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To: Alisha DSouza,Ph.D

Senior Review Editor JoVE

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Authors: Slézia et al.

Dear Dr. Alisha DSouza, 20. December 2018

Enclosed is the revised manuscript "Electrophoretic delivery of GABA into epileptic focus prevents epileptic seizures in mice" with the responses to the editorial comments.

Sincerely yours,

Adam Williamson PhD, Aix Marseille Université , INS, UMR_S 1106, Marseille, France

and

George G. Malliaras, PhD, University of Cambridge, Cambridge CB3 0FA , Cambridge, UK

TITLE:

Electrophoretic Delivery of γ-Aminobutyric Acid (GABA) into Epileptic Focus Prevents Seizures in Mice

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KEYWORDS:

microfluidic ion pump, µFIP, electrophoresis, epilepsy, seizure, epileptic focus, GABA, hippocampus, silicon probe, 4-aminopyridine, 4AP, mouse

SUMMARY:

The challenge of epilepsy research is to develop novel treatments for patients where classical therapy is inadequate. Using a new protocol—with the help of an implantable drug delivery system—we are able to control seizures in anesthetized mice by the electrophoretic delivery of GABA into the epileptic focus.

ABSTRACT:

Epilepsy is a group of neurological disorders which affects millions of people worldwide. Although treatment with medication is helpful in 70% of the cases, serious side effects affect the quality of life of patients. Moreover, a high percentage of epileptic patients are drug resistant; in their case,

neurosurgery or neurostimulation are necessary. Therefore, the major goal of epilepsy research is to discover new therapies which are either capable of curing epilepsy without side effects or preventing recurrent seizures in drug-resistant patients. Neuroengineering provides new approaches by using novel strategies and technologies to find better solutions to cure epileptic patients at risk.

As a demonstration of a novel experimental protocol in an acute mouse model of epilepsy, a direct in situ electrophoretic drug delivery system is used. Namely, a neural probe incorporating a microfluidic ion pump (µFIP) for on-demand drug delivery and simultaneous recording of local neural activity is implanted and demonstrated to be capable of controlling 4-aminopyridine-induced (4AP-induced) seizure-like event (SLE) activity. The γ-aminobutyric acid (GABA) concentration is kept in the physiological range by the precise control of GABA delivery to reach an antiepileptic effect in the seizure focus but not to cause overinhibition-induced rebound bursts. The method allows both the detection of pathological activity and intervention to stop seizures by delivering inhibitory neurotransmitters directly to the epileptic focus with precise spatiotemporal control.

As a result of the developments to the experimental method, SLEs can be induced in a highly localized manner that allows seizure control by the precisely tuned GABA delivery at the seizure onset.

INTRODUCTION:

Epilepsy is the fourth most common neurological disorder: about 1% of the population suffers from epilepsy, and about one-third of the affected have recurrent seizures. In most cases, seizures can be controlled with medication. However, drug treatment needs to be set for every patient individually, where proper dosing can take years to find[1,2]. Additionally, most of the medication has serious side effects that reduce the quality of life[3–7]. Finally, in 30% of the cases patients are resistant to medication, and in case of a constant single seizure generator locus, only resective neurosurgery can attenuate the occurrence of seizures[8]. Therefore, a major initiative in modern epilepsy research is to discover new strategies which can prevent recurrent seizures in patients at risk, while reducing the necessity of strong drug therapies and invasive resective surgeries.

Epileptic seizures occur when there is an imbalance within excitatory and inhibitory circuits either throughout the brain (generalized epilepsy) or in a localized part of the brain (focal epilepsy), such that neurons discharge in an abnormal fashion[9–11]. Antiepileptic drugs can act in two different ways in seizure prevention: either decreasing excitation or enhancing inhibition[12]. Specifically, they can either modify the electrical activity of neuronal cells by affecting ion channels in the cell membrane[13] or act on chemical transmission between neurons by affecting the inhibitory neurotransmitter GABA or the excitatory glutamate in the synapses[14,15]. For some medications, the mode of action is unknown[18]. Also, drug treatments have a continuous effect on patients and cannot adapt to the prevalence dynamics of seizures. Ideally, drugs with specific mechanisms of action would act on the underlying epileptic processes. An optimal treatment would not touch the brain interictally but would act immediately when a seizure starts

developing. In contrast to that, in all cases of epilepsy, medication now means a systematic treatment, affecting the whole brain and the whole body of the patient[9].

Epileptic seizures can appear many years after the initial insult such as brain trauma. The period between the initial insult and the occurrence of the first spontaneous seizures is characterized by considerable molecular and cellular reorganizations, including neuronal death with the disappearance of neuronal network connections and axonal sprouting/neosynaptogenesis with the appearance of new connections[19–21]. Once seizures become recurrent, their frequency and severity tend to increase, involving more brain regions. It is important to distinguish the sites of seizure onset (epileptogenic regions) from propagation networks, as the rules of seizure genesis and propagation may differ. Research performed on human tissue and experimental models of epilepsy have provided important data regarding the reorganization of circuits and their ability to generate seizures[20–23]. However, it is difficult to determine if these reorganizations are adaptive responses or whether they are causally related to epileptogenesis or seizure genesis and propagation[12].

Therefore, localizing the epileptic focus and applying antiepileptic drugs locally are one of the main challenges in contemporary epilepsy research. Several experiments using animal models of epilepsy and some clinical studies aimed to find the onset of the seizure events and define the underlying mechanisms in the brain[24–27]. To this end, we developed a new experimental protocol using the 4AP-induced epilepsy model[28–31] in an acute mouse preparation, which allows the precise insertion of three devices into the given area of the hippocampus, where network activity in vivo is manipulated in a highly localized manner. Localized 4AP injection by a glass micropipette helps to induce epileptic SLEs in a localized spot in the hippocampus, while with the help of the novel polymer-based µFIP probe the control of the seizure activity is achieved simultaneously by recording the neuronal electrical activity with the device's recording sites. Hippocampal local field activity is also monitored with a multichannel silicon probe in a layer-specific manner in the cortex and in the hippocampus simultaneously.

The recently invented µFIP probes work by using an applied electric field to push charged drugs stored in a microfluidic channel across an ion exchange membrane (IEM) and out to the surrounding tissue (**Figure 1**). The IEM selectively transports only one type of ion (cation or anion) and, thus, works to limit both passive diffusion in the "off" state and transport of oppositely charged species from the surrounding tissue into the device. The electric field is created on demand by applying a small voltage (<1 V) between the source electrode which is internal to the microfluidic channel and a target electrode which is external to the device (in this case, the head screw on the animal model). The rate of drug delivery is proportional to the applied voltage and the measured current between the source and target electrodes. The precise tunability of drug delivery is one of the primary advantages of the µFIP. Another critical advantage, compared to fluidic or pressure-based drug delivery systems, is that in the µFIP there is only a negligible pressure increase at the drug delivery outlet as drugs are delivered across the IEM without their carrier solution.

There is a small amount of passive leaking of GABA when the µFIP is "off", but this was found not to effect SLEs. The µFIP are custom-made following conventional microfabrication methods that we reported previously[31].

