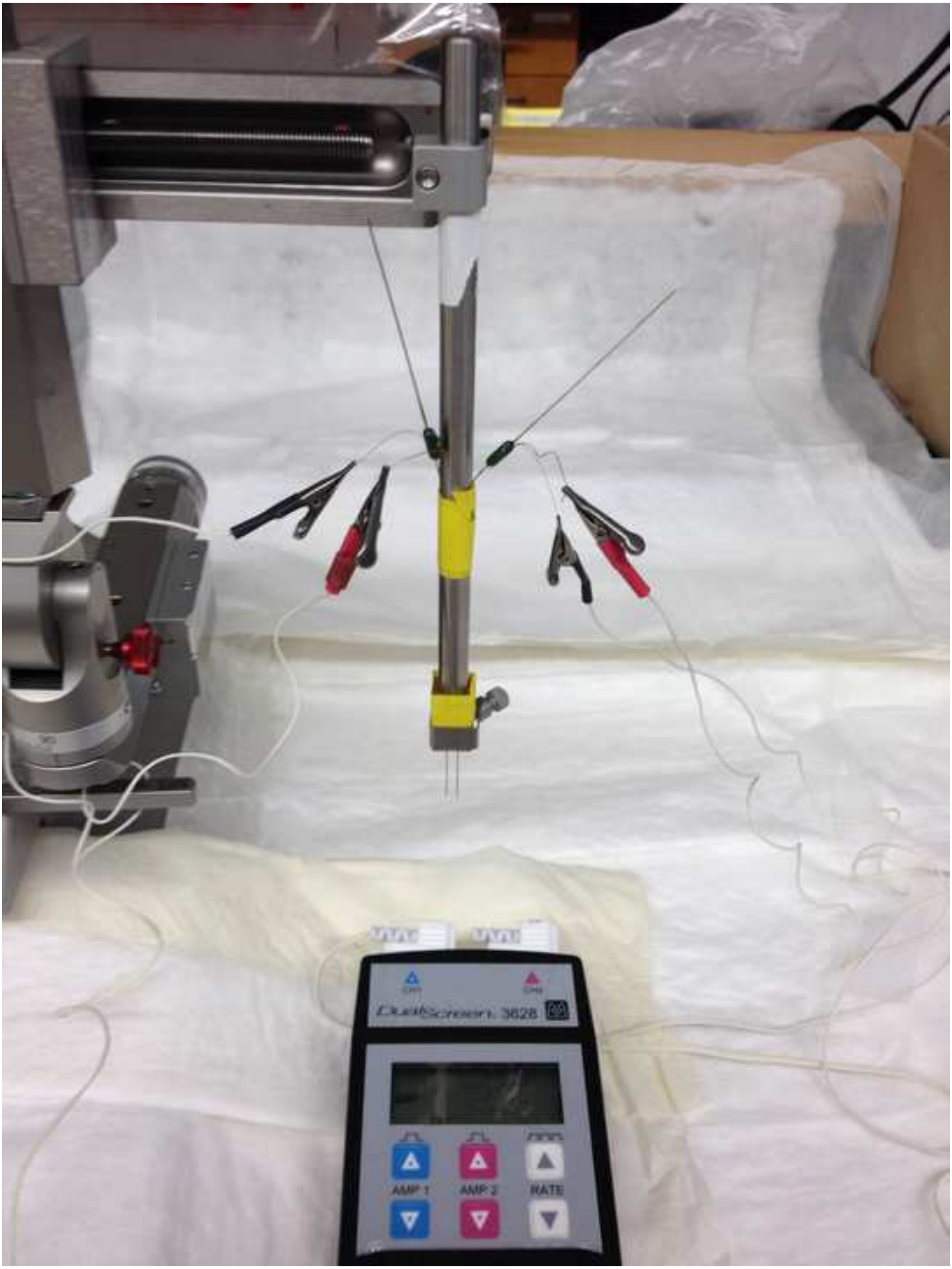

Table 1
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PCR Cycles			
Stage 1:	Initial Denaturation	95 °C	15 mins
Stage 2:	Denaturation Annealing Extension	95 °C 60 °C 72 °C	15 secs 30 secs 30 secs
	40 cycles of Step 2		
Note: The annealing temperature varies according to the primer melting temperature. Primers are typicallydesigned to have an optimal annealing temperature of 60 °C			

