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Microdialysis of Excitatory Amino Acids During EEG Recordings in Freely Moving Rats

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*Direttore: Prof**. Dario Di Luca***

July 13, 2018

Dr. Vineeta Bajaj
Editorial Manager

I am submitting the revised manuscript entitled "Microdialysis of excitatory amino acids during EEG recordings and their liquid chromatography determination" (JoVE58455R1). All authors have approved the final manuscript.

Following the indications that you provided in your email dated July 2, 2018, we have made text changes (highlighted in yellow) and enclose to the submission a detailed point-by-point response to the Editorial comments.

Sincerely,

Marie Soukupova, Ph.D.
Department of Medical Sciences, Section of Pharmacology
University of Ferrara, Italy

TITLE:

Microdialysis of Excitatory Amino Acids During EEG Recordings in Freely Moving Rats

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KEYWORDS:

microdialysis, glutamate, aspartate, epilepsy, pilocarpine model, stereotaxic surgery, potassium stimulation, electroencephalography, liquid chromatography

SHORT ABSTRACT:

Here, we describe a method for *in vivo* microdialysis to analyze aspartate and glutamate release in the ventral hippocampus of epileptic and non-epileptic rats, in combination with EEG recordings. Extracellular concentrations of aspartate and glutamate may be correlated with the different phases of the disease.

LONG ABSTRACT:

Microdialysis is a well-established neuroscience technique that correlates the changes of neurologically active substances diffusing into the brain interstitial space with the behavior and/or with the specific outcome of a pathology (*e.g.,* seizures for epilepsy). When studying epilepsy, the microdialysis technique is often combined with short-term or even long-term video-electroencephalography (EEG) monitoring to assess spontaneous seizure frequency, severity, progression and clustering. The combined microdialysis-EEG is based on the use of several methods and instruments. Here, we performed *in vivo* microdialysis and continuous video-EEG recording to monitor glutamate and aspartate outflow over time, in different phases of the natural history of epilepsy in a rat model. This combined approach allows the pairing of changes in the neurotransmitter release with specific stages of the disease development and progression. The amino acid concentration in the dialysate was determined by liquid chromatography. Here, we describe the methods and outline the principal precautionary measures one should take during *in vivo* microdialysis-EEG, with particular attention to the stereotaxic surgery, basal and high potassium stimulation during microdialysis, depth electrode EEG recording and high-performance liquid chromatography

analysis of aspartate and glutamate in the dialysate. This approach may be adapted to test a variety of drug or disease induced changes of the physiological concentrations of aspartate and glutamate in the brain. Depending on the availability of an appropriate analytical assay, it may be further used to test different soluble molecules when employing EEG recording at the same time.

INTRODUCTION:

To provide insight into the functional impairment of glutamate-mediated excitatory and GABAergic inhibitory neurotransmission resulting in spontaneous seizures in temporal lobe epilepsy (TLE), we systematically monitored extracellular concentrations of GABA[1] and later the levels of glutamate and aspartate[2] by microdialysis in the ventral hippocampus of rats at various time-points of the disease natural course, *i.e.*, during development and progression of epilepsy. We took advantage of the TLE pilocarpine model in rats, which mimics the disease very accurately in terms of behavioral, electrophysiological and histopathological changes[3,4] and we correlated the dialysate concentration of amino acids to its different phases: the acute phase after the epileptogenic insult, the latency phase, the time of the first spontaneous seizure and the chronic phass[5-7]. Framing the disease phases was enabled by long-term video-EEG monitoring and the precise EEG and clinical characterization of spontaneous seizures. Application of the microdialysis technique associated with long-term video-EEG monitoring allowed us to propose mechanistic hypotheses for TLE neuropathology. In summary, the technique described in this manuscript allows the pairing of neurochemical alterations within a defined brain area with the development and progression of epilepsy in an animal model.

Paired devices, made up of a depth electrode juxtaposed to a microdialysis cannula, are often employed in epilepsy research studies where changes in neurotransmitters, their metabolites, or energy substrates should be correlated to neuronal activity. In the vast majority of cases, it is used in freely behaving animals, but it can be also conducted in a similar way in human beings, *e.g.*, in pharmaco-resistant epileptic patients undergoing depth electrode investigation prior to surgery[8]. Both EEG recording, and dialysate collection may be performed separately (*e.g.*, implanting the electrode in one hemisphere and the microdialysis probe in the other hemisphere or even performing the microdialysis in one group of animals while performing the sole EEG in another group of animals). However, coupling the electrodes to probes may have multiple advantages: it simplifies stereotaxic surgery, limits tissue damage to only one hemisphere (while leaving the other, intact, as a control for histological studies), and homogenizes the results as these are obtained from the same brain region and the same animal.

On the other hand, the preparation of the coupled microdialysis probe-electrode device requires skills and time if it is home-made. One could spend relatively high amounts of money if purchased from the market. Moreover, when microdialysis probes (probe tips are typically 200-400 µm in diameter and 7-12 mm long)[9], and EEG electrodes (electrode tips are usually of 300-500 µm in diameter, and long enough to reach the brain structure of interest[10]) are coupled, the mounted device represents a bulky and relatively heavy object on one side of the head, which is troublesome for animals and prone to be lost especially when it is connected to the dialysis pump and the hard-wire EEG recording system. This aspect is more relevant in epileptic animals that are difficult to handle and less adaptive to the microdialysis sessions. Proper surgical techniques and appropriate post-operative care can result in long-lasting

implants that cause minimal animal discomfort and should be pursued for combinatory microdialysis-EEG experiments[10-12].

The advantages and limitations of the microdialysis technique have been reviewed in detail by many neuroscientists. Its primary advantage over other *in vivo* perfusion techniques (*e.g.*, fast flow push-pull or cortical cup perfusion) is a small diameter of the probe which covers a relatively precise area of interest[13-15]. Second, the microdialysis membrane creates a physical barrier between the tissue and the perfusate; therefore, high-molecular weight substances do not cross and do not interfere with the analysis[16-17]. Moreover, the tissue is protected from the turbulent flow of the perfusate[18]. Another important advantage is the possibility to modify the perfusate flow for maximizing the analyte concentration in the perfusate (*i.e.*, the process of microdialysis can be well defined mathematically and can be modified to yield high concentrations of the analyte in the sample)[19]. Finally, the technique may be used to infuse the drugs or pharmacologically active substances into the tissue of interest and to determine their effect at the site of intervention[20]. On the other hand, microdialysis has a limited resolution time (typically more than 1 min due to the time needed for collecting samples) in comparison to electrochemical or biological sensors; it is an invasive technique that causes tissue damage; it compromises the neurochemical balance within the space around the membrane due to the continuous concentration gradient of all soluble substances which enters the perfusate together with the analyte of interest. Finally, the microdialysis technique is highly influenced by the limits of the analytical techniques employed for the quantification of substances in the perfusate[9,21-23]. The high-performance liquid chromatography (HPLC) after derivatization with orthophthaldialdehyde for glutamate and aspartate analysis in biological samples has been well validated[24-27] and its extensive discussion is out of the scope of this manuscript, but the data produced by using this method will be described in detail.

When performed properly and without modifications of the perfusate composition, microdialysis can provide reliable information about the basal levels of neurotransmitter release. The largest portion of the basal levels is likely the result of the transmitter spillover from the synapses[9]. Because in many instances the simple sampling of the neurotransmitter in the extra synaptic space is not sufficient to pursue the goals of an investigation, the microdialysis technique can be also employed to stimulate neurons or to deprive them of important physiological ions such as K^+ or Ca^{2+}, in order to evoke or prevent the release of the neurotransmitter.

High K^+ stimulation is often used in neurobiology to stimulate neuronal activity not only in awake animals but also in primary and organotypic cultures. The exposure of a healthy central nervous system to solutions with high concentrations of K^+ (40-100 mM) evokes the efflux of neurotransmitters[28]. This ability of neurons to provide an additional release in response to high K^+ may be compromised in epileptic animals [1] and in other neurodegenerative diseases[29,30]. Similarly, the Ca^{2+} deprivation (obtained by perfusing Ca^{2+} free solutions) is used to establish calcium-dependent release of most neurotransmitters measured by microdialysis. It is generally believed that Ca^{2+} dependent release is of neuronal origin, whereas Ca^{2+} independent release originates from glia, but many studies raised controversy over the meaning of Ca^{2+}-sensitive measurements of e.g. glutamate or GABA[9]: thus, if possible, it is advisable to support microdialysis studies with microsensor studies, as these latter have higher spatial resolution and the electrodes allows to get closer to synapses[31].

Regarding microdialysis studies in epileptic animals, it is important to stress that the data obtained from most of them rely upon video or video-EEG monitoring of seizures, *i.e.*, of the transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain[32]. There are some specifics of electrographic seizures in pilocarpine treated animals which should be considered when preparing the experiment. Spontaneous seizures are followed by depressed activity with frequent EEG interictal spikes[3] and occur in clusters[33,34]. Sham operated non-epileptic animals may exhibit seizure-like activity[35] and therefore the parameters for EEG recordings evaluation should be standardized[36] and, if possible, the timing of microdialysis sessions should be well defined. Finally, we highly recommend following the principles and methodological standards for video-EEG monitoring in control adult rodents outlined by experts of International League Against Epilepsy and American Epilepsy Society in their very recent reports[37,38].

Here, we describe microdialysis of glutamate and aspartate in parallel with the long-term video-EEG recordings in epileptic animals and their analysis in the dialysate by HPLC. We will emphasize the critical steps of the protocol that one should take care of for best result.

PROTOCOL:

All experimental procedures have been approved by the University of Ferrara Institutional Animal Care and Use Committee and by the Italian Ministry of Health (authorization: D.M. 246/2012-B) in accordance with guidelines outlined in the European Communities Council Directive of 24 November 1986 (86/609/EEC). This protocol is specifically adjusted for glutamate and aspartate determination in rat brain dialysates obtained under EEG control of microdialysis sessions in epileptic and non-epileptic rats. Many of the materials described here may be easily replaced with those that one uses in his laboratory for EEG recordings or microdialysis.

1. Assembly of the Microdialysis Probe-electrode Device

1.1. Use a 3-channel two-twisted electrode (with at least a 20 mm cut length of the registering electrode and a 10 cm long grounding electrode) and couple it to a guide cannula to prepare the device. See examples of 3-channel electrode and guide cannula for dialysis in **Figures 1A-1B.**

1.2. Remove (**Figure 1C**) and insert (**Figure 1D**) the metal guide cannula into the dummy plastic cannula a few times prior to the usage in order to ease its removal at the moment of its switch for microdialysis probe in animal.

1.3. Bend the twisted wires of registering electrode two times (**Figures 1E-1F**) in order to align the wires with the dummy cannula of the guide and cut the electrode tip (**Figure 1G**) to be 0.5 mm longer than the tip of the guide cannula (**Figure 1H**) using the digital caliper.

1.4. Have ready the 1 mm long silicon circlet (O.D. 2 mm, thickness 0.3 mm; **Figure 1I**) and insert the tip of the guide cannula and the tip of the twisted electrodes into the silicon circlet using the tweezers (**Figure 1J**). Fix it onto the foot of the guide cannula pedestal with polymer

glue of rapid action or resin (**Figure 1K**). See the example of the completed devices in **Figure 1L and Figure 2A**.