Since one way of preventing recurrent seizures is the blockade of network discharges at the very beginning or even before the first seizure event, the presented method for delivering the inhibitory neurotransmitter GABA into the epileptic focus has great therapeutic potential for seizure control in patients with focal epilepsy. Since GABA is an endogenous substrate, it leaves intrinsic neuronal properties unchanged in physiological concentrations. The local application of low levels of GABA will only affect cells naturally responsive to inhibition, and will only cause similar effects to physiological inhibition, contrary to deep brain stimulation (DBS), which has unspecific actions by stimulating all cells of the neuronal network in its environment, causing a mixed response involving both excitation and inhibition. In conclusion, the proposed method provides a more specific approach to seizure control than DBS.

PROTOCOL:

All experimental procedures were performed according to the ethical guidelines of the Institut de Neurosciences des Systèmes and approved by the local Ethical Committees and Veterinary Offices.

NOTE: Seventeen adult male *OF1* mice were used for the experiments. Mice were entrained to a 12 h light/dark cycle with food and water available ad libitum.

1. Anesthesia

1.1. Inject intraperitoneally a mixture of ketamine and xylazine (100 mg/kg body weight and 10 mg/kg body weight, respectively) to anesthetize the animal.

1.2. Check the level of anesthesia by observing the respiratory rate and whisking and by checking the mouse's response to pain.

NOTE: When the mouse's breathing becomes regular, no whisking can be observed, and the animal does not react to tail pinches, the anesthesia is deep enough to continue.

1.2.1. Place the animal on an electrically programmable heating pad. Cover the rectal temperature probe with a petroleum jelly-based product (see **Table of Materials**) and place it gently into the rectum (1–2 cm deep) of the mouse to monitor its body temperature. Maintain a body temperature between 36.5 and 37.5 °C during the surgical procedures and experimental recordings.

1.2.2. Monitor the anesthesia level by checking the mouse's reflexes, whisker movements, and its frequency of breathing. Taking into account the level of anesthesia recorded at least every 30 min, give a small dose of a ketamine-xylazine cocktail (20–50 µL, the same concentration used as before) intramuscularly.

2. Surgery/craniotomy

2.1. Fix the head of the mouse in a stereotaxic frame. Using a 30 G needle, inject local analgesic ropivacaine (5 µL, 7.5 mg/mL, see **Table of Materials**) subcutaneously at the planned incision site. Allow 5 min for it to take effect.

2.2. Make a straight cut midline in the skin above the skull with a scalpel. Gently pull the skin toward the sides with fine forceps and clamp it aside with bulldog serrefine clamps to leave the skull exposed for further work.

2.3. Clean the skull of fascia with a scalpel or any similar tool. In case of superficial bleeding, remove the blood with cotton swabs or small pieces of paper towel.

2.4. Take a poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS)-coated ground screw (size: #00, diameter: 0.047 in, length: 1/8 in, see **Table of Materials**) with a soldered wire and connect it with a connector to the amplifier headstage.

2.5. Moisten the skull at the desired hole site and drill a hole at high speed using a fine, round drill bit (with a 0.4 mm diameter) on the skull above the cerebellum until the dura is visible. Put the ground screw into the hole and screw it in with a precision screwdriver until it reaches the top of the cerebellum**.**

NOTE: The head screw was dip-coated with a PEDOT:PSS solution containing 1% 3-glycidyloxypropyl)trimethoxysilane (GOPS) by weight followed by baking at 140 °C for 90 min. PEDOT:PSS is a conjugated polymer with a volumetric capacitance that is known to be biocompatible. GOPS is a cross-linker mixed with PEDOT:PSS to increase stability in aqueous media (**Figure 2**).

2.6. With the help of the stereotaxic frame, measure the stereotaxic coordinates for the desired brain region. For example, the region of interest is the hippocampus, anteroposterior (AP) -1.8 mm and mediolateral (ML) 1.8 mm from the Bregma point based on the brain atlas for mice[32].

NOTE: These are coordinates for the right hemisphere (**Figure 2**).

2.7. Thin an approximately 500 µm diameter area of the skull above the target region using a reliable dental drill (see **Table of materials**) set at a fast speed until a thin, well-polished, transparent bone membrane remains.

2.8. Then, if the thickness of the bone membrane is thin enough (<200 µm), make a small hole with thin forceps and gently remove the thin layer of the bone[33]. Use sharp-point forceps to remove the dura. Minimize the size of craniotomy and durotomy to prevent the development of edemas and to minimize cardiac and/or respiratory pulsations of the brain.

NOTE: The craniotomy must be filled with a droplet of saline solution to prevent drying and then regularly refilled during the experiment (**Figure 2**).

3. Insertion of the multichannel silicon probe

3.1. Use stereotaxic arms in a slight AP angle (20°) for the silicon probe to leave ample space for the positioning of the other two implants and to have the recording and the injection sites of the electrode, ion pump, and micropipette as close as possible.

NOTE: Electrodes, syringes, and ion pumps were covered with a drop of DiI stain solution (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate [DiI]), for the post hoc visualization of the implantation traces (0.5 mg/ml DiI in dimethyl sulfoxide).

3.2. Place the silicon probe on the stereotaxic arm attached to a magnetic holder and place it next to the stereotaxic frame. Set the AP angle (20°), and then connect the probe to the headstage and to the ground screw.

3.3. Slowly lower the silicon probe into the hippocampus with the help of the micron-precise stereotaxic arm or a motorized micromanipulator to avoid lateral movements (**Figure 2** and **Figure 3**).

3.3.1. Initiate the recording software and record—with the headstage, the connected amplifier, and a computer—electric neuronal signals while moving the multichannel silicon probe from the top of the cortex until the targeted dorsoventral (DV) position is reached (-1,800 µm from the cortical surface). Record and watch the local field potential signal (LFP) during penetration on the computer screen.

NOTE: Control the descent of the probe so that it is moving slowly and continuously while recording, to have better visual control for the penetration and for reaching the target zone.

3.3.2. Use the ripple activity in the stratum pyramidale of the hippocampal formation in the recorded LFP as a marker of the target zone.

NOTE: Ripple activity is visible on one or two neighboring channels of the multichannel silicon (Si) probe having a 100 µm distance between recording sites (**Figure 4**).

3.3.3. Record LFP signals from the layers of the cortex and the hippocampus simultaneously through the multichannel amplifier's software (see **Table of Materials**) with the help of the multichannel Si probes (**Figure 4**).

4. Insertion of µFIP

4.1. Connect tubes (see **Table of Materials**) to the inlet of the µFIP and fill the probe with 0.05 M GABA solution. Remove the tubes and close the inlet with paraffin film wrapping. Connect electrical leads to the source measurement unit.

4.2. Insert the µFIP with the help of the stereotaxic arm at a mediolateral (MP) angle (20°). The Si probe remains inserted during the whole process.

NOTE: µFIP is very flexible and may benefit from the support of a small and clean paintbrush to keep it straight until it reaches the brain surface. After that step, µFIP can be lowered gently with axial movements.

4.3. Lower the µFIP slowly with axial movements and never let it bend during the trajectory until it reaches the dorsoventral (DV) coordinate (-1,200 µm from the cortical surface).

NOTE: Try to put the two devices (µFIP and silicon probe) as close to each other as possible, considering the 300 µm distance of the outlet from the µFIP tip.

CAUTION: Avoid any mechanical issues among the devices and their connectors during insertion (**Figure 2B** and **Figure 3B**).