Excel Spreadsheet- Table of Materials/Equipment

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Deep Brain Stimulation Surgery		
Reagent/Equipment	**Vendor Name**	**Catalog No.**
Stereotactic frame	Kopf Instruments	Model 900
Drill	Dremmel	7700, 7.2 V
Scalpel	BD	372610
Ketamine	Patterson Veterinary	07-803-6637
Xylazine	Patterson Veterinary	07-808-1947
Buprenorphine	Patterson Veterinary	07-850-2280
Surgical staples	ConMed Corporation	8035
Sutures (3-0)	Harvard Apparatus	72-3333
Syringe (1 ml, 29 1/2 G)	BD	329464
Syringe (3 ml, 25 G)	BD	309570
Needles	BD	305761
Ethanol	Fisher Scientific	S25309B
Eye Lubricant	Fisher Scientific	19-898-350
Stimulator	Medtronic	Model 3628
DBS electrodes	Rhodes Medical Instruments, CA	SNEX100x-100mm
Betadine (Povidone-Iodine)	PDI	S23125

Brain Dissection and Hippocampal tissue isol		
Reagent/Equipment	**Vendor Name**	**Catalog No.**
Acrylic Rodent Brain Matrix	Electron Microscopy Sciences	175-300
Razor Blade	V W R	55411-050
Guillotine Scissors	Clauss	18039
Scissors	Codman Classic	34-4098
Forceps	Electron Microscopy Sciences	72957-06
Phosphate Buffered Saline	Boston Bioproducts	BM-220

RNA Extraction and cDNA Preparation		
Reagent/Equipment	**Vendor Name**	**Catalog No.**

Tri Reagent	Sigma	T9424
Syringe (3 ml, 25 G)	BD	309570
Chloroform	Fisher Scientific	BP1145-1
Isopropanol	Fisher Scientific	A416-1
Glycogen	Thermo Scientific	R0561
Dnase I Kit	Ambion	AM1906
Superscript First Strand Synthesis Kit	Invitrogen	11904-018
Tabletop Microcentrifuge	Eppendorf	5415D

Quantitative PCR		
Reagent/Equipment	**Vendor Name**	**Catalog No.**
SYBR Green PCR Kit	Qiagen	204143
Custom Oligos	Invitrogen	10668051
PCR Plates (96 wells)	Denville Scientific	C18080-10
Optical Adhesive Sheets	Thermo Scientific	AB1170
Nuclease free Water	Thermo Scientific	SH30538-02
Real Time PCR Machine	Applied Biosystems	7500

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Sterile, use for Anesthesia administration intraperitoneally
Sterile, use for Analgesia administration subcutaneously
Sterile, use for clearing broken bone pieces from the burr holes
Use for general sterilization
Electrodes are platinum, concentric and bipolar
Single use swabsticks, use for sterilizing the scalp before making incision

ation

Comments
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For decapitation, make sure these scissors are maintained in clean and working condition
Use for removing the brain from the skull
Use for removing the brain from the skull and for handling during dissection

Comments

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Use for tissue homogenization
Always use in a fume hood and wear protective goggles while handling; avoid contact with skin

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A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

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Article Title:	Deep Brain Stimulation of the anterior thalamic nucleus in rat.
Signature:	Travis Tierney
Date:	6/27/2014

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Response for editorial review comments
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Dear JoVE Editorial Board,

I would like to thank the editors for a careful review for our manuscript titled 'Deep Brain Stimulation of the Anterior Thalamic Nucleus in Rat'. I appreciate your comments and we have paid due attention to addressing your points. Please find below a list of changes as response to your comments. Please note that I have highlighted the changes in red in the manuscript document.

Sincerely,
Travis S. Tierney

LIST OF CHANGES

1. Editor modified the formatting of the manuscript and made minor copy-edits. A few protocol steps were changed to the imperative tense. Please maintain the current formatting throughout the manuscript. You can find the updated manuscript attached to this e-mail.

   We have scanned the whole manuscript and made sure that the steps in the protocol are in the imperative tense.

2. Please add City and State/Country for each author affiliation.

   Added on Pg 1

3. Please provide an email address for each author.

   Added on Pg 1

4. Please re-word the Short Abstract to more clearly state the goal of the protocol within the 50 word limit. For example, "This protocol/manuscript describes…"

   Rewrote the short abstract (Pg 1) in the above format requested.