1.5. Sterilize the device under germicidal UV light for 4 h. Turn over the device four times so as each of its sides is exposed to the light for 1 h.

Note: Many home-made electrodes and microdialysis probes may be assembled in a similar way. The head of above described implant for rats has the following dimensions: 7 mm width x 5 mm depth x 10 mm length from the top to pedestal toe; the implant tip is about 11 mm long, 600 µm in diameter and all the device weights about 330-360 mg. The device may be reused two or three times if (i) a sufficient length of the ground electrode is left on the skull during the surgery for the next use and (ii) when the animal is killed, and the device recovered together with the dental cement it is left in acetone overnight, such that the cement may be mechanically disaggregated, and the device washed and sterilized again.

2. Stereotaxic Surgery

2.1. Use a stereotaxic apparatus and probe clip holder (**Figure 2B**) for the device implantation following the contemporary standards for aseptic and pain-free surgeries[39-41].

2.1.1. Anesthetize the adult Sprague-Dawley rats with ketamine/xylazine mixture (43 mg/kg and 7 mg/kg, i.p.) and fix it onto the stereotaxic frame. Add isoflurane anesthesia (1.4% in air; 1.2 mL/min) to initial ketamine/xylazine injection as it allows to control the depth of anesthesia in time. Shave the fur on animal's head.

2.2. Swab the head skin surface by iodine-based solution followed by 70% ethanol to prepare it for aseptic surgery[39].

2.2.1. Implant the guide cannula-electrode device prepared in precedence (1.1 – 1.5) into the right ventral hippocampus using the following coordinates: nose bar + 5.0 mm, A – 3.4 mm, L + 4.5 mm, P + 6.5 mm to bregma[1,2]. Follow standard techniques for stereotaxic surgeries[10-12].

2.2.2. Ensure that it does not cover the anchoring screws. When mounting it onto the stereotaxic apparatus, grasp the device for the guide cannula head as this may be easily aligned to the probe holder.

2.3. Anchor the device to the skull with at least four stainless screws screwing them into the skull bone (1 screw into the left and 1 screw into the right frontal bone plates, 1 screw into the left parietal and 1 screw into the interparietal bone plates). Add a drop of tissue glue to further fix each screw to the skull bone.

2.3.1. Cover half of screw threads with methacrylic cement. Promote the binding of the cement by making shallow grooves in the bone to increase the adherence.

2.4. Once the tip of the device is positioned into the brain tissue, twist the wire of the ground electrode around 3 anchoring screws. Cover all mounted screws and the device with the dental cement[12,42,43].

2.5. Monitor the animals during the surgery and for about 1 h thereafter until upright and moving around the cage. Keep them on a warming pad to avoid hypothermia. Allow the rats to recover for at least 7 days after the device implantation.

2.6. Monitor the animals at least once daily for 3 days after the surgery for signs of pain or distress. Give the animals with the antibiotic cream (gentamycin 0.1%) close to the incised site to prevent the infection and an analgesic (tramadol 5 mg/kg, i.p.) for 3 days to prevent the post-surgical pain.

3. Temporal Lobe Epilepsy Induction by Pilocarpine and Assignment of Animals to Experimental Groups

3.1. After a week of post-surgical recovery, assign the animals randomly to groups: (i) control animals receiving vehicle and (ii) epileptic animals that will receive pilocarpine. Use a proportionally higher number of animals for epileptic group since not all of the pilocarpine administered rats will develop the disease.

3.2. Inject a dose of methylscopolamine (1 mg/kg, s.c.) and 30 min after, a single injection of pilocarpine (350 mg/kg, i.p.) to induce the status epilepticus (SE). Inject methylscopolamine and the vehicle (saline) to the control rats. Use 1 mL syringe with 25G needle for all i.p. administrations.

3.2.1. Check visually the animals to start to have behavioral seizures (moving vibrissa within 5 min, nodding head, cloning the limbs) and within 25 min to clone continuously all the body (SE).

3.3. Arrest the SE 2 h after the onset to have a mortality about 25% and a mean latent period of approximately 10 days by administration of diazepam (20 mg/kg, i.p.). Observe and record any seizure behavior beginning immediately after the pilocarpine injection and continue for at least 6 h thereafter.

3.4. Give the animals saline (1 mL, i.p.) using 1 mL syringe with 25G needle and sucrose solution (1 mL, p.o.) using 1 mL syringe and flexible feeding 17G needle for 2-3 days after SE to promote the recovery of body weight loss.

3.4.1. Exclude the animals that do not achieve the initial body weight within the first week after pilocarpine SE from the study (except for the acute group killed 24 h after SE, where the body weight follow up is not possible).

3.5. Assign post-SE animals randomly to different experimental groups (**Figure 3**): acute phase (where the microdialysis takes place 24 h after SE), latency (7-9 days after SE), first spontaneous seizure (approximately 11 days after SE), and chronic period (starts about 22-24 days after SE, i.e. about 10 days after the first seizure). Monitor the animals for the occurrence of spontaneous seizures.

Note: Use the following inclusion/exclusion criteria for further experiments in epileptic rats:

development of convulsive SE within 1 h after pilocarpine administration; weight gain in the first week after SE and the correct positioning of the microdialysis probe and electrode.

4. Epileptic Behavior Monitoring and Analysis

4.1. Long-term monitoring of epileptic behavior

4.1.1. Approximately 6 h after pilocarpine administration (*i.e.*, at the end of direct observation by the researchers), place the animals into the clean home cages and start the 24 h video monitoring.

4.1.2. Continue the 24 h video monitoring until day 5, using a digital video surveillance system.

4.1.3. Beginning at day 5, connect the rats in their home rectangular cages to tethered EEG recording system and continue the 24 h video monitoring.

4.1.4. Set the parameters on the amplifier positioned outside of the Faraday cage (set amplification factor on each channel according to the specificity of the EEG signal of each single animal) and start the EEG acquisition observing the EEG signal produced by unconnected cables. Use sampling rate 200 Hz and low pass filter set to 0.5 Hz.

4.1.5. Connect the animal to cables holding an animal's head between two stretched fingers of one hand and screwing down the connectors to the electrode pedestal using the other free hand. Start the acquisition.

Caution: Ensure that the signal is free of artifacts. Common artifacts are the spikes greatly exceeding the scale.

4.1.6. A day before the microdialysis experiment, transfer the animals into the tethered EEG system equipped with plexiglass cylinders for microdialysis. Disconnect the animals from the EEG tethering system in home cage screwing up the connectors from the electrode without restrain the animal. Place the animal into the high plexiglass cylinders.

4.2. Monitoring of epileptic behavior a day before and during the microdialysis session

4.2.1. Switch on the amplifier positioned outside of the Faraday cage. Open the EEG software. Start the EEG acquisition observing the EEG signal produced by unconnected cables.

4.2.2. Connect the animal to the tethered EEG recording system holding an animal's head between two stretched fingers of one hand and screwing down the connectors to the electrode pedestal using the other free hand. Set an amplification factor (gain) on each channel of amplifier according to electrode signal of single animal so the EEG signal is in scale. Let the animal explore the new cage (cylinder) for at least 1 h under the direct observation of the researcher.

Note: 24 h before the microdialysis experiment, the rats are briefly anesthetized with

isoflurane for the switch of guide cannulas to microdialysis probe. Take advantage of the moment when they are anesthetized to connect them to the EEG recording system.

4.2.3. Shorten or prolong pendulous cable according to animal's commodity. Make sure that the cables do not interfere with animal's movements and lying posture.

4.2.4. Check for the correct image framing of the video cameras. Start the video-EEG recording.

4.3. Identification of seizures and EEG activity

4.3.1. Use a software player to watch the videos. Scroll the movie 8 times faster than the real time playing and individuate the generalized seizures (see animal to rear with forelimb clonus or animal rearing and falling with forelimb clonus). Slow down the video and note the precise time of the beginning and of the end of the behavioral seizure.

4.3.2. Process the data by counting the number of generalized seizures observed in 24 h of video records and express them in terms of seizure frequency and duration as mean values of all seizures observed in 24 h.

4.3.3. Define the EEG seizures as the periods of paroxysmal activity of high frequency (>5 Hz) characterized by a >3-fold amplitude increment over baseline with progression of the spike frequency that lasts for a minimum of 3 s[2,44] or similar[28]. Use EEG software to process the raw EEG recordings. Split the EEG traces into 1 h fractions. Copy the EEG tracing fractions to file for software automatic spike analysis.

4.3.4. Analyze the EEG activity data using EEG software and predefined parameters (4.3.3.). Conduct all video analyses in two independent investigators who are blind for the group of analyzed animals. In case of divergence, make them re-examine the data together to reach a consensus [45].

5. Microdialysis

5.1. *In vitro* probe recovery

5.1.1. Prepare the probe for its first use according to the manufacturer's instructions, handling it in its protective sleeve.

5.1.2. Run the experiment in triplicate: prepare three 1.5 mL test tubes loading them with 1 mL of Ringer's solution containing the mixture of standards (2.5 µM of glutamate and 2.5 µM of aspartate). Put three loaded 1.5 mL test tubes into the block heater set to 37 °C and position it on the stirrer.

Note: Use the same standard solutions for chromatography calibrations.

5.1.3. Seal the 1.5 mL test tubes with paraffin film and puncture it by sharp tweezers to make a hole of about 1 mm in diameter.

5.1.4. Take the probe and insert it into the hole made in paraffin film. Immerse the membrane at least 2 mm under the solution level. Fix further the probe to 1.5 mL test tube by paraffin film.

Caution: Ensure that the tip does not touch the walls of the 1.5 mL test tube.

5.1.5. Connect the probe inlet to the syringe mounted on the infusion pump using FEP-tubing and tubing adapters. Optionally, use fine bore polythene tubing of 0.28 mm ID and 0.61 mm OD and colored tubing adapters (red and blue tubing adapters) for connections.

5.1.6. Start the pump at 2 µL/min and let the fluid appear at the outlet tip. Connect the probe outlet to the 0.2 mL collecting test tube using FEP-tubing and tubing adapters.

Note: Use FEP-tubing for all connections. Cut the desired length of tubing by using a razor blade. Use tubing adapters of different color for inlet and outlet of the probe. Let the pump run for 60 min. Check for leaks and air bubbles. These should not be present.

5.1.7. Set the pump to the flow rate 2 µL/min and start to collect the samples on the outlet side of the tubing.

5.1.8. Collect three 30 min perfusate samples and three equal volume samples of the solution in the 1.5 mL test tube. Take the equal volume samples from the 1.5 mL test tube every 30 min using the microsyringe immersed into the standard solution in the 1.5 mL test tube.

5.1.9. Repeat the experiment (5.1.1-5.1.8) setting up the pump to the flow rate 3 µL/min (5.1.7) to have a probe recovery comparison when using two different perfusion flow rates.

5.1.10. When finished, stop the pump and rinse the tubing with distilled water, 70% ethanol and push the air into it. Store the probe in a vial filled with clean distilled water. Rinse the probe thoroughly by perfusing it at 2 µL/min by distilled water prior the storage.