5. Preparation of devices for seizure induction

5.1. Change the metal needle of the syringe (10 µL) (see **Table of Materials**). Remove the needle-holding metal part, place and fix the micropipette (outer diameter [OD]: 1.2 mm, inner diameter [ID]: 0.75 mm, tip diameter: 20–50 µm with ± 0.5 cm of tapering of the shank), and then replace the needle-holding element.

5.2. Position the syringe and the attached borosilicate micropipette at a 20° lateromedial (LM) angle for the injection of 4AP (50 mM in artificial cerebrospinal fluid [ACSF]).

CAUTION: Do not use the metal needle of the syringe or a micropipette with a tip bigger than 50 µm.

5.3. Draw 500 nL–1 µL of 50 mM 4AP with the help of an automated microinjection pump.

6. Insertion of the glass pipette attached to a syringe for 4AP injection

6.1. Lower the glass micropipette attached to the syringe to the aimed DV position (-1,500 µm), and then inject 250 nL of the 4AP solution (**Figure 2** and **Figure 3**). Start recording with the recording software. Watch the screen and wait for the first interictal spike to appear.

6.2. Start the GABA delivery by µFIP immediately with the appearance of the first interictal spike. Deliver GABA by applying 1 V between source and target for 100 s followed by 1 s off, for 30 cycles. With the help of the recording software, record for a minimum of 2 h.

NOTE: The total mass of the delivered GABA is around 1 nmol (**Figure 5**).

6.3. At the end of the experiment, gently remove the inserted probes and the ground screw, and remove the animal from the stereotaxic equipment. After overanesthetizing the animal with a lethal dose of anesthetics, sacrifice it.

7. Evaluation of the placement of the implants

7.1. After euthanizing the animal, perfuse it transcardially, first with 50 mL of saline and then with 150 mL of an ice-cold fixative solution containing 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB)[34].

CAUTION: PFA is hazardous and must be handled with care.

7.2. Decapitate the animal, and then remove the skin and the muscle from the top and sides of the skull. Starting from the foramen magnum, make lateral incisions in the skull toward the ears and a sagittal midline incision, taking great care not to damage the brain. Gently remove the skull with a bone trimmer. Remove the brain, and then cut a tissue block from the region of interest (from the Bregma point, -1 to -3 mm AP) with the help of a brain matrix (see **Table of Materials**).

7.3. Glue the tissue block to the specimen holder of a vibratome, put the stand into it, and set the vibratome to 40 µm thickness in a PB bath make 40 µm coronal sections.

7.4. Wash extensively with 0.1 M PB. Follow the histological protocol for glial fibrillary acidic protein (GFAP) staining[31].

7.5. Mount sections on slides and cover them with a mounting medium containing 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) (see **Table of Materials**).

8. Confocal microscopy

8.1. Place the slides with the stained coronal sections under a 20x objective of a confocal microscope. Select the target region.

8.2. Choose the optimal excitation and emission (exc/ems) filter sets for the dyes as follows: DAPI = 358/461 nm, DiI = 551/569 nm, and fluorescein (see **Table of Materials**) = 490/525 nm.

NOTE: Since staining varies per section, a proper range of minimal and maximal excitation and detection needs to be determined for each section, where the least dense and most dense regions both show emission.

8.3. Choose the least dense region and set the laser intensity and detection to high values, and then verify at the densest regions whether these values cause oversaturation of detected

emission. If so, lower the values and recheck them with the least dense region. Iterate these steps until arriving at the highest possible detection at low staining levels and proper, not oversaturated levels at highly stained areas. Repeat this process for all dyes.

8.4. Use the tile scan function of the microscope with 512 x 512 pixels per tile to obtain a large overview of the probe insertion sites with an adequate resolution for post hoc processing.

REPRESENTATIVE RESULTS:

Using the procedure presented here with a 4AP epilepsy model in anesthetized mice, control of epileptic seizures can be achieved in the epileptic focus. The precise localization of the implants (**Figure 2**) helped to record hippocampal local field potentials (LFPs, **Figure 4**), to induce small hippocampal seizures and to deliver GABA at the seizure onset. The localization of the implants was verified after each experiment by post hoc histology (**Figure 3**).

In the case when SLEs were present only in the hippocampus, epileptic activity was put under complete control. **Figure 5** shows a representative example of when SLEs could be stopped with the delivery of GABA by the novel neuronal probe incorporating a µFIP. When 4AP was injected into a larger area or on the top of the cortex, epileptic seizures became generalized, so the delivered GABA was not able to modify the extent of the epileptic seizures (**Figure 6**). The delivery of sodium ions did not have a notable effect on 4AP-induced activity (**Figure 7**).

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the µFIP probe. Schematic view and real size of the µFIP probe. (**a**) Schematic of the implanted end of a µFIP probe showing primary features. (**b**) Photo of a µFIP probe with a needle-like implanted end pointing up. The red block is for fluidic connections. The scale bar = 1 cm. (**c**) Microscope image of a µFIP probe tip without the IEM. The scale bar = 100 µm.

Figure 2: Cranio-durotomy and localization of the implants in the mouse brain. (**A**) Schematic view of the surgery, the craniotomy, and the target of the implants in the mouse brain. (**B**) Used stereotaxic coordinates and angles for the implantation of the three devices. µFIP = 20° mediolateral (ML), -1,200 µm DV (green). Multichannel silicon probe = 20° AP, -1,800 µm DV (blue). Micropipette with syringe = 20° LM, -1,500 µm DV (red). The center of the craniotomy is 1.8 mm ML, -1.8 mm AP for the right hemisphere. This figure has been modified from Proctor et al.[31] (copyright distributed under the Creative Commons Attribution CC BY-NC).

Figure 3: Histological evaluation of the implantation placement. Panel (**A**) Shows a schematic 3D figure on the localization of the hippocampal formation in the mouse brain (green). Panels (**B**, **C** and **D**) show the traces of the implanted devices—µFIP, micropipette with syringe and multichannel silicon probe (DiI, red, arrows), respectively[31]. Panels (**Ba**, **Ca**, **Da**) show a high magnification image; panels (**Bb**, **Cb**, **Db**) show the corresponding page of the Paxinos and Franklin mouse brain atlas, whereas (**Bc**, **Cc**, **Dc**) show a low magnification image on the whole coronal section of the right hemisphere. The scale bar = 500 µm.

Figure 4: Multichannel recording from the cortex and the hippocampus. LFP recording with a multichannel silicon probe with a 100 µm distance between recording sites, from the layers of the cortex (white) and the hippocampal formation (purple, CA1 = Cornu Ammonis area 1; blue, DG = dentate gyrus. Note the ripple activity of the CA1 stratum pyramidale.

Figure 5: Control of a 4AP-induced seizure by GABA. Representative electrophysiology recordings from the hippocampus. (**A**) Recording in the absence of µFIP treatment with SLEs starting approximately 30 min after a 4AP injection, followed by status epilepticus. (**B**) Recording of a case in which the µFIP treatment was initiated immediately following the first SLE. The recording shows no further pathological events after the treatment starts. (**C**) Recording in which µFIP treatment was initiated before a 4AP injection, showing no pathological events. The red arrows indicate a 4AP injection. Solid green arrows indicate the start of the µFIP treatment, and open green arrows mark the end of the µFIP treatment. Sharp peaks at 100 s intervals following a green arrow are artifacts from the µFIP treatment[31].