5. JoVE is unable to film steps involving anesthetization/euthanasia therefore, in step 1.3, the following text was un-highlighted: "Use a Ketamine/Xylazine mix (Ketamine 75mg/kg and Xylazine 10 mg/kg) to anesthetize the rats. Note: Isoflurane can also be used as the anesthetizing agent."

   Agree with the above change.

6. In step 2.10, please re-write the following sentence in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.): "If so, the electrodes are inserted to a depth of 5.2mm from the surface of the skull." Alternatively, this text may be added as a "Note" following step 2.10.

   Rewrote step 2.10 in imperative tense as *"If so, insert the electrodes to a depth of 5.2mm from the surface of the skull".*

7. Please also re-write steps 2.12, and 7.5 in the imperative tense.

Rewrote step 2.12 in the imperative tense *as* *"Deliver high frequency stimulation for an hour (or for a desired period of time as per experimental setup). Perform unilateral or bilateral stimulation based on one's experimental needs. Include controls such as low frequency stimulation (for e.g., 10Hz) and unstimulated animals (inserting electrodes with no subsequent stimulation)."*

Removed contents from what was previously Step 7.5. In the updated manuscript step 7.5 refers to 'Data analysis'.

8. It would be beneficial to provide the PCR conditions in step 7.5 in the form of an Excel table uploaded to the "Table" section of the JoVE submission site.

Step 7.5 indicating PCR cycles was deleted and merged with the previous step. In the updated manuscript, step 7.5 refers to 'Data analysis'. A separate table in excel spreadsheet for the PCR parameters will be uploaded in JoVE submission site

9. JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove all commercial sounding language from your manuscript. Examples of commercial sounding language in your manuscript are "Kopf Instruments Model 1770", "Dremmel" etc. All commercial products should be sufficiently referenced in the table of materials/reagents.

Removed trade name 'Kopf' from Step 1.4. Rewrote as *"Mount and secure two electrodes on the electrode holder of a stereotactic surgical frame".*

Removed 'Dremmel' from Step 2.6. Rewrote as *"Use a hand-held drill to make the burr holes".*

Removed 'Medtronic' from Step 2.11. Rewrote as *"Connect the electrodes via leads to a stimulator set at..."*

Inserted "from DNaseI kit" in steps 5.1 and 5.2 for clarity.

Removed 'Invitrogen' from Step 6.1 . Replaced with *"Superscript First Strand Synthesis Kit".*

10. Please minimize use of the pronoun "our" in the manuscript.

Removed 'our' from step 7.5

11. Please add at least one paragraph of results text that explains your representative results in the context of the technique you describe; i.e. how do these results show the technique, suggestions about how to analyze the outcome etc. This text should be written in paragraph form under a "Representative Results" heading and should refer to all of the results figures. You may include the figure captions under this heading but the captions and figure text must be separate entities.

    I have added the requested information in pages 9 and 10. Text is highlighted in red. Please note insertion of references 25-29 as part of this change. In the second inserted paragraph where I describe the data analysis methodology using the ΔΔCt method, I was not able to use the imperative tense because it seemed to affect the flow of the sentences in the paragraph. If this is a problem, please let me know.

8/28/2014

Dear JoVE Editors and Reviewers,

I appreciate the effort and all the input we have received with regards to our manuscript submission. I have taken into account all the valuable feedback and comments from the reviewers. I appreciate the time and effort from the reviewers in critical reading of our manuscript. We have tried our best in answering the reviewers' queries and have modified our manuscript according to the reviewer's feedback. We have included additional information where it was needed. Please find below, our response to the reviewers' comments. We have bolded our responses for ease of reading.

I sincerely hope that the JoVE editorial board as well as the reviewers will find our resubmission satisfactory and we hope our article will be of help to the DBS scientific community. I am happy to answer any further questions.

Sincerely

Dr. Travis Tierney
Director, Stereotactic and Functional Neurosurgery
Department of Neurosurgery,
Brigham and Womens Hospital,
Harvard Medical School
Boston, MA.

Editorial comments:

1) All of your previous revisions have been incorporated into the most recent version of the manuscript. Please download this version of the Microsoft word document from the "file inventory" to use for any subsequent changes.
We have downloaded this file and used it for subsequent changes.