5.1.11. Analyze the concentration of the glutamate and aspartate in the samples by chromatography (see the details below; 6.3).

5.1.12. Calculate the recovery using the following equation:

Recovery (%) = ($C_{perfusate}$ /$C_{dialysed\ solution}$) x 100.

5.2. Microdialysis sessions in freely moving rats

5.2.1. Preparative procedures: probe insertion and testing, infusion pump setting and start

5.2.1.1. Prepare the microdialysis probes for the first use according to the manufacturer user's guide and fill them with Ringer's solution. Cut about 10 cm long pieces of FEP-tubing and connect them to inlet and outlet cannulas of the probe using the tubing adapters of different colors.

424
425 5.2.1.2. Make sure that the tubing touches the adapters with no dead space in all
426 connections.
427
428 5.2.1.3. 24 h before the microdialysis experiment, anesthetize briefly the animal with
429 isoflurane (5% in air) in an induction chamber until recumbent. Remove the dummy cannula
430 from its guide using the tweezers and holding the animal's head firmly. Insert the microdialysis
431 probe, endowed with a dialyzing membrane, into the guide cannula and firm further the
432 microdialysis cannulas inserted in their guide by modeling clay.
433
434 **Caution:** Do not let the probe touch the walls of the protective probe sleeve when extracting.
435
436 5.2.1.4. Put the animal into the plexiglass cylinder and let it explore the new ambience.
437 Connect the animal to the tethered EEG recording system as described above (follow the
438 points 4.2.2 and 4.2.3).
439
440 5.2.1.5. Follow the awake and freely moving rat movements and connect the inlet of
441 the probe to the 2.5 mL syringe with blunted 22G needle containing Ringer's solution using
442 the tubing adapters. Push Ringer's solution inside the probe ejecting 1 mL of Ringer's solution
443 in 10 s pushing continuously the piston of 2.5 mL syringe. Check for the drop of the liquid
444 appearing on the outlet. The probe is now ready for use.
445
446 5.2.1.6. Fill up the 2.5 mL syringes connected to FEP-tubing by tubing adapters with
447 Ringer's solution and mount them onto the infusion pump. Start the pump at 2 µL/min. Let it
448 run overnight.
449
450 **Note:** Use the desired length of all FEP-tubing but calculate the tubing dead volume to know
451 when the high K^+ stimulation should be started and to correlate the quantification data with
452 neurochemical changes in animal brain. Use the air bubble created in the tubing under the
453 working flow to calculate this time.
454
455 5.2.2. Collection of samples during EEG recording and potassium stimulation
456
457 5.2.2.1. Verify the absence of seizures in the 3 h preceding the onset of sample
458 collection (video-EEG recordings) and continue to monitor seizure activity during
459 microdialysis.
460
461 5.2.2.2. Stop the pump carrying the FEP-tubing cannulated syringes filled up with
462 Ringer's solution. Mount onto the pump another set of 2.5 mL syringes connected to FEP-
463 tubing with tubing adapters filled up with a modified Ringer's solution containing 100 mM K^+
464 solution.
465
466 5.2.2.3. Start the pump at 2 µL/min and let it run. For more rapid filling of the tubing,
467 set the pump at 5 µL/min for the time of filling. Check for the absence of air bubbles in the
468 system. Ensure that the tubing touches the adapters with no dead space in all connections.
469
470 5.2.2.4. Test the probe if ready for use in animal as described above (5.2.1.5).

Note: If for some reason the probe does not work, change it. For these cases, keep a few prepared microdialysis probes ready to use near the microdialysis-EEG workstation. Disconnect the animal from EEG cables and anesthetize it briefly with isoflurane if necessary to realize the change.

5.2.2.5. Connect the FEP-tubing of syringes filled up with Ringer's solution to the inlet cannula of the probe in each animal and wait for the appearance of the liquid drop on the tip of the outlet.

5.2.2.6. Connect the outlet of the probe to the FEP-tubing, which leads to collection in the test tube. Insert the FEP-tubing into the closed 0.2 mL test tube with a perforated cap. Ensure that the tube stays in the place by fixing with a piece of modeling clay.

5.2.2.7. Continue to run the pump at 2 µL/min for 60 min without collecting samples to equilibrate the system (zero sample).

5.2.2.8. Collect 5 consecutive 30 min dialysate samples (60 µL respective volume) under baseline conditions (perfusion with normal Ringer's solution). Store samples on ice.

5.2.2.9. Calculate the time it takes liquid to pass from the pump into the animal's head (it depends on dead volume of tubing, *i.e.*, air bubble time) and switch the FEP-tubing that goes from the syringes containing normal Ringer's solution to syringes containing modified (100 mM K^+) Ringer's solution at this time without stopping the pump. Check for the absence of the air bubbles in the system. Let the pump run for 10 min.

Caution: In 10 min of high K^+ stimulation, the animals tend to move themselves frenetically and usually present a great number of wet dog shakes (control and out of seizure cluster animals) or behavioral seizures (epileptic animals), so be ready to intervene to protect the tubing and cables from twisting.

5.2.2.10. After 10 min, switch the tubing from the syringes containing 100 mM K^+ Ringer's solution to normal Ringer's solution and let the pump run. Do not turn off the pump during the solution changes so that that there will be a drop of the liquid at the end of the tubing to be connected in line.

5.2.2.11. From the moment at which the dialysate contains high potassium, *i.e.*, after collection of the fifth post-equilibration dialysate, collect the dialysate fractions every 10 min (20 µL) for 1 h. Collect 3 additional 30 min dialysate samples and stop the pump. Store the samples on ice.

5.2.2.12. Store the samples at -80 °C after the experiment until HPLC analysis.

5.2.2.13. Repeat the microdialysis experiment for 3 consecutive days, except for the acute (24 h) and first seizure group, in which only one microdialysis session takes place 24 h after SE or within 24 h after the first spontaneous seizure (**Figure 3**).

5.2.2.14. On completion of each experiment, euthanize the animal with an anesthetic overdose and remove the brain for verification of probe and electrode placement.

5.2.3. Post-microdialysis procedures

5.2.3.1. Rinse the used microdialysis probes with distilled water and store them in a vial filled with clean distilled water until next use.

Note: The reused membranes may have increased permeability; check for the probe recovery before its repeated use.

5.2.3.2. Rinse the entire microdialysis set up (tubing, connectors and syringes) with distilled water followed by 70% ethanol. Replace ethanol with air and store the set up in a sterile environment.

5.2.3.3. Split the basal dialysate samples into 20 µL fractions and use only one 20 µL fraction for amino acid basal concentration analysis. Store the remaining sample volume for further or confirmatory analysis at -80 °C.

5.2.3.4. Fix the brains in 10% formalin and preserve them by paraffin-embedding[1]. Coronally section the brains into slices and stain them with hematoxylin and eosin. Examine the brains for correct probe and electrode placement[1,2].

Note: Fix the brains in cooled 2-methylbutane and store them at -80 °C. Use any other proven staining on the nervous tissue sections which permits to visualize the probe and electrode tract.

6. Chromatographic Analysis of Glutamate and Aspartate

6.1. Preparation of derivatizing agent

6.1.1. Mix the respective volumes 20:1 (v/v) of orthophthaldialdehyde reagent (OPA) and 2-mercaptoethanol (5-ME) in the vial. Close the vial using the cap and air tight septum.

Caution: Work under the chemical hood.

6.1.2. Vortex the prepared solution and put it into the autosampler into the position for derivatizing agent.

6.2. Preparation of dialysate samples

6.2.1. Put the glass insert with bottom spring into the 2 mL brown autosampler vial. Prepare the vials for all samples measured in one batch.

6.2.2. Take the 20 µL dialysis samples from -80 °C freezer and let them melt. Remove 1 µL of the solution and add 1 µL of internal standard (IS) L-Homoserine (50 µM) to 19 µL of the sample, thus the sample contains 2.5 µM of IS. Pipette 20 µL of the dialysis sample into the

565 glass insert in vial and seal it with an air tight septum.
566
567 6.2.3. Place the vials containing the samples into the autosampler using the chromatographic
568 software to label the samples in their positions.
569
570 6.3. Chromatographic analysis of samples to determine glutamate and aspartate
571 concentration
572
573 6.3.1. Run the analyses on the liquid chromatograph system with spectrofluorometric
574 detection. Detect the amino acids after 2 min pre-column derivatization with 20 µL of
575 orthophthaldialdehyde/5-mercaptoethanol 20:1 (v/v) added to 20 µL of sample.
576
577 6.3.2. Prepare the system for amino acids analysis. Switch on the autosampler, the pump,
578 the degasser, the detector and controlling unit together with computer.
579
580 6.3.3. Immerse the siphons into the bottles containing the mobile phase and purge the
581 channels of the chromatographic pump to be used for the analysis.
582
583 6.3.4. Start to increase the flow of the mobile phase checking the pressure on the column
584 (*e.g.*, start at 0.2 mL/min and increase the flow for additional 0.2 mL/min every 5 min until
585 achieving the working flow). Let the system run at working flow 0.8 mL/min for at least 1 h to
586 equilibrate the column.
587
588 **Caution:** Unstable pressure indicates the presence of the air in the system. The pressure
589 should not exceed 25 MPa.
590
591 6.3.5. Set the gain, high sensitivity and the excitation and emission wavelengths on the
592 detector to 345/455 nm respectively. Reset the detector signal (AUTOZERO).
593
594 6.3.6. Using the chromatographic software, send the method to the instrument. Now, the
595 chromatograph should be ready to measure.
596
597 6.3.7. Separate the dialysate samples, standard spiked dialysate samples as well as standards
598 (0.25 µM – 2.5 µM aspartate and glutamate in Ringer's solution) on the appropriate
599 chromatographic column. Calibrate the chromatographic method and establish the detection
600 and quantification limits before any dialysis samples analysis.
601
602 6.3.8. Activate the single analysis or create the sequence of the samples to be analyzed using
603 the chromatography software and run the sequence.
604
605 **Caution:** Run more than one blank sample and different standard samples within the
606 sequence of dialysate samples in order to control the method accuracy.
607
608 6.3.9. Once the chromatograms are acquired, analyze them with chromatography software.
609 Check the integration of the peaks of the interest into the calibration plot. Use peak height or
610 peak area for quantification.
611