Figure 6: Failure of the control of a 4AP-induced seizure by GABA. Representative electrophysiological recording from the hippocampus in a case where 4AP was injected with the metallic needle of the syringe, so epileptic seizures affected a larger brain area. Note that GABA delivery did not affect the seizure intensity.

Figure 7: Vehicle experiment. Control experiments delivering an equivalent dose of sodium ion (Na+) in the place of GABA did not have a notable effect on 4AP-induced activity, demonstrating that it is not the applied current from the ion pump that modulates electrophysiological activity but rather the delivered molecules.

DISCUSSION:

By developing a new experimental protocol in an acute mouse model of epilepsy, SLEs could be successfully controlled with the help of a µFIP implanted in the epileptic focus. Thanks to its capability to deliver GABA with temporal and spatial precision, 4AP-induced SLEs were controlled at the onset of the seizures. Treatment of epilepsy is theoretically possible if the control of the neural network discharges is achieved at the place of the seizure start. The presented protocol proved this possible if the localization of the injected inhibitory neurotransmitter, GABA, is precise enough to reach the epileptic focus in time. However, in those cases where epileptic seizures affect larger areas of the brain, seizure control by precisely localized GABA delivery is not possible. The area of influence of the µFIP probe was estimated with a radius of approximately 550 µm from the outlet. It was proven that SLEs could only be affected if the 4AP injection was localized to this area[31].

Therefore, the method is useful when epileptic seizures are localized, but it was not possible to control or stop epilepsy when the seizures became multifoci or generalized. Also, the method has been proven in anesthetized rodents; in freely moving animals, chronic applications are still necessary to investigate the efficiency of this protocol.

While epilepsy has several forms and is caused by different underlying mechanisms, it is proven that approximately 60% of patients have a single epileptic focal point[35]. Therefore, the advantage of the local administration of an endogenous inhibitory neurotransmitter provides a useful tool for further experimental animal studies and presents a new strategy in the treatment of focal epilepsy. The described method proved the utility of µFIP treatment in an acute mouse study and opened the way for further technological developments to ensure its chronic application. We believe that precisely targeted electrophoretic drug delivery devices can be further adapted to treat not only epilepsy but other neurodegenerative diseases as well.

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DISCLOSURES:

The authors have nothing to disclose.

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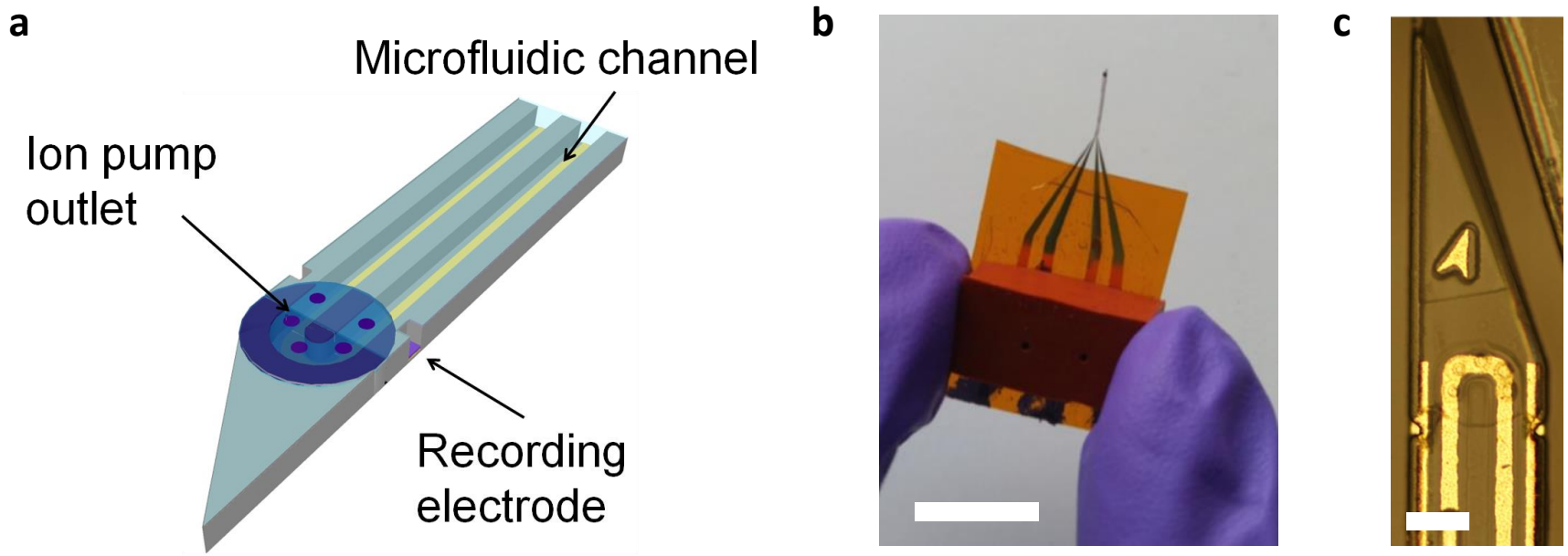

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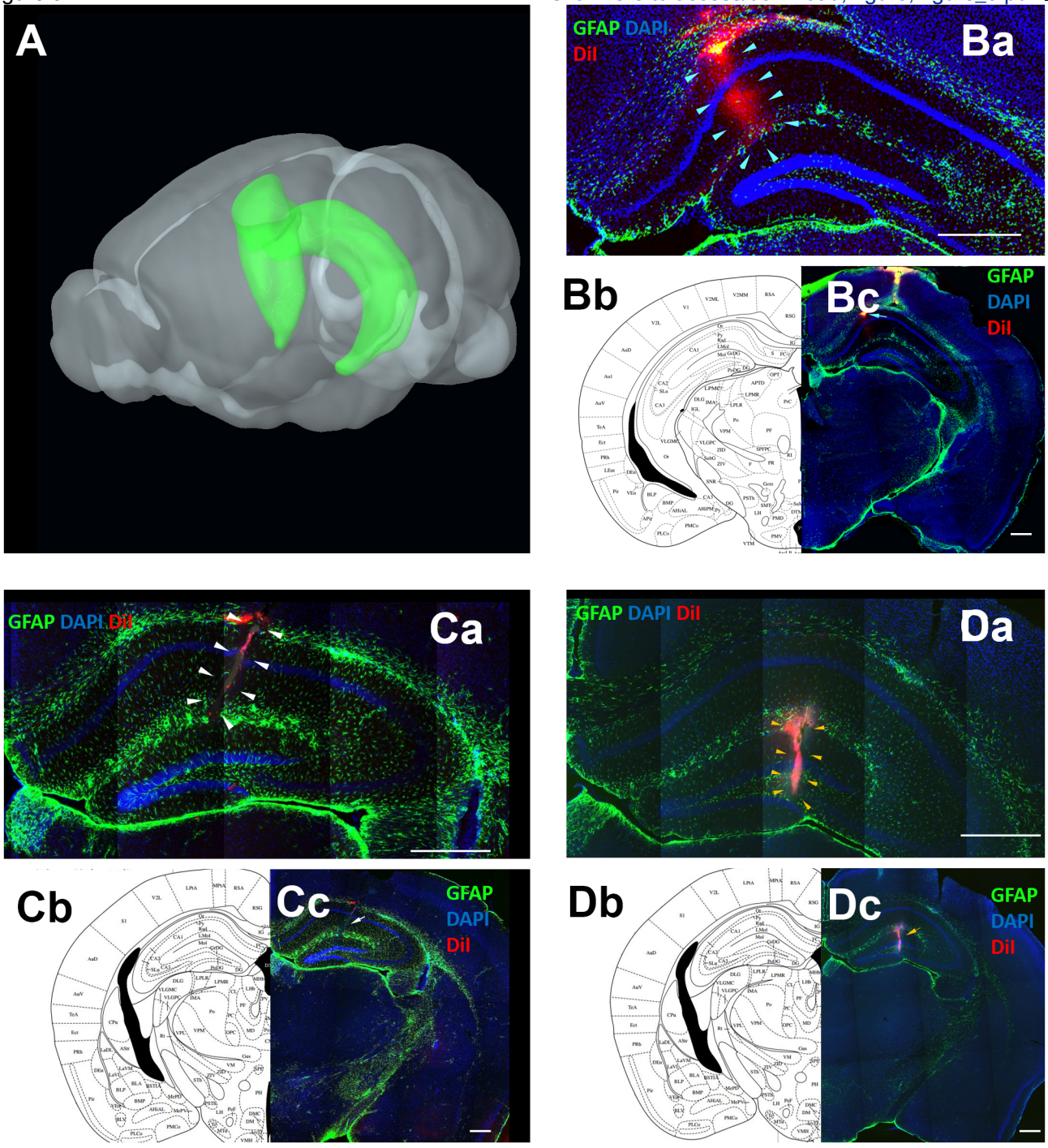

