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 find1,2. Additionally, most of the medication has serious side effects that reduce the quality of life3–7. 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 seizures8. 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 fashion9–11. Antiepileptic drugs can act in two different ways in seizure prevention: either decreasing excitation or enhancing inhibition12. Specifically, they can either modify the electrical activity of neuronal cells by affecting ion channels in the cell membrane13 or act on chemical transmission between neurons by affecting the inhibitory neurotransmitter GABA or the excitatory glutamate in the synapses14,15. For some medications, the mode of action is unknown18. 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 patient9.

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 connections19–21. 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 seizures20–23. However, it is difficult to determine if these reorganizations are adaptive responses or whether they are causally related to epileptogenesis or seizure genesis and propagation12.

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 brain24–27. To this end, we developed a new experimental protocol using the 4AP-induced epilepsy model28–31 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 previously31.

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 mice32.

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 bone33. 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) staining31.

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 NonCommercial License 4.0 (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), respectively31. 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 treatment31.

**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 area31.

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 point35. 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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