2) JoVE is unable to film protocol steps describing anesthesia/euthanasia. Step 2.16 describes euthanasia therefore this step was un-highlighted.
Agreed

3) In the JoVE protocol, steps should be short, consisting of 2-3 related actions and a maximum of 4 sentences per step. Step 2.6 contained more than 4 sentences, therefore it was split into two steps (2.6 and 2.7).
Agreed

4) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.
We have checked our manuscript for spelling or grammatical errors.

5) Please disregard the comment below if all of your figures are original.
If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."
Figures are original.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript describes very well the steps and techniques necessary to run gene expression studies. The author used the Anterior Thalamus Nucleus (ATN) DBS as a tool to induce differential gene expression, once it has been successfully used as treatment for refractory epilepsy and it resulted in altered pattern of hippocampal neurogenesis. So, the manuscript describes the stereotactically-guided implant of electrodes, stimulation parameters (used for epilepsy-treatment), tissue extraction and RT-PCR.

Major Concerns:
1. There is no novelty in terms of technical procedures in spite of the value of described results that are good indeed. On his title, the author did not mention the used techniques, which were

described in his manuscript - the actual title does not give any meaning to the study as methodological one.

The title has been modified to 'Analysis of gene expression changes in the rat hippocampus after deep brain stimulation of the anterior thalamic nucleus'.

Minor Concerns:

2. From the RT-PCT description, it is not clear how the author designed the used primer for BDNF or GABA receptor gene expression.

The number of animals used should be given.

Details about primer design were included in Section 7.1. We are happy to provide the primer sequences used for BDNF and GABRD amplification upon request.

We have used one animal per time-point in these representative results. These results are similar to many such trials we have performed previously in the lab. The data presented here has not been published yet, so we decided to withhold our data figures comprising more animals per timepoint for our publications which are currently under preparation. Despite the lack of more animals per datapoint, we believe that the data presented here is a good representation of the results that can be expected using the approach described in the manuscript and that it serves the purpose of demonstrating the methodologies involved in gene expression analysis using the qPCR technique and the subsequent data analyses. We sincerely acknowledge the reviewer's interest in the sample size, and are hopeful that he/she would agree with our explanation.

Reviewer #2:

Manuscript Summary:

In this protocol, Selvakumar and colleagues describe an experimental approach to study DBS-induced changes in gene expression. The protocol is overall well-described, and potentially of good value to the growing community of pre-clinical DBS researchers. This stated, there are several minor weaknesses and omissions that should be corrected.

Major Concerns:

None.

Minor Concerns:

1. Both the Introduction and Discussion sections are too broadly focused on DBS history and general applications. The Reviewer strongly suggests focusing more on the current state of knowledge regarding ATN-DBS for epileptic disorders, as well as the rationale, strengths and limitations of their gene expression approach.

In the introduction section we have added details (line# 101-112) about a few more studies that focused on ATN-DBS for treating epilepsy.

Further discussion about the limitations, strengths and rationale of the gene expression approach is described in the discussion section between lines 468-474 and lines 480-490.

[**Editorial comment:** Please keep JoVE's manuscript guidelines in mind as you address the above comment(s).] The discussion should cover the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.]
We have adhered to JoVE manuscript guidelines while making inclusions/modifications in the manuscript.

2. The use of a needle to remove bone fragments in the burr hole seems like it may cause additional tissue damage. Is the needle blunted? Are alternative methods available (e.g., saline wash, small blunt forceps, etc.)?
Based on our experience in the lab, we have used this technique to remove bone fragments that are loosely lodged in the burr hole as well in the edges on the burr hole. We perform this carefully so as to avoid direct contact of the needle with the underlying dura and the brain tissue beneath. However, the reviewer's suggestion of using a blunt needle or blunt forceps is excellent and could be used successfully if one is worried about potentially damaging the brain tissue. In our experience, using saline wash has not been successful in removing the bone fragments.

Additional text was introduced in line# 196-198 to address this point by the reviewer.

[**Editorial comment:**The above comment may be addressed in the Discussion]
We found it easier to address this in the protocol section 2.7 where this point is mentioned. We hope this is acceptable.

3. It does not appear that electrode implantation accuracy is histologically verified in this protocol. This is particularly perplexing as reproducible electrode targeting is acknowledged as a challenge of this experimental procedure. If the nature of these experiments precludes verification of accurate targeting, this represents a limitation of the procedure that should be described.
We agree with the reviewer's comment and we have addressed this limitation in the discussion section line # 446-451.

[**Editorial comment:**The above comment may be addressed in the Discussion]
We have addressed this comment in the Discussion section.