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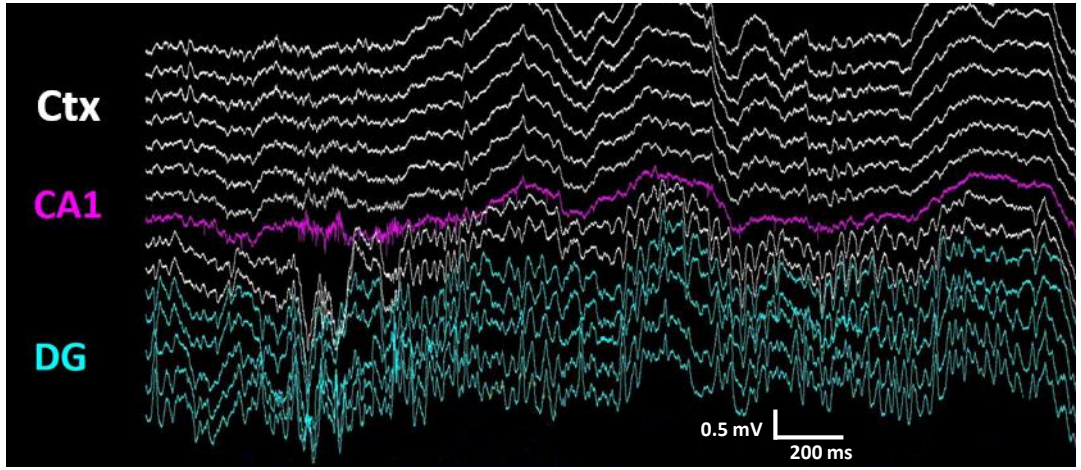

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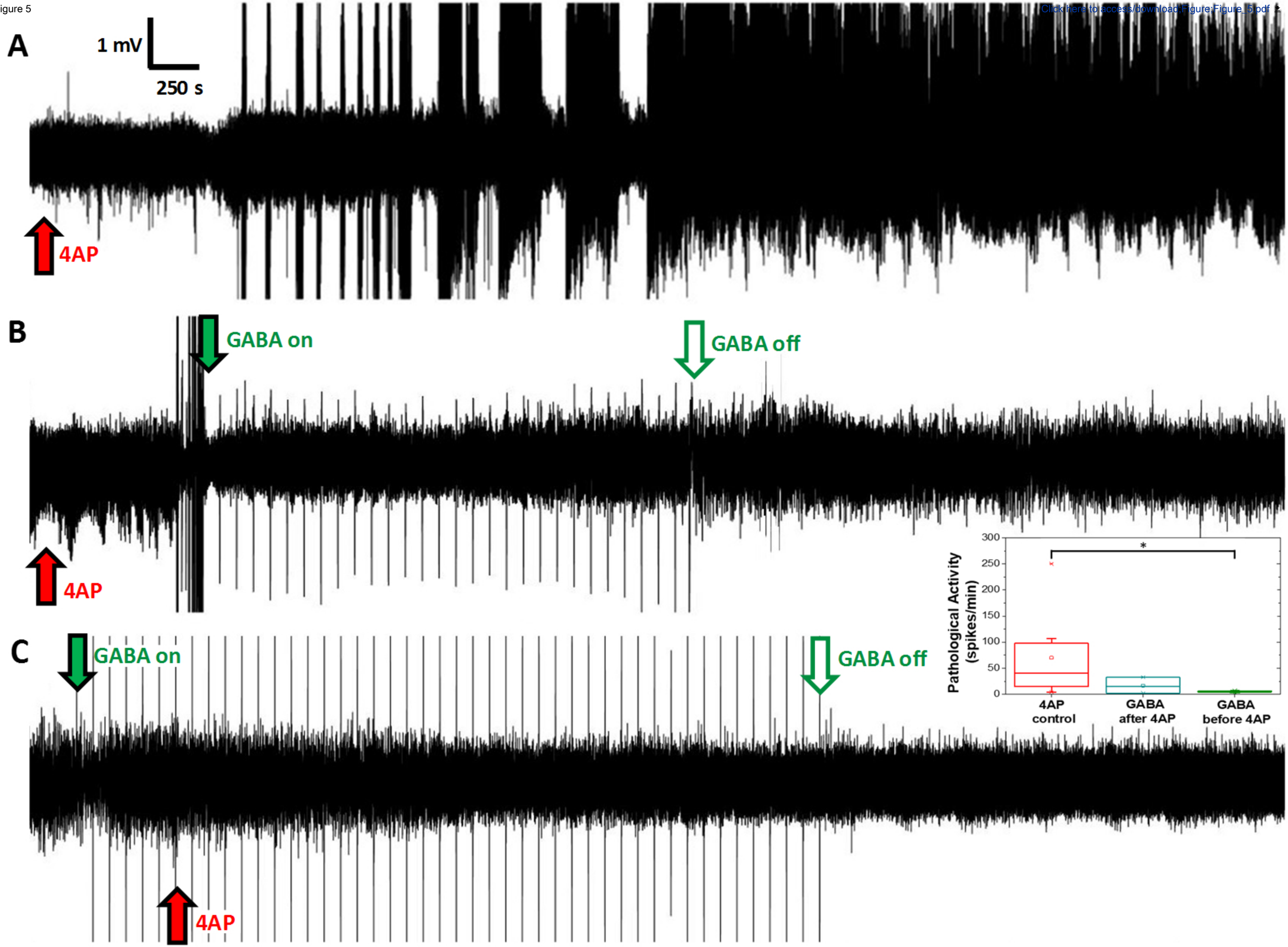