612 6.3.10. Once the sample recording is finished fill the column and the system with an organic
613 solvent (*e.g.*, 50% acetonitrile in ultrapure water) to prevent its aging and mold growth in it.
614
615 6.3.11. Shut down the system.
616
617 **REPRESENTATIVE RESULTS:**
618
619 **Probe recovery**
620 The mean recovery (*i.e.,* the mean amino acid content in the perfusate as a percentage of the
621 content in an equal volume of the vial solution) was 15.49 ± 0.42% at a flow rate of 2 µL/min
622 and 6.32 ± 0.64 at 3 µL/min for glutamate and 14.89 ± 0.36% at a flow rate of 2 µL/min and
623 10.13 ± 0.51 at 3 µL/min for aspartate when using the cuprophane membrane probe. If using
624 the polyacrylonitrile membrane probe, the mean recovery was 13.67 ± 0.42% at a flow rate of
625 2 µL/min and 6.55 ± 1.07 at 3 µL/min for glutamate and 14.29 ± 0.62% at a flow rate of 2
626 µL/min and 8.49 ± 0.15 at 3 µL/min for aspartate (**Figures 4A-4B**). As it can be clearly seen in
627 **Figure 4A**, the slower flow rate (2 µL/min) enhances the dialyzing performance of both probes.
628 For the following experiments the cuprophane membrane endowed probe perfused at a flow
629 rate 2 µL/min was chosen, because its mean recovery was higher (even if not significantly) at
630 this flow rate for both analytes and because of experimental continuity (these probes were
631 used for analyzing GABA in precedence[1]).
632
633 **Seizures development and progression of the disease after status epilepticus**
634 The behavioral and EEG monitoring of seizures, their evaluation, was done in all the animals
635 employed in this study to confirm the development and progression of TLE disease in these.
636 The robust convulsive SE, that was interrupted after 3 h using diazepam, occurred 25±5 min
637 after the pilocarpine injection. Then, all the animals entered a latency state in which they were
638 apparently well and they were continuously video-EEG monitored in order to verify that no
639 spontaneous seizures occurred in the first 9 days or to identify the first spontaneous seizure,
640 respectively for the latency and the first seizure group. The first spontaneous seizure occurred
641 11.3 ± 0.6 days after SE (mean ± SEM, n=21). Thereafter, seizures occurred in clusters, and
642 aggravated in time. In late chronic phase (days 55-62 after SE) the epileptic rats experienced
643 3.3±1.2 (mean ± SEM, n=12) generalized seizures daily. There was a clear progression of the
644 disease. Many, but not all EEG seizures, corresponded with behavioral seizure activity. **Figure**
645 **5B** shows the recorded paroxysmal epileptiform activity that was observed about 500 ms
646 before and during behavioral seizures. **Figure 5A** shows control traces in non- epileptic rats.
647
648 **Representative basal values of amino acids found in microdialysis perfusate and potassium**
649 **stimulated release of glutamate**
650 Basal glutamate concentration found in chronic epileptic rats (0.87 ± 0.06 µM) was
651 significantly higher than in control animals (0.59 ± 0.03 µM; $p < 0.05$ vs. controls; one-way
652 ANOVA and post hoc Dunnett's test). There was no statistically significant difference between
653 chronic epileptic (0.31 ± 0.04 µM) and non-epileptic animals (0.30 ± 0.05 µM) in basal or high
654 K^+ evoked aspartate concentrations. See the original article for details[2]. The reported basal
655 levels of glutamate in control rats are in line with those found by others in similar studies (*i.e.*,
656 about 0.75 µM when using a 2 µL/min flow and membranes of 2 mm effective length)[46-53].
657 However, many different factors can influence the results of microdialysis, for example the
658 effective length of the probe and the membrane cut off.

High K^+-evoked an additional release of glutamate for about 30 min in control rats and for about 60 min in chronic epileptic rats (**Figure 6**). See the original article for details [2]. As can be seen from the depicted time course, the 10 min time resolution of microdialysis was sufficient to capture the variances in glutamate release found in both groups of animals.

HPLC calibration and limits

The data were calculated based on calibration curves obtained with standard solutions of glutamate and aspartate and the internal standard L-homoserine. The concentration of the neurotransmitters glutamate and aspartate in the perfusates was expressed in absolute values (µmol/L). Each calibration plot was constructed by analysis of solutions of glutamate and aspartate at four concentration levels (five replicates at each level). Regression coefficients were calculated for calibration plots: $y = \mathbf{k}x + \mathbf{q}$, where x was the concentration ratio of aspartate or glutamate to L-homoserine (IS) and y was the corresponding peak-area ratio of aspartate or glutamate to L- homoserine (IS). The coefficient of determination (r^2) was calculated. The applicability of HPLC method was within the limits; the lower limit of quantification was determined as the lowest concentration in the standard calibration curve and the upper limit of quantification as the highest used concentration of amino acid analytes for calibration, respectively. Limit of detection (LOD) was also calculated. Some of these values are delineated in **Table 1**. A model chromatogram of blank sample, standards sample and collected dialysis sample obtained with above described method are shown in **Figure 7**.

Probe localization

Microdialysis probe and recording electrode were implanted into the right ventral hippocampus and their correct placement was verified. Only those animals where the implantation was maximally in 500 µm distant from stabilized coordinates (see **Figure 8**) were included in analysis.

FIGURE AND TABLE LEGENDS:

Figure 1. Step-by-step preparation of the device to be implanted. (A) 3-channel electrode with 10 cm long grounding electrode in its protective sleeve on the left and guide cannula for microdialysis on the right needed to assemble the device. (B). The bare electrode and guide cannula in detail. The first step is to remove (C) and insert (D) the metal guide cannula from and into its plastic dummy few times to ease its release once implanted into the animal's head. The second step is to bend two times the twisted registering electrode to be aligned to dialysis guide cannula (E, F). (G) The electrode tip should be cut to be 0.5 mm longer than the tip of the metal guide cannula. (H) Check for the precision of the cut using the digital caliper. Subsequently, about 1 mm long silicon circlet should be used to fix the alignment of electrode to guide cannula foot (I). (J) The photograph showing how to ring the electrode and guide cannula shaft. The final step is to put a drop of resin or glue onto the guide cannula pedestal fixing the silicon circlet to it (K). (L) Assembled device ready to be sterilized.

Figure 2. Photographs of different types of devices for microdialysis-EEG in rats used (A) and the photograph of the probe clip holder (B) used to implant these devices. (A) The guide cannula (in green) is replaced by a microdialysis cannula typically 24 h before the experiment. The electrical connector of the device (first left was used for the recordings described in this

706 manuscript) permits the attachment of wires that conduct electrical signal to amplifier and
707 data collection equipment. The device is surgically attached to the skull of anesthetized rats
708 and recordings may be obtained later without causing pain or discomfort in freely behaving
709 rats.
710
711 **Figure 3. Experimental design.** The week before status epilepticus (SE) induction, the rats are
712 implanted with the device. SE is induced by pilocarpine and animals (if not dialyzed and killed
713 at 24 h after SE; the rats from acute group) are video monitored for 5 days (blue line), then
714 video-EEG monitored to assess the seizure frequency and duration in their home cages (green
715 line). For the microdialysis experiment, the epileptic and respective non-epileptic control rats
716 are transferred to another EEG set up equipped with cylinder cages in 24 h before the
717 microdialysis session and still video-EEG monitored (light green line). The vertical red lines
718 represent the dialysis sessions at different time-points of epileptic disease development. The
719 horizontal red lines represent the different groups of epileptic animals (and respective non-
720 epileptic animals), where the arrow indicates the last day of the microdialysis and the day of
721 animal's death.
722
723 **Figure 4. *In vitro* recovery of two dialysis probes.** Mean *in vitro* recovery (%) of aspartate and
724 glutamate using two different commercially available microdialysis probes (both endowed
725 with 1 mm long dialyzing membrane) at (A) 2 µL/min and (B) 3 µL/min flow rate. Data are the
726 mean ± SEM of 3 independent experiments run in triplicates. There are not statistically
727 significant differences between the efficiency of various probes (Student's unpaired t-test,
728 $p<0.05$). Using a flow rate 2 µL/min the glutamate recovery increased about 5% compared to
729 3 µL/min flow rate, thus the slower flow rate was used for microdialysis experiments.
730
731 **Figure 5. Illustrative EEG recordings from ventral hippocampus of paraoxystic activities as**
732 **can be seen at chronic phase in control and epileptic rats.** (A) Two representative traces
733 recorded in two saline treated non-epileptic rats. (B) Traces recorded in two epileptic rats.
734 Epileptiform discharges correspond with class 3 behavioral seizures in these rats.
735
736 **Figure 6. Time-course of the effect of potassium stimulation on glutamate release from the**
737 **rat hippocampus.** Representative result of the microdialysis experiment performed in 6
738 control (open circles) and 6 chronic epileptic rats (black circles). The graph shows the temporal
739 changes of dialysate glutamate concentration in the course of microdialysis experiments and
740 during high 100 mM K^+ stimulation. The time of high K^+ stimulus (10 min) is indicated by the
741 black bar on bottom of the graph. The data are the means ± SEM of 6 animals per group.
742
743 **Figure 7. Illustration of chromatograms.** Known peaks are labeled. Pink trace: chromatogram
744 of Ringer's solution without intentionally added amines after OPA/5-ME derivatization (blank
745 sample). Blue trace: chromatogram of dialysate sample after derivatization showing the peaks
746 of amino acids: aspartate (t_R 4.80 min), glutamate (t_R 6.75 min) and glutamine (t_R 9.19 min)
747 and the peak of IS L-homoserine (2.5 µM, retention time, t_R 9.83 min). Red trace:
748 Chromatogram of standard of aspartate (2.5 µM) and glutamate (2.5 µM) in Ringer's solution.
749 Azure and yellow background of the picture stands for mobile phase A (azure) and mobile
750 phase B (yellow) portion used for analytes elution. A red rectangle indicated area (t_R 10.41 min
751 and further) shows the peaks of unknown substances and OPA degradation products. All
752 injection volumes were 20 µL. The derivatives were separated at a flow rate of 0.8 mL/min.

Figure 8. Representative image of combined electrode-probe placement within the ventral hippocampus. (A) Photograph shows the scare left by the device tip in detail (black arrow). (B) Schematic illustration of the electrode-probe tip positions within the implanted ventral hippocampus of 12 rats. The solid squares (some overlapping) indicate correctly localized probe-electrode tips. Open squares indicate incorrectly localized probe-electrode tips in animals excluded from the study (n=3). Coronal brain slices containing probes and recording sites were processed after experiments for histological analysis. The numbers above the illustration show the distance from Bregma (according to Pellegrino *et al.* 1979 atlas of rat brain; nose bar + 5.0 mm, co-ordinates used: A -3.4 mm, L+5.4 mm; P + 7.5 mm from dura).

Table 1. Quantification characteristics of HPLC method used for amino acids determination. Concentration range of standards (c), slope (k), intercept (q), coefficient of determination (r^2) and limit of detection (LOD) describing the calibration plots obtained with standard solutions of glutamate and aspartate (0.25, 0.5, 1.0 and 2.5 µM) and internal standard L-homoserine (2.5 µM) using the described HPLC method with spectrofluorometric detection.

DISCUSSION:

In this work, we show how a continuous video-EEG recording coupled with microdialysis can be performed in an experimental model of TLE. Video-EEG recording techniques are used to correctly diagnose the different phases of the disease progression in animals and the microdialysis technique is used to describe the changes in glutamate release that occur in time (no changes have been found for aspartate in a previously published study[2]). We strongly recommend the use of a single device/implant to perform them both in each animal for the reasons discussed in the Introduction.