4. Reference 38 is incorrectly described in the Discussion.
Removed reference 38 (Welter et al). Introduced 'Porta et al' (Now Reference # 55) as the appropriate reference to fit the discussion.

5. Reagents and Materials: The electrode should be included on this list, including electrode material, type (bipolar?), and dimensions.
Included this information in the 'Reagents and Materials' table which will be uploaded to the JoVE website with the submission. The electrodes are platinum, bipolar and concentric from Rhodes Medical Instruments, CA (Catalog# SNEX100x-100mm)

Reviewer #3:
In this paper the authors give detailed information on the procedures of DBS of the anterior thalamus, and preparing tissue for quantitative PCR assays. The paper provides detailed information and is sufficient for other researchers who would like to conduct similar experiments.

I do have some suggestions the authors may include.

1. At the end of the Introduction the authors may give some more background with respect to the relation between neurogenesis and epilepsy. Also, some references would be very helpful. On basis of their study I assume the authors expect that increased neurogenesis would be favorable for the treatment of epilepsy. If possible the authors substantiate this claim (references).
We have included results from studies that link decreased neurogenesis with chronic stages of epilepsy and have cited the relevant references in lines 121-129.

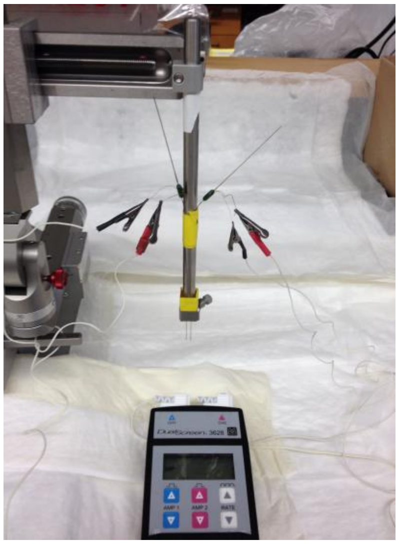
2. The sentence in 1.3 "Weigh the rat (typically 200-250 gm)" is somewhat strange. I assume that the authors suggest that the rats should weigh about 200-250 g (not gm) when using this protocol.
Yes, that is correct. The rats are purchased such that they weigh between 200-250 g at the time of surgery. Although they generally weigh within this range, we still weigh them before surgery for accurate calculation of anesthesia dose. Between 200-250 g is optimal for fixing the animals in the stereotactic frame we use as well as for accurate electrode targeting based on the coordinates suggested by the rat brain atlas we use.
The sentence in 1.3 was modified for clarity.

3. For anesthesia the use of ketamine/xylazine is not preferred in our lab. Our veterinarian strongly encourages us to use isofluran (inhalation) because it the dose can be regulated much better, also during the anesthesia.
We have consistently used ketamine/xylazine anesthesia for all our studies so far. So to avoid any additional changes we have preferred to use ketamine/xylazine mix. Moreover, we have avoided using isoflurane because it has been shown to influence the calcium signaling, MAP kinase, BDNF pathways which we are investigating in our studies. (Reference: Bickler et al., Anesthesia and Analgesia (2006) 103(2): p419-429).

4. The toe-pinch is not sufficient to examine the depth of the anesthesia. One should also carefully check the depth/regular breathing pattern of the animal, especially when pinching the skin between the toes.
Introduced text indicating the need to check for respiratory rate and breathing pattern in line# 166-167.

5. On what atlas are the ATN coordinates based?
"The stereotactic coordinates are based on the Paxinos and Watson (6th edition) rat brain atlas."
Included this information in line#178-180.

6. I would very much encourage including a picture of the two electrodes in the holder and how the electrodes look like when they are ready for stimulation (connected to stimulator).
Picture uploaded in JoVE website with submission (Copied here for reference)



7. The authors should provide some additional information why these stimulation parameters should be used (references?).
These stimulation parameters were based on an earlier study by Toda et al (Ref#1). The reference was cited in line# 216-217.

8. The authors suggest various time points after which the animal can be sacrificed. This is of course clearly related to the biological assay one wants to perform. May be the authors could state this more explicitly.
We added the following comment in Figure legend 1 as wells line#230 to address this point.
"Note: The timepoints selected here are with respect to a particular study and is subject to change according to the hypothesis and experimental plan."

9. The final paragraph of the Discussion could easily be deleted.
Final paragraph was deleted.