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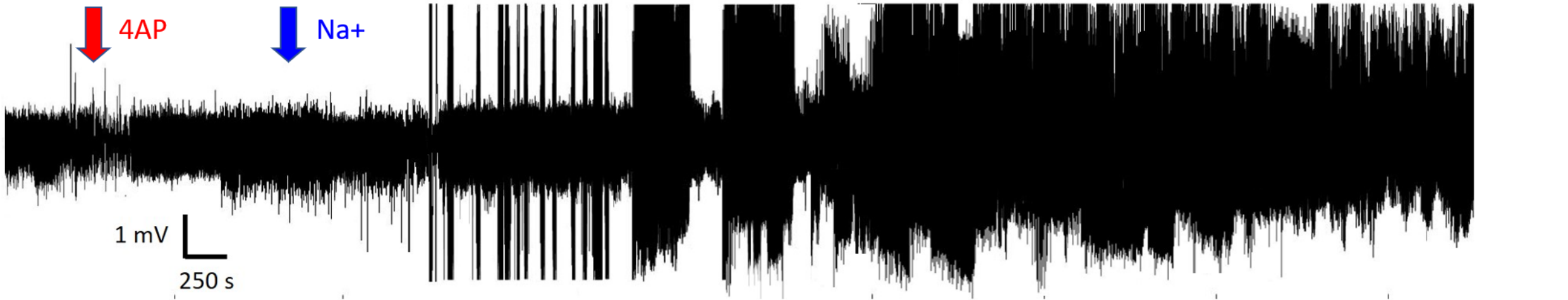

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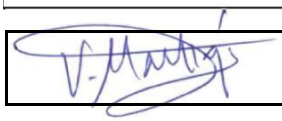
Signature:	
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To: Phillip Steindel ,Ph.D

Science Editor JoVE

MS#: ADr. Williamson-932

Authors: Slézia et al.

Dear Dr. Phillip Steindel 10. December 2018

Enclosed is the revised manuscript "Electrophoretic delivery of GABA into epileptic focus prevents epileptic seizures in mice" which we are submitting for consideration to JoVE as an invited manuscript.

Please find our responses to the editorial comments and reviewer remarks and questions below.

Sincerely yours,

Adam Williamson PhD, Aix Marseille Université , INS, UMR_S 1106, Marseille, France

and

George G. Malliaras, PhD, University of Cambridge, Cambridge CB3 0FA , Cambridge, UK

Editorial comments:

The authors thank for all the observations, corrections and suggestions of the editors.

Find our responses to the editor and reviewer comments in italic.

Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We carefully checked the manuscript to minimize the number of spelling and grammar issues.

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We asked explicit copyright permission for the reused materials and are waiting for the response. We linked the editorial policy and appropriately cited figures in the text.

4. Please revise lines 54-58 and 60-61 to avoid previously published text.

We revised lines 54-58 and 60-61 and changed the text:

"Epileptic seizures occur when there is an imbalance within excitatory and inhibitory circuits either throughout the brain - generalized epilepsy - or in a localized part of the brain - focal epilepsy - , such that neurons discharge in an abnormal fashion 10-12. Antiepileptic drugs can act in two different ways in seizure-prevention: either decrease excitation or enhance inhibition. Specifically, they can either modify electrical activity of neuronal cells by affecting ion channels in the cell membrane 14 or act on chemical transmission between neurons by affecting either the inhibitory neurotransmitter GABA or the excitatory glutamate in the synapses 15,16"

5. Figure 6: Please describe the different panels of the figure in the figure legend.

We described the different panels of Figure 6.

6. Please provide an email address for each author.

We provided email addresses for each author.

7. Please define all abbreviations before use.

We defined all abbreviations we used.

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and

Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Vaseline, Hamilton, SuperFrost, VECTASHIELD, etc.

We used generic terms instead of commercial language in all cases and added (see table of materials) into the text.

9. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

We added more details in protocol steps. In some cases, we added references which specify how to perform the action.

10. 1.2: Please mention how proper anesthetization is confirmed.

We added details to confirm that:

“Check the level of anesthesia by observing the respiratory rate, whisking and by checking response to pain. Note: When the breathing becomes regular, no whisking can be observed and the animal does not react to tail pinch, the anesthesia is deep enough to continue.”

11. 2.6, 2.8: What is the diameter of the hole?

We have added this information to the text:

“Drill an approximately 500 micrometer(µm) diameter hole on the skull for craniotomy, then remove gently the dura mater for duratomy with a fine forceps.”

12. 5.1: Please provide advice on the exact volume of 4AP to draw.

We added the recommended volume of 4AP in the text:

“Redraw 500 nl to 1 µl of 50 mM 4AP with the help of an automated injection pump”

13. 7.1: At what temperature is the perfusion done?

We suggested the temperature for the perfusion:

“Perfuse the mouse transcardially first with saline, then with 150 ml of ice-cold fixative solution containing 4% PFA in 0.1 M phosphate buffer (PB).”

14. 7.2: Please specify all surgical equipment used. Please specify the region of interest where a tissue block is cut and describe how.

We added the names of surgical equipment and specified the region of interest:

“Remove the brain, then cut a tissue block with the help of a brain matrix (see list of materials) from the region of interest (from the Bregma point AP: -1 mm till AP: -3 mm).”

15. 7.5: Please describe the histological protocol or provide a relevant reference.

We provided a relevant reference for the histological protocol used.

16. 9.2: Please specify the maximal and minimal excitation and detection parameters used.

We specified the maximal and the minimal excitation and detection parameters in the text:

“Choose the optimal excitation and emission (exc/ems, nm) filter sets for the dyes as follows: DAPI 358/461, DiI 551/569, fluorescein (see list of materials) 488 490/525. Since staining varies section by section, for each section a proper range for minimal and maximal excitation and detection needs to be determined, where the least dense and most dense regions both show emission.”

“Choose the least dense region and set laser intensity and detection level values to high levels, then verify at most dense regions whether these values cause oversaturation of detected emission. If yes, lower values and re-check with least dense region. Iterate these steps until arriving to highest possible detection at low staining levels and proper, non-oversaturated levels at highly stained areas. Repeat the process for all dyes. “

17. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We have combined the steps where it was possible.

18. Please include single-line spaces between all paragraphs, headings, steps, etc.

We set single-line spaces in the required parts of the text.

19. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We highlighted the required amount of pages in the Protocol.

20. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

We highlighted whole sentences. We did not highlighted steps describing anesthetization and euthanasia.

21. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We included all relevant details that are required to perform the step in the highlighting.

22. Please number the figures in the sequence in which you refer to them in the manuscript text. For figures showing the experimental set-up, please reference them in the Protocol.

We numbered the figures in sequence in the referred text.

23. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

We added modifications, troubleshootings and limitation of technique sentences.

24. References: Please do not abbreviate journal titles.

We changed abbreviations to full titles.

25. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

We sorted materials in alphabetical order.

Reviewers' comments:

We would like to thank the reviewers for careful and thorough reading of this manuscript and for the thoughtful comments and constructive suggestions, which help to improve the quality of this manuscript. Our response follows the reviewer's comments.

Reviewer #1:

The authors thank for all the observations, corrections and suggestions of Reviewer #1.