Whenever available, radiotelemetry should be preferred to tethered systems for chronic EEG recording as it minimizes interferences with behavior and reduces harm risk and distress for the animals[54]. However, the tethered EEG recording is much less expensive than telemetry.
In our laboratory, we use the connectors to the EEG recording system and microdialysis tubing in parallel, such that wires and tubings are attached to two different swivels. This is the most critical issue for these experiments: the wires and tubing tend to cross frequently due to the animal's movements. Therefore, we use connectors and tubing long enough to let the animal chase its own tail (a behavior that is typically observed with potassium stimulation) or fall down and roll during generalized epileptic seizures. It is advisable to firm further the microdialysis cannulas inserted in their guide by modeling clay, in order to strengthen their contact with the guide (sometimes, microdialysis cannulas are bumped against the walls of the cage during generalized seizures and may slip off). On the other hand, it is advisable to keep the tubing as short as possible, to minimize the delay between neurochemical time and collection time. This is particularly important when collection periods are short. In general, the microdialysis tubing should be of adequate length and capacity to ensure that the sampling time does not exceed the time between dialysis outlet and collection. It was observed that the solutes tend to diffuse more between some plugs if the tubing dead time is superior to the sampling rate[55]. Therefore, the experimental dead time/volume of microdialysis tubing should be reduced as much as possible and determined very precisely in order to correlate the neurochemistry data with the animal’s behavior. Finally, it is important to note that both swivels and electrodes coupled with cannula for combined EEG and microdialysis studies are

commercially available. Therefore, whenever possible, set up the EEG system with the option to perform the microdialysis experiments.

The minor recommendations are: (i) before beginning any experiment, check that the EEG recording system and/or microdialysis set up are functioning properly and troubleshoot any problem; we suggest that having one reserve set up ready (another pump with syringes mounted on and completed of tubing filled up with working solutions) when performing the experiment, as well as a sufficient number of ready to use microdialysis probes for changing broken ones; (ii) when transferring animal into the working EEG-microdialysis cage it is helpful to have a second person assisting and starting the acquisitions; (iii) make sure that the column and autosampler reached the appropriate temperatures before chromatography; in addition, use standards and construct the calibration plots before any dialysate samples are injected on the chromatographic column; (v) whenever needed, try to develop the chromatographic or other analytical method to measure multiple analytes at the same time.

Alterations in neurotransmission have implications in many CNS disorders (including epilepsy) and there has been a great interest over the decades to quantify these changes during the progression from a healthy to a diseased phenotype. Today, only a few techniques allow the measurement of changes in neurotransmitter levels over days or months. Microdialysis is one of these techniques. In a large number of cases, like that described here, it is performed in freely moving animals and coupled to conventional offline analytical assays like high performance liquid chromatography (HPLC) or capillary electrophoresis (CE), with which it reaches 5-30 min temporal resolution[31,56]. Clearly, these sampling intervals do not reflect the rapid neurotransmitter dynamics in the vicinity of synapses, but may be convenient for some long term microdialysis applications (*e.g.*, disease development or drug effect studies) which require coupling neurochemical, EEG and behavioral data. However, other studies are primarily concerned with measuring real-time or close to real-time changes in neurotransmitter release. For these, the microdialysis technique must be refined to increase the speed of sampling (therefore decreasing sample volumes). Indeed, the classic microdialysis technique is often criticized for its poor temporal (minutes) and spatial resolution (the conventional probe is much larger than the synaptic cleft)[9,21,56,57]. However, it is the mass sensitivity of the analytical method coupled to microdialysis what determines the microdialysis time resolution (*i.e.*, its resolution is equal to the time required to have enough sample to be detected by an analytical technique[56]). Thus, when the microdialysis produces tiny amounts of samples, the sensitivity of quantification techniques must be increased. To date, such improvements in temporal resolution of the microdialysis technique followed 3 different lines. One of these is represented by miniaturization of the columns and/or detection cells of classic HPLC methods; these are called UHPLC (ultra-performant HPLC) techniques and allow to achieve 1-10 min time resolution[58-60]. Another approach is to couple a classic HPLC to mass spectrometry (MS) or tandem (MS/MS) for multiplex analysis of neurotransmitters in brain dialysates. Combined HPLC-MS assays have an excellent sensitivity and reach about 1-5 min time resolution[56,61-63]. A third line of improvement exists in modifications of capillary electrophoresis (CE). If CE uses laser induced fluorescence detection (CE-LIFD), it enables the determination of submicromolar concentrations of various neurotransmitters in nanoliter fractions obtained every 5 min[55,64,65] or even at 10 s intervals[56]. A clear advantage that emerges from UHPLCs or advanced CEs analytical approaches is that the sampling may be done in freely moving animals, not compromising experiments in which spontaneous behavior

must be observed and analyzed. On the other hand, there are methods that permits the brain dialysate sampling at even hundred milliseconds temporal resolution (*e.g.,* enzyme reactor based on-line assays or droplet collection of dialysate coupled to MS techniques), but these are typically used in restrained animals[66] or under general anesthesia[67-69], not allowing to couple microdialysis with behavioral studies.

When considering the second most important weakness of microdialysis, *i.e.,* relatively low spatial resolution due to the membrane dimensions (often about 0.5 mm in diameter and 1-4 mm long), an alternative may be the microprobes developed with low-flow push-pull sampling. These probes consist of two silica capillaries (of 20 µm ID and 200 µm OD) fused side-by-side and sheathed with a polymeric tubing. During the experiment, these capillaries are perfused at very low flow rates, such that fluid is pulled out of one capillary and a sample is retrieved from the other at the same flow rate. Because the sampling occurs only at the probe tip, the spatial resolution is greater than with the probe for microdialysis[70]. Another possibility is to switch from miniaturized probes to microelectrode arrays (biosensors) for real-time neurotransmitter evaluation. Different electrochemical techniques (based principally on voltammetry or amperometry) permit analyte sampling very close to the synapse (micron scale) and in less than 1 s[31,70,71]. These devices can measure the concentration of multiple analytes from multiple brain regions. However, they also require some refinements, for example to avoid artifacts and a relatively rapid deterioration.

Considering the latest advances in *in vivo* neurochemical monitoring, it seems likely that the different transmitter sampling methods will be combined in one sensor in the near future. The work on microfabricated sampling probes has already started, and we believe that further progress in microfabrication technologies together with analytical advances will further facilitate *in vivo* neurochemical monitoring investigation. At this time, however, the conventional microdialysis correlated to EEG remains a valid method for many neuroscience applications.

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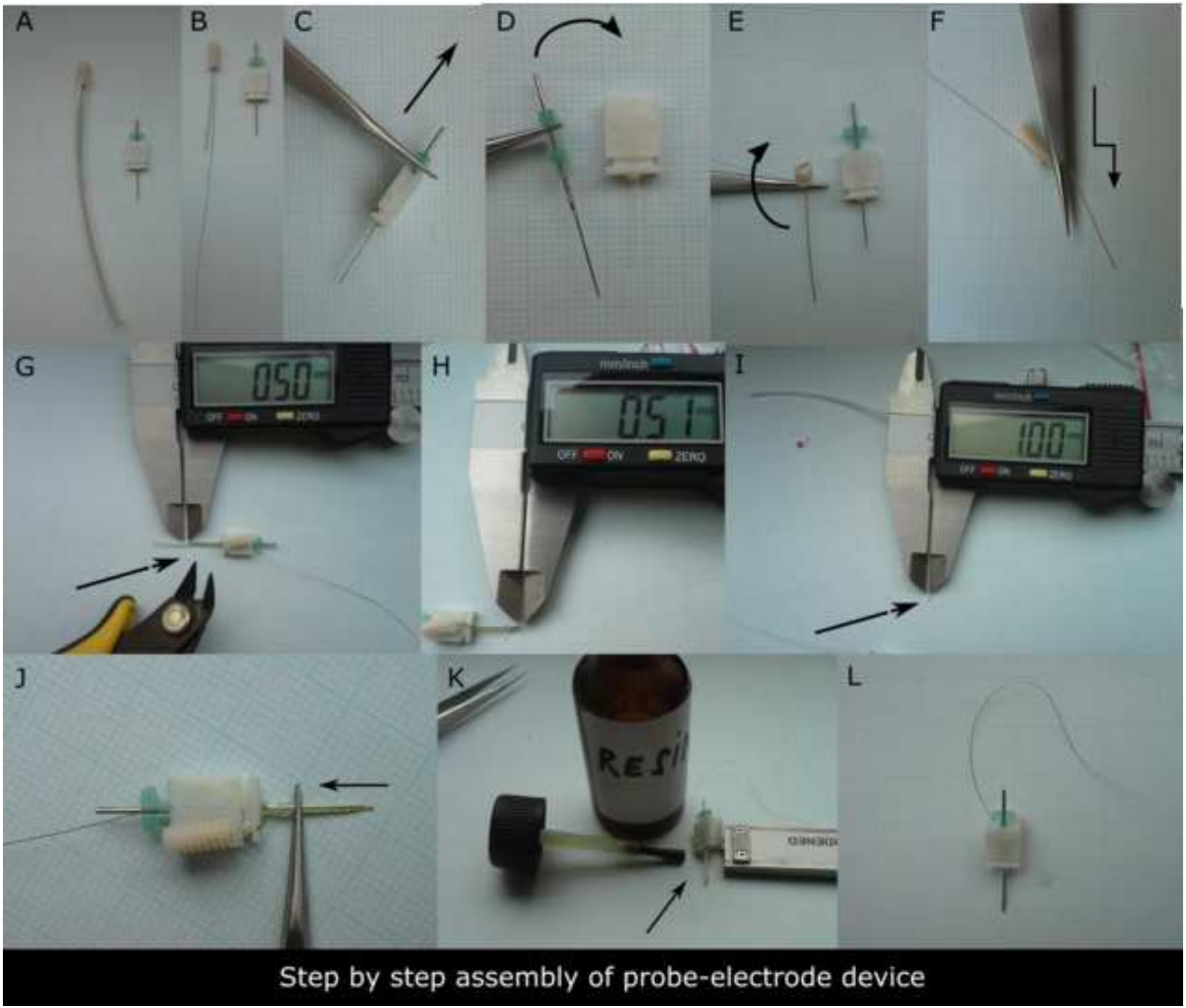
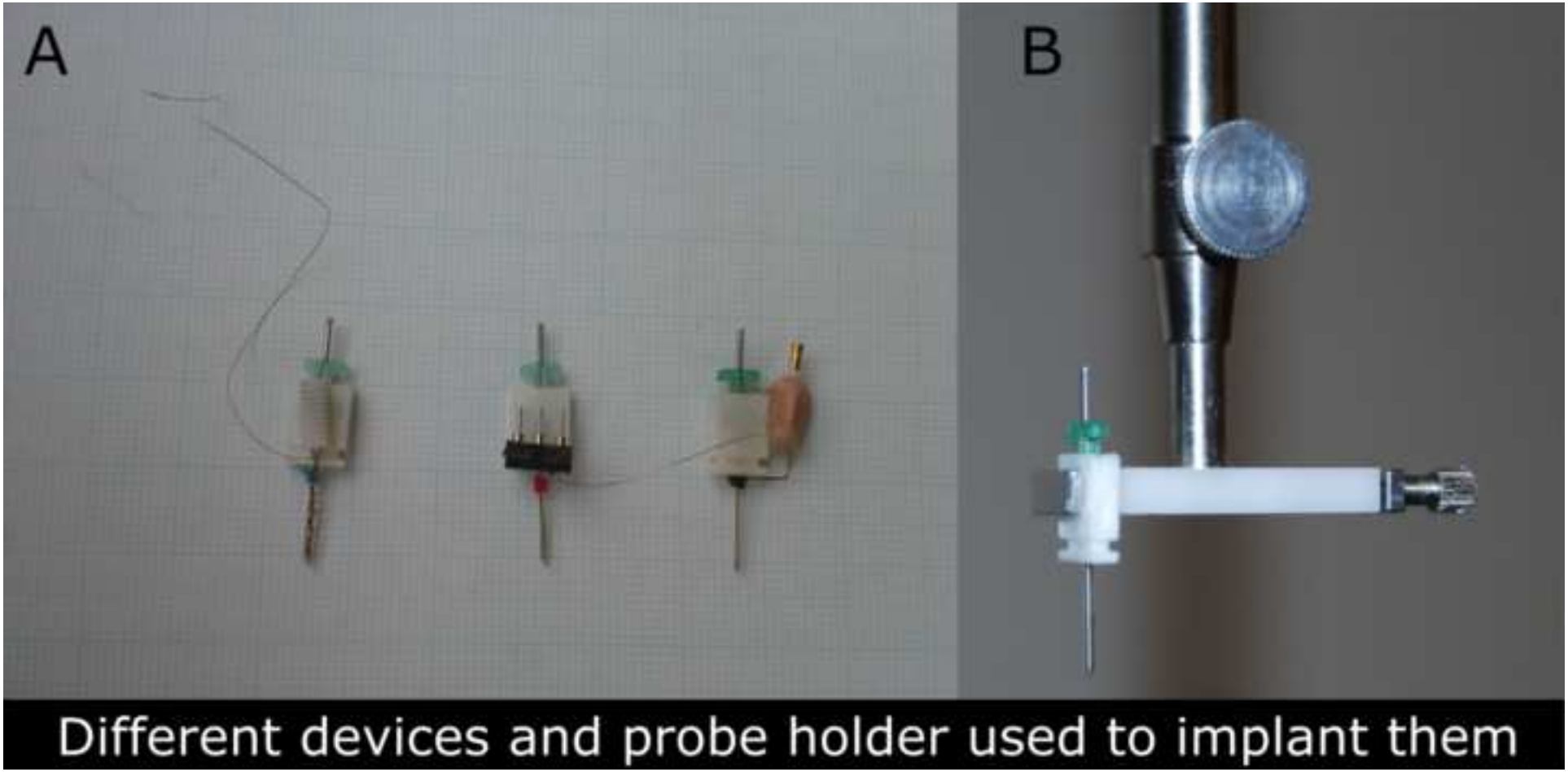