Manuscript Summary:
Slezia, Proctor, and co-workers present a very useful protocol for implementing their electrophoretic substance delivery tool for epilepsy research. Such drug delivery technologies are often proposed for in vivo application and new therapies, but are not always shown to be actually effective. In this regard, this JoVE submission is a very useful contribution to the field. Furthermore, most demonstrations of such bioelectronic tools do not include the protocol details as presented here. This would like be a great resource for all people working in the field.

I had difficulty in deciding between minor and major revision, but chose to go with major based on the points below.

Major Concerns:
First, the manuscript needs a significant review for the quality of the English and the flow of the writing. While the focus of the manuscript is "visual", the text should still be of the highest possible quality.

We carefully checked the manuscript regarding spelling and grammar issues and improved the quality of English language.

Second, the µFIP and the electrophoretic delivery concept are not sufficiently introduced. For a reader who doesn't already understand the mechanism, it's not at all clear why applying a voltage would cause GABA delivery. Likewise, the mention of "source and target" in step 6.3 are unclear without more introduction. Also, there is little or no mention of the placement of the µFIP relative to the epileptic focus and how this placement could effect the results (similar to how larger-scale 4AP treatment made the µFIP unable to turn off SLEs). Is there passive delivery of GABA when the µFIP is "off"? Does this need to be controlled for? And why is the µFIP better than, for example, an intrathecal pump? How is the µFIP made (or purchased)? I suggest that the authors revise their introductory text to address the µFIP in more detail.

We added a paraghraph into the introductory text regarding µFIP to explain all these points.

"The recently invented µFIP probes work by using an applied electric field to push charged drugs stored in a microfluidic channel across an ion exchange membrane (IEM) and out to the surrounding tissue. The IEM selectively transports only one type of ion (cation or anion) and thus works to limit both passive diffusion in the "off" state and transport of oppositely charged species from the surrounding tissue into the device. The electric field is created on demand by applying a small voltage (< 1 V) between the source electrode which is internal to the

microfluidic channel and a target electrode which is external to the device (in this case the head screw on the animal model). The rate of drug delivery is proportional to the applied voltage and the measured current between the source and target electrodes. The precise tunability of drug delivery is one of the primary advantages of the µFIP. Another critical advantage compared to fluidic or pressure-based drug delivery systems is that in the µFIP there is only negligible pressure increase at the drug delivery outlet as drugs are delivered across the IEM without their carrier solution.

"There is a small amount of passive delivery of GABA when the µFIP is "off", but this was not found to effect seizure like activity. The µFIP were custom made following conventional microfabrication methods that we reported previously.

We added these sentences regarding µFIP placement and epileptic focus:

"The area of influence of the µFIP probe was estimated with a radius of approximately 550 µm from the outlet. It was proven that SLEs could only be affected if the 4AP injection was localized to this area. If the seizure focus or foci were outside of this region, the blockade of SLEs was not possible."

Minor Concerns:
Line 48: "it needs to be set". The "it" is not clear here.

We changed the text: "drug treatment needs to be set".

Line 67: what do the authors mean, specifically, by "connections"?

We changed "conncetions" to " neuronal network connections"

Line 122: this is the first mention of PEDOT:PSS (and GOPS). These crucial materials may need some introduction?

We have added an introduction and the full name of the materials as well.

"PEDOT:PSS is a conjugated polymer with a volumetric capacitance that is known to be biocompatible.

GOPS is a cross linker that is mixed with PEDOT:PSS to increase stability in aqueous media."

Line 210 and following: the use of colons is really confusing here.

We rewrote and restructured this section to avoid confusion.

Line 225: "among". Do the authors mean "between"?

We changed "among" to "between".

Figure 3: "Ctx" has a spell-check squiggle underneath it.

We corrected it.

Figure 6: the scale-bar color (dark purple) is very hard to make out, particularly in the microscope image.

We changed the color of the scale bar in Figure 6.

Reviewer #2:

The authors thank for all the observations, corrections and suggestions of Reviewer #2.

In their manuscript, Slezia and colleagues are reporting on their protocol about the simultaneous microinjection, electrophoretic injection and extracellular electrical recording of neuronal activity in rodent brains. They demonstrate it by inducing and terminating seizure-like activity in the hippocampus of intact, anesthetized mice.

Although automated drug-injection is a routine approach nowadays even in clinical settings, the precise, non-pressure mediated microvolume deposition of pharmaceuticals/chemicals to a given brain regions is not properly established. Microfluidic channels designed in microfabricated electrodes gives a good option to maintain an optimal size/function balance, and the iontophoretic control of substance release allows for the precise, millisecond timing of the intervention.

Conclusively, the demonstration and a step-by-step hands-on tutorial on the use of such a methodology is very timely and will be beneficial for a very broad audience in the field of neuroscience.

Although I like the presented work a lot, given that the target audience will be most likely those people working in neuroscience already, I think the protocol in its current form needs some shift in its focus. My opinion is that the community would profit from this description way much more if it would focus on the details of how to use such a uFIP electrode, as this is not an obvious or standard skill that every neuroscientist would gain during their training. So I suggest to fine-tune the protocol in a way, that in addition to (or instead of) detailing the specifics of the anesthesia, surgery and histology, the emphasis would be put on how to get/handle/fill/use such a uFIP electrode.
I think that if the authors can comply with this request, the result is going to be an extremely useful video-manual on how to perform micro-iontophoresis in rodents.
My specific comments in order of their appearance are:

Long Abstract:
-Please spell out 4-AP at its first appearance.

We corrected this.

-How does the GABA induced overinhibition comply with the possible seizure mechanism based on overinhibition induced rebound bursts?

We added the sentence:

“GABA concentration was kept in physiological range by the precise control of GABA delivery to reach antiepileptic effect in seizure focus but not to cause overinhibition induced rebound bursts.”

Introduction:
-„only neurosurgery can attenuate the occurrence of seizures"
This is only true in the case of seizures with a constant single seizure generator locus, which is at a resecable part of the brain.

We modified the text:

“Worse yet, in 30 % of the cases patients are resistant to medication, and in case of constant single seizure generator locus, only neurosurgery can attenuate the occurrence of seizures.”

-„For some drugs, the mode of action is unknown": Please give examples, references.

We gave a reference.

-Please mention in the Introduction that drugs have a continuous effect and cannot adapt to the prevalence dynamics of the seizures. An optimal treatment would not touch the brain interictally but would act immediately when a seizure starts developing.

We added:

“.Thus, drug treatments have a continuous effect on the patients and cannot adapt to the prevalence dynamics of the seizures.”

“ An optimal treatment would not touch the brain interictally but would act immediately when a seizure starts developing.”

-"…It is difficult to determine if these reorganizations are adaptive responses or whether they are causally related to epileptogenesis or seizure genesis and propagation": This is the exact problem with the primarily generalized seizures: there is no definite seizure onset (epileptogenic) zone. Please discuss that to what extent is the proposed method superior to e.g. DBS which is also only suitable for seizures with a single generator focus (not talking about e.g. thalamic stimulation)

We added:

“Since GABA is an endogenous substrate, it leaves intrinsic neuronal properties unchanged in physiological concentrations. Local application of low levels of GABA will only affect cells naturally responsive to inhibition, and will only cause similar effects to physiological inhibition, contrary to DBS, which has unspecific actions by stimulating all cells of the neuronal network in its environment, causing a mixed response involving both excitation and inhibition. In

conclusion, the proposed method provides a more specific approach to seizure control than deep brain stimulation (DBS). “

-Please add a short introduction on the uFIP probes, by mentioning why are they a superior choice compared to standard pressurized drug injection system, and cite references on their manufacturing, handling, etc.