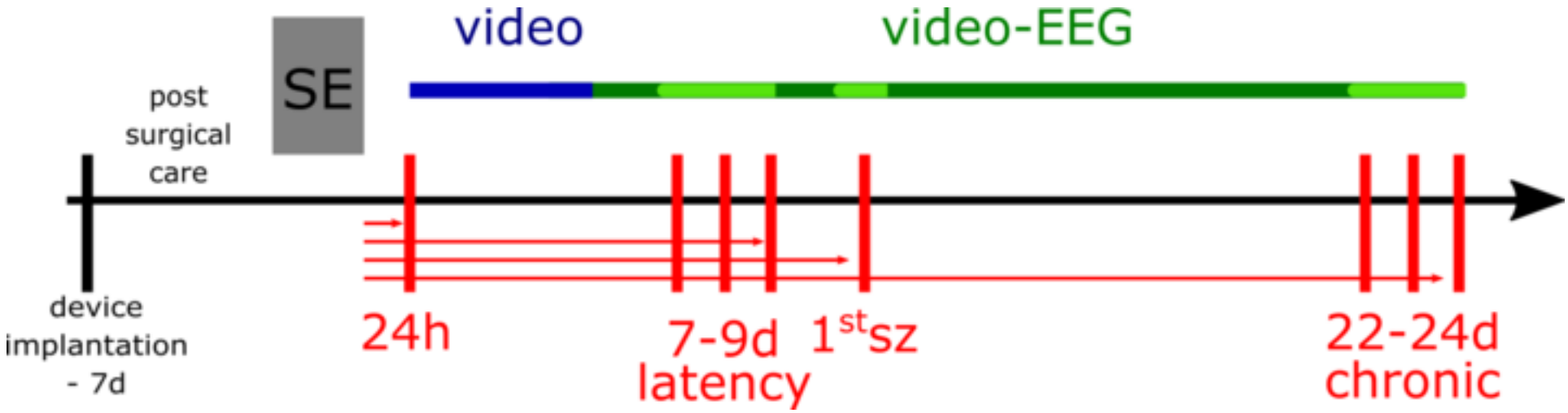
Step by step assembly of probe-electrode device

Figure 2



Different devices and probe holder used to implant them

Figure 3



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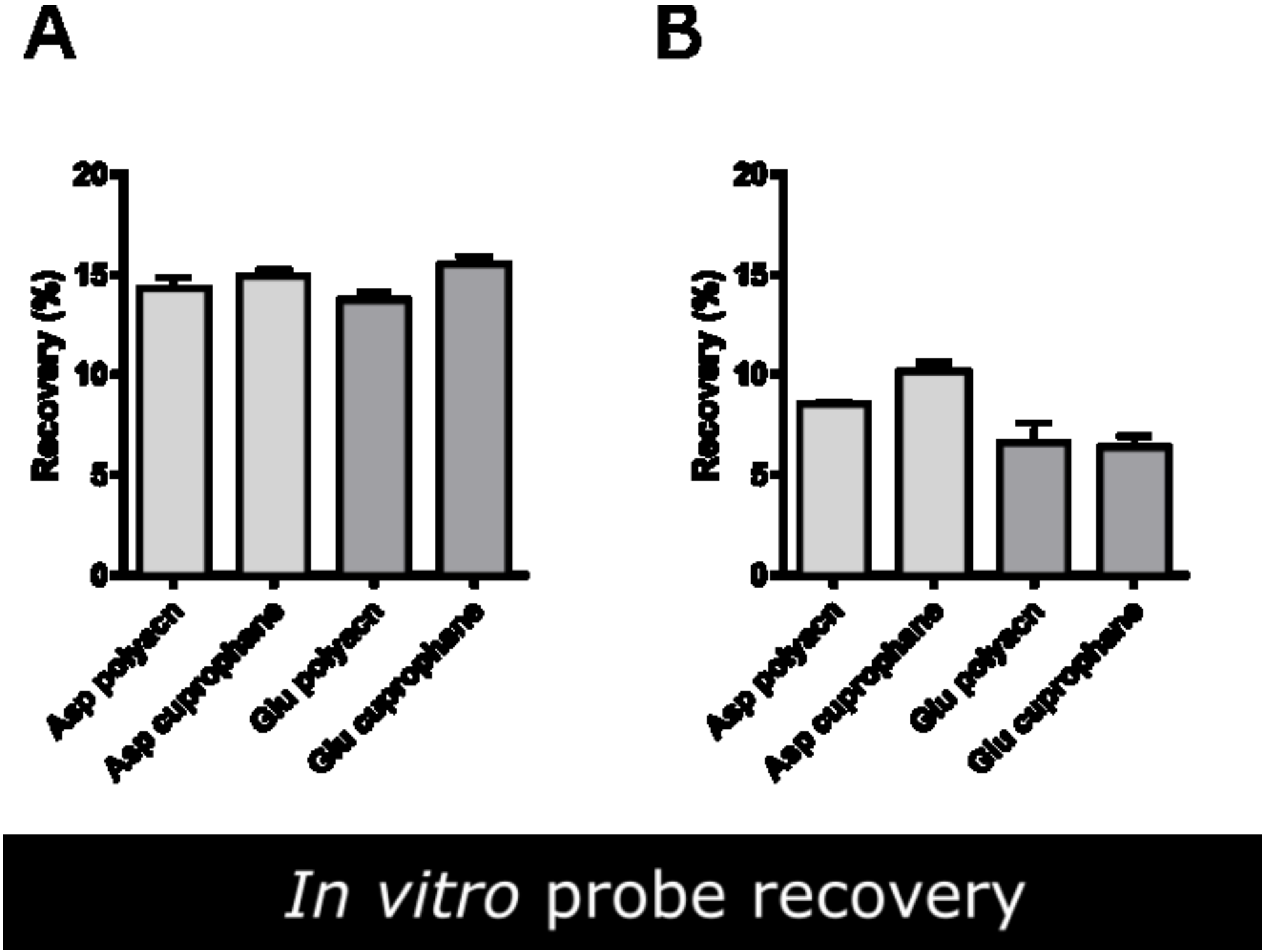

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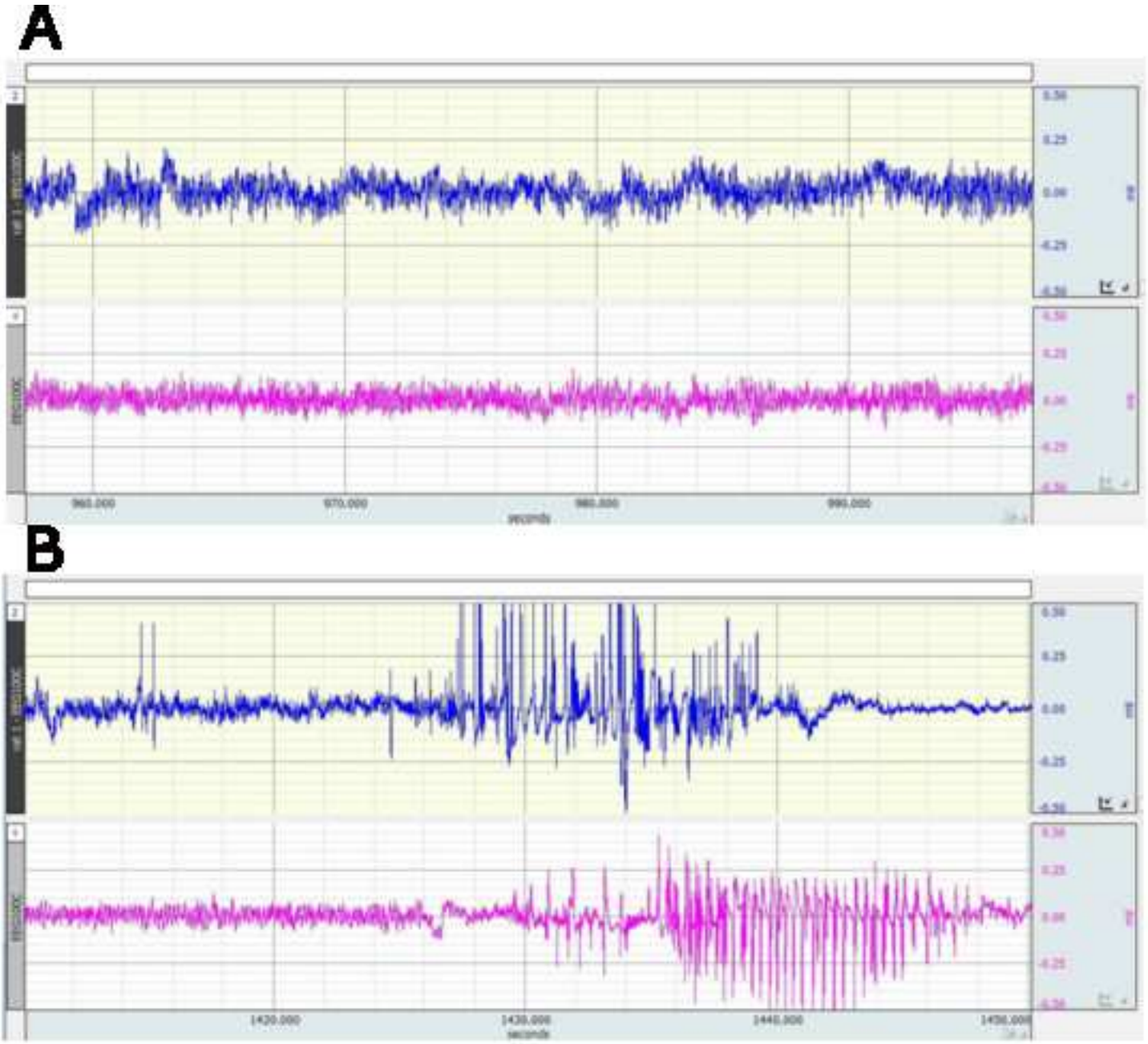
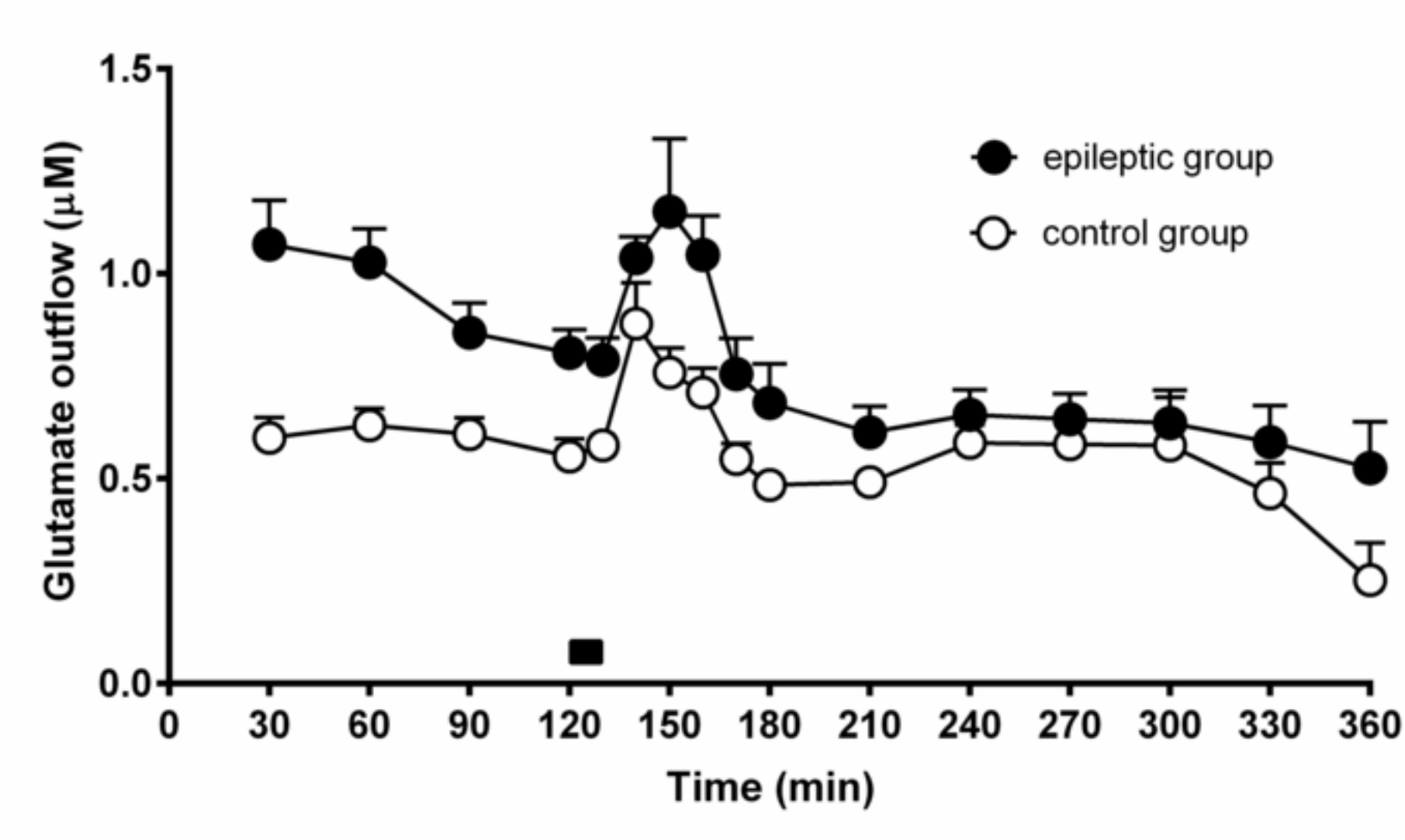


Examples of baseline and epileptiform activity

Figure 6



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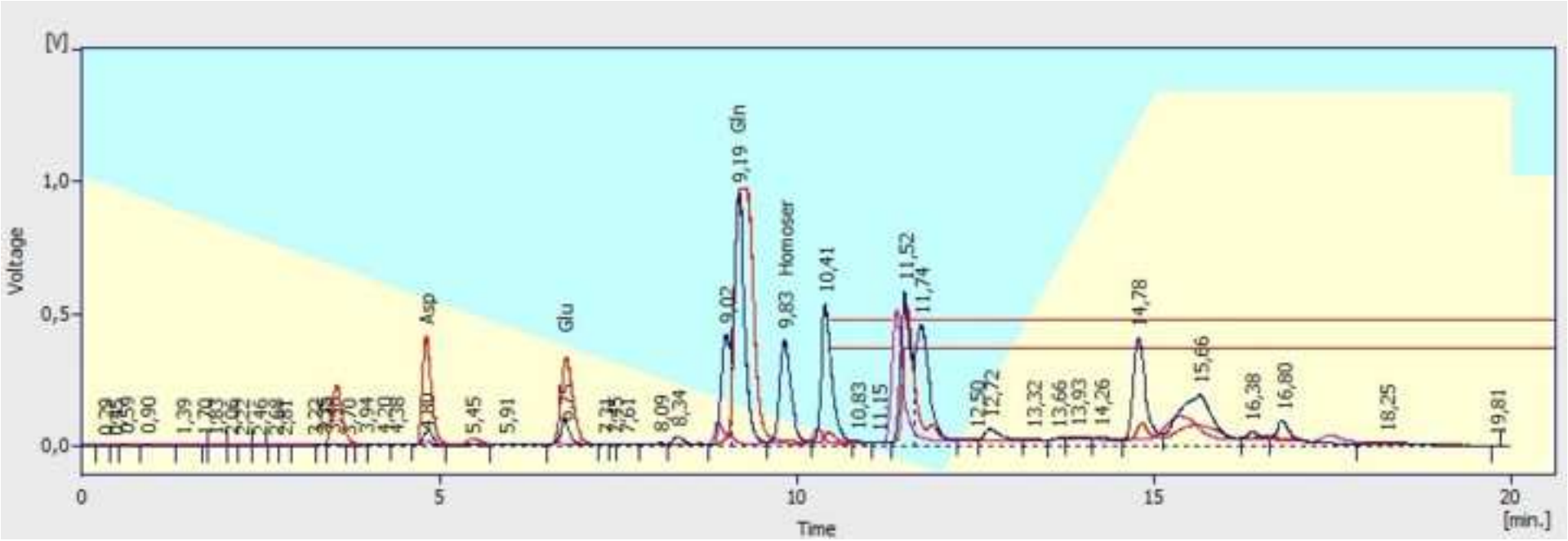

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Analyte	c (µmol/l)	*k*	*q*	r^2
Glutamate	0.25-2.5	5.215	1043.79	0.999
Aspartate	0.25-2.5	2.258	1994.72	0.998

LOD (pmol/l)
19.4
31.7

Name of Material/ Equipment
3-channel two-twisted electrode
guide cannula
Resin KK2 Plastik
Super Attack gel Loctite
Imalgene-Ketamine
Xylazine
Isoflurane-Vet
Altadol 50 mg/ ml - tramadol
Gentalyn 0.1% crm - gentamycine
simplex rapid dental cement
GlasIonomer CX-Plus Cement
probe clip holder
Histoacryl® Blue Topical Skin Adhesive
Valium 10 mg/2 ml - diazepam
1 mL syringe with 25G needle

rat flexible feeding needle 17G

Grass Technology apparatus

modular data acquisition and analysis system MP150

digital video surveillance system

microdialysis probe

microdialysis probe

block heater

stirrer

infusion pump

fine bore polythene tubing

blue tubing adapters

red tubing adapters

2.5 mL syringe with 22G needle

vial cap

septum

glass insert with bottom spring

autosampler vial

Smartline manager 5000 system controller and degasser u

Smartline 1000 quaternary gradient pump

spectrofluorometric detector

chromatogrphic column

chromatogrphic pre-column

mobile phase solution A

mobile phase solution B

Ringer solution

modified Ringer solution

saline

sucrose solution

Company

Invivo1, Plastic One, Roanoke, Virginia, USA

Agn Tho's, Lindigö, Sweden

Elettra Sport, Lecco, Italy

Henkel Italia Srl, Milano, Italy

Merial, Toulouse, France

Sigma, Milano, Italy

Merial, Toulouse, France

Formevet, Milano, Italy

MSD Italia, Roma, Italy

Kemdent, Associated Dental Products Ltd, Swindon, United Kingdom

Shofu, Kyoto, Japan

Agn Tho's, Lindigö, Sweden

TissueSeal, Ann Arbor, Michigan, USA

Roche, Monza, Italy

Vetrotecnica, Padova, Italy

Agn Tho's, Lindigö Sweden

Grass Technologies, Natus Neurology Incorporated, Pleasanton, California, USA

Biopac, Goleta, California, USA

AverMedia Technologies, Fremont, California, USA

Agn Tho's, Lindigö Sweden

Synaptech, Colorado Springs, Colorado, USA

Grant Instruments, Cambridge, England

Cecchinato A, Aparecchi Scientifici, Mestre, Italy

Univentor, Zejtun, Malta

Smiths Medical International Ltd., Keene, New Hampshire, USA

Agn Tho's, Lindigö Sweden

Agn Tho's, Lindigö Sweden

Chemil, Padova, Italy

Cronus, Labicom, Olomouc, Czech Republic

Thermo Scientific, Rockwoood, Tennessee, USA

Supelco, Sigma, Milano, Italy

National Scientific, Thermo Fisher Scientific, Monza, Italy

Knauer, Berlin, Germany

Knauer, Berlin, Germany

Shimadzu, Kyoto, Japan

Knauer, Berlin, Germany

Knauer, Berlin, Germany

0.1 M sodium phosphate buffer, pH 6.0

40% 0.1 M sodium phosphate buffer, 30% methanol, 30% acetonitrile, pH 6.5

composition in mM: $MgCl_2$ 0.85, KCl 2.7, NaCl 148, $CaCl_2$ 1.2, 0.3% BSA

composition in mM: $MgCl_2$ 0.85, KCl 100, NaCl 50.7, $CaCl_2$ 1.2, 0.3% BSA

0.9% NaCl, ph adjusted to 7.0

10% sucrose in distilled water

Catalog Number	Comments/Description
MS333/3-B/SPC	Material
MAB 4.15.IC	Material
KK2	Material
2047420_71941	Material
221300288 (AIC)	Solution
X1251	Material
103120022 (AIC)	Solution
103703017 (AIC)	Solution
20891077 (AIC)	Material
ACR811	Material
PN1167	Material
p/n 100 5001	Equipment
TS1050044FP	Material
019995063 (AIC)	Material
11.3500.05	Material