We added a short introduction:

The recently invented μFIP probes work by using an applied electric field to push charged drugs stored in a microfluidic channel across an ion exchange membrane (IEM) and out to the surrounding tissue. The IEM selectively transports only one type of ion (cation or anion) and thus works to limit both passive diffusion in the “off” state and transport of oppositely charged species from the surrounding tissue into the device. The electric field is created on demand by applying a small voltage (< 1 V) between the source electrode which is internal to the microfluidic channel and a target electrode which is external to the device (in this case the head screw on the animal model). The rate of drug delivery is proportional to the applied voltage and the measured current between the source and target electrodes. The precise tunability of drug delivery is one of the primary advantages of the μFIP. Another critical advantage compared to fluidic or pressure-based drug delivery systems is that in the μFIP there is only negligible pressure increase at the drug delivery outlet as drugs are delivered across the IEM without their carrier solution.

“There is a small amount of passive delivery of GABA when the μFIP is "off", but this was not found to effect seizure like activity. The μFIP were custom made following conventional microfabrication methods that we reported previously.”

Protocol:
-1.2.1: "Place it gently into the rectum": How deep?

We have added the depth as required: “(1-2 cm deep)”

-1.2.2: What concentrations:

The concentration is in the text:

“Inject intraperitoneally a mixture of ketamine and xylazine (100 mg/kg and 10 mg/kg body weight, respectively) to anesthetize the animal.”

-2.6: Give stereotaxic coordinates

The stereotaxic coordinates are given in the text:

“With the help of the stereotaxic frame, measure the stereotaxic coordinates for the desired brain region.; For example, our region of interest was the hippocampus, anteroposterior (AP) -

1.8 mm and mediolateral (ML) 1.8 mm from the Bregma point on the basis of the brain atlas for mice. Note: These are coordinates for the right hemisphere."

-2.8.: Mention that the craniotomy must be filled with a droplet of saline solution to prevent drying. Please also state that the saline droplet must be regularly refilled.

We added the sentence:

"Note: the craniotomy must be filled with a droplet of saline solution to prevent drying, and then regularly refilled during the experiment".

-3.3: State that the probe must be lowered with axial movements relative to the shank, otherwise it brakes.

We added the sentence:

"Note: µFIP is very flexible, it may benefit from the support of a small and clean paint brush to keep it straight until it reaches the brain surfaces. After that step, µFIP can be lowered gently with axial movements."

-4 If these uFIPs are not commercially available refer to a protocol how to prepare them.

The µFIP were custom made following conventional microfabrication methods that we reported previously. These points are discussed in more detail in the referenced work.

-4.1. How to perform filling?

The uFIP probes are filled with drug solution using standard fluidic tubing and connectors (seen in Fig 6b).

-5. Might worth to introduce how to attach a glass micropipette to the Hamilton Syringe, as this is a delicate step which needs some good manuality and tweaking

We added the sentences:

"Change the metal needle of the syringe (see materials). Remove the needle holding metal part, place and fix the micropipette, then replace the needle holding element."

-6. Better to use the word insertion instead of implantation.

We changed "implantation" to "insertion".

-7. Add step before: "Remove the inserted probes, overanesthesize the animal…"

We added the requested step:

“ Gently remove the inserted probes and the headscrew, remove the animal from the stereotaxic equipment. After overanesthesizing the animal with a lethal dose of anaesthetics, the mouse is ready for the perfusion.”

-7.1. Cite some reference for the transcardial perfusion (Is there any JoVE video for this?), as this is not obvious either, and requires a good skillset.

We cited a reference of a JoVE video about transcardial perfusion.

Representative results:
-Should test the injection of vehicle as a control, too, to show that it's not the injection current or volume excess causing the seizure suppression.

We prepared Figure 7 as a vehicle control.

Discussion:
-"mostly these are the patients where conventional drug treatments are inadequate": This seems counterintuitive to me, and I cannot find this statement in the cited reference. Please give a reasoning and support it with solid evidence from robust studies. This gets back to the same problem I mention in my remark regarding the long abstract.

We thank the comment of the Reviewer. We removed the sentence from the discussion.

Reviewer #3:

The authors thank for all the observations, corrections and suggestions of Reviewer #3.

Andrea Slezia and co-authors present an electrophoretic GABA delivery system into epileptic focus that can prevent epileptic seizures in mouse. In the presented protocol, which acutely induces seizure in a localized spot in the hippocampus, the neural activity was recorded with incorporated electrodes and multichannel silicon probe and 4AP-induced seizure was successfully controlled in the epileptic focus by the microfluidic ion pump (μFIP) filled with GABA solution. Additionally, the authors show histological data to evaluate the implantation placement of three different implants (silicon probe, μFIP, and micropipette). In my opinion, as its characterizations were well demonstrated, this manuscript is sufficient to be published in Journal of Visualized Experiments after minor revision.

1. In the manuscript Page 6, line 165 (6.3), although the authors stated that the μFIP is filled with 0.05 M GABA solution, there is no mention about the dosage of GABA release (GABA volume) to prevent epileptic seizure in mouse.

We added the dosage of GABA in the text.

As we previously reported, we delivered 1 nmol of GABA in the seizure prevention experiments.

We added the sentence:

“Note: The delivered GABA concentration is around 1 nmol.”

And I wonder that if GABA more than necessary is released in the epileptic focus, is there any side effect to their body and brain?

We added the sentence:

“GABA concentration was kept in physiological range by the precise control of GABA delivery to reach antiepileptic effect in seizure focus but not to cause overinhibition induced rebound bursts.”

2. The authors mentioned that two devices (μFIP; mediolateral, dorsoventral coordinate: 1200 μm and micropipette with Hamilton syringe; lateromedial, dorsoventral coordinate: 1500 μm) were implanted into specific hippocampus region. Then, there is a difference of 300 μm at the dorsoventral coordinate. What's the general distance between μFIP outlet and pipette tip? In my opinion, the release of GABA closer to the pipette tip is likely to affect the epilepsy focus more directly.

As we previously reported, we estimate the distance between the μFIP outlet and pipette tip within a few hundred microns (< 300 μm).

In our experiments we tried to put the three devices as close as possible, considering this 300 μm distance of outlet from the μFIP tip. On the other hand, it was necessary to avoid any mechanical issues among the devices and their connectors during insertion. In addition, mechanical issues on the neural tissue regarding brain movements in the consequence of breathing, blood pressure changes during maintenance of anesthesia, etc. were also necessary to avoid. We chose the closest placing possible of our devices for the closest GABA release site relative to epileptic focus.

And I recommend that fully-implanted mouse photographs are attached on figure set to easily understand.

Unfortunately, we don’t have any publish-quality photos but we can take these photos during the the filming and can add later to the manuscript if it is possible.

3. Please add the full name. e.g. PEDOT: PSS, GOPS and ACSF.

We added the full name of the materials.

4. Please check if it's a typing error;
Page 4, line 113, '2.1' instead of '2.2'

We corrected numbering.

Page 7, line 213 (Fig. 1) and 219 (Fig. 2), 'reference # 31' instead of '#32'

We changed references and in Fig1 and Fig2.