7206	Material
M665G08	Equipment (AS40 amplifier, head box, interconnecting cables, telefactor mode
MP150WSW	Equipment
V4.7.0041FD	Equipment
MAB 4.15.1.Cu	Material
S-8010	Material
QBD2	Equipment
711	Equipment
864	Equipment
800/100/100/100	Material
1002	Material
1003	Material
S02G22	Material
VCA-1004TB-100	Material
National C4013-60 8 mm TEF/SIL septum	Material
27400-U	Material

C4013-2	Material
V7602	Equipment
V7603	Equipment
RF-551	Equipment
25EK181EBJ	Material
P5DK181EBJ	Material
	Solution
	Solution
	Solution
	Solution
	Solution
	Solution

l RPSA S40)

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JoVE58455R1 POINT–BY-POINT RESPONSE TO EDITOR:

Editorial comments

GENERAL COMMENTS (e-mail dated July 2nd, 2018):

1. The editor has formatted the manuscript to match the journal's style, please retain the same.

Now we maintained the journal's style.

2. Please address all specific comments marked in the manuscript.

These were addressed and the text changes evidenced in yellow and further indicated at the end of this document.

3. All optional steps, caution, and notes need to start from a new line number.

Thank you, the formatting of all optional steps, caution, and notes was corrected.

4. Please describe exactly how you did the experiment rather than generalizing.

The generalizing terms and descriptions were now limited.

5. Presently the protocol is more than 10 pages. Please reduce the same.

The protocol is now 9 pages.

6. Once rewritten, please ensure that the highlight is no more than 2.75 pages including heading and spacings. Also, this should form a cohesive story and logically flow from one step to another. Presently, it is disconnected.

The part of the document to be filmed is now of about 1.5 pages. To have a cohesive story we decided to further cut the protocol and only the 1 day microdialysis under EEG recording was maintained because it is core of our work.

Importantly, we decided to add a flow chart (Figure 3) to the work for better comprehension of experimental design (lines 268, 707-717) hoping that this can help.

SPECIFIC COMMENTS (manuscript track changes):

7. You are not filming the chromatographic determination of the glutamate and aspartate. Either reword the title or recheck the highlights to ensure it is in line with the title of the manuscript. Additionally,

highlights should form a cohesive story and should not be more than 2.75 pages including heading and spacings.

We agree. Now, the chromatographic determination does not appear in the title. We added the detail on animals used in protocol instead.

8. line 58 - Please be more crisp.

We used the term monitored in the phrase (line 58).

9. line 72 - Not clear, please check the grammar.

The phrase was substantially modified (lines 72-74).

10. line 98 - Provide citations to show how the technique has been used for the purpose mentioned here.

Relative citations are now provided (page 2, 2nd paragraph, lines 101, 103, 104, 107, 109, 116, 118 and lines 914-934) and the numbers of following citations were updated, the reference list was update equally (within lines 935-1098).

11. line 131 - Behaving animals? Please reword. Animal behavior studies maybe?

We replaced the term for awake animals (line 131).

12. lines 135-136 - Is the usage of to correct here?

Thank you, this was not correct. To was deleted (lines 135-136).

13. line 160 - Please do not generalize the protocol. Please explain how to perform your experiments with specifics. Please ensure that all the steps have required details, volume, concentration, time, how specifics etc. Please use imperative tense throughout as if directing someone how to do the experiments.

We reformulated the text according to suggestions of the editor.

14. line 171 - Please provide step by step figure highlighting important steps to show how the assembly of probe-electrode device is happening to bring out more clarity. Label this as figure 1 and refer to different panels wherever possible in the steps.

Thank you. We added Figure 1 showing how to assemble the device to the manuscript and we refer to it (lines 175, 177, 181-183, 185, 187-189).

15. line 178 - So you remove the electrode while performing microdialysis?

No, only the guide cannula is removed and substituted for microdialysis probe 24h before the dialysis session. Electrode is firmly attached to the guide cannula sleeve and once the device is cemented onto the animal's skull it cannot be removed. We hope that with Fig. 1 the shift information is more clear.

16. line 191 - Please reword: Sterilize the device under germicidal UV light…. What is meant by grilling it on its 4 sides?

Thank you, indeed it is not precise. We reworded the point (lines 191-192).

17. line 203 - Please write this in the order of how you performed the steps. First you anesthetize the rats, fix it on the stereotaxic frame and then implant the device (where is it being implanted- in the brain, which part), etc. Just exactly write the order of the steps in imperative tense as if directing someone to do the experiment.

Stereotaxic surgery is now described in more details (lines 206-210, lines 212-218, lines 220-223).

18. line 213 - Where is the device being implanted.

As above (lines 214-215).

19. line 207 - What is meant by adult handled? Also, how do you implant. If it is done using standard techniques, please provide a reference.

The term "handled" was deleted as superfluous. How we implant: as above (point 2.2). References are present.

20. line 216 and followings - We cannot have two caution statements together. Hence merged. Please check.

We checked the text, it is fine, thank you (lines 216-218).

21. line 220 – Where and how?

The point 2.3 was rephrased and the information regarding the screw positions was provided (lines 220-223).

22. line 227 - Please reference the videos here instead of making optional statement.

We referenced to videos in point 2.4. (lines 229-230).

23. line 236 - This is not written in imperative tense. Hence converted to a note. If this needs to be a part of the step above, please write in imperative tense and provide the accurate dosage of what antibiotic was given in your experiment.

The imperative was used and the note comprising the names of used drugs and relative doses is now part of the point 2.6. (lines 237-239). The table of Materials was updated to refer the used drug products.

24. line 249 - Reworded please check. Also please be crisp while writing steps. Please also provide the needle and syringe size for subcutaneous/ i.p injection

We checked the text, it is fine, thank you (lines 249-250). The needle and syringe size for i.p injection is now provided (lines 251-252) and Table of Materials updated for these.

25. line 250 - How do you visually check the induction of SE? include a note stating this.

The note was added to the point 3.2. (lines 252-254).

26. line 259 - Aint the first and the last sentence same? Please write exactly as to how you performed your experiment. How and when did you interrupt/arrest the SE. This will make it easier for someone to follow the protocol. Presently the difference between interrupting and arresting is not coming out well.

Thank you for your suggestion. The point was reorganized (lines 256-257).

27. line 261 - Is this through injection or orally. Please reword. Only 1 ml of the solution is given every day? Don't you decide the amount as per the body weight?

The information regarding the type of administration is now included (lines 261-262). Yes, only 1 ml of saline and 1 ml of sucrose solution is given/animal/day and the animals recover well.

28. line 279 - Please explain how to perform this? Also please remove the redundancy. Looks like step 4.1.1 is just a combined step of 4.1.2-4.1.7. please exactly write how you did the experiment. So you divided the rats into different groups? Did you process the groups differently or all the group undergo same monitoring and analysis?

The chapter 4.1. would not be part of the video, thus we did not evidence points 4.1.4. and 4.1.5. The precedent point 4.1.1 was deleted because redundant and the following points rearranged (lines 289, 292-293, 297-299, 304-307). We also included Figure 3 (experiments flow) into the manuscript.

29. line 297 – How?

Previous option is now part of the point 4.1.5. explaining how to connect the animal (lines 297-299).

30. line 305 - So EEG and microdialysis is not done together? Please clarify? How do you know when to do what? Again it be nice if you write the specifics of your protocol as to how you did your experiment rather than writing a general protocol.

The decision is depending on the experimental group investigated. We included the Figure 3 with flow chart to bring more clarity into all story.

31. line 309 – We cannot have paragraph of text in the protocol section. Please consider moving some to the discussion section.

The part of the precedent note text was now integrated in protocol point 4.1. (lines 304-307).

32. line 331 - So many substeps are not necessary.

This chapter was substantially rearranged (lines 334-335, lines 344-346, lines 348-350) to be shorter.

33. line 342 - How?

The description is provided (lines 342-343).

34. line 348 and 349 – Recorded file to one for analysis? What does that mean. Also, how do you copy or transform. Please ensure that you answer how question how is this step performed.

The was rearranged (lines 348-350).

35. line 355 - We cannot have paragraph of text in the protocol section. Please only include what is relevant to the current protocol described here and please consider moving some parts to the discussion section.

We eliminated the in vitro recovery testing form video. The chapter 5.1. was substantially rearranged (lines 361-363, 397-398).

36. line 368 - This is commercial. Please use generic term instead. Maybe paraffin film.

The term was changed according editor's suggestion (lines 368, 371-373).

37. line 361 - How do you do so? (run the experiment in triplicate).

The information is now provided (lines 361-363).

38. line 390 - What are the testing conditions in your case?

The text was rearranged and point 5.1.9. added to 5.1. section to be more comprehensive (lines 397-398).

39. line 394 - Optional, notes and caution steps cannot be filmed. Please remove the highlight from these. Also start all these three from new lines leaving a blank space between step and optional step.

Option was avoided, the precedent optional text is now part of the point 5.1.8. (lines 394-395).

40. line 400 - This is not an option. This is what you do. Removed the option word. Please check.

You are right, thank you. The step is part of point 5.1.10.

41. line 405 – Step number please.

The number was indicated (line 405).

42. line 423 - What amount of isoflurane is used?

The information is now provided (line 423).

43. line 428 - Caution, notes and options cannot be filmed.

Yes, thank you. This caution is usually reported on manufacturer user's guide of dialyzing probe if these are endowed with protective sleeve thus may not be filmed.

44. line 430 – Ambient or ambience? Please check the grammar throughout.

Yes, thank you, the ambience is better (line 430). The phrase was anticipated because of work logic flow.

45. line 436 - What is the low pressure used. How much ringer solution and how is it pushed in?

We added the information, but now we rather refer to velocity of pushing the syringe piston than pressure, because we do not know this precisely (lines 436-437). Also the previous point 5.2.1.8. is part of point 5.2.1.6. now (lines 441-442).

46. line 484 – What is the right moment?

The step was reformulated to indicate the moment (lines 484-485, 487).

47. line 496 - As above. (Option).

The previous option was included into step 5.2.2.10. and will be a part of the film (lines 496-498).

48. line 514 - So the microdialysis is performed in live animals?

Yes, this was now addressed in the title of section 5.2. (line 410).

49. line 530 - Provide citation.

The citation was now provided (line 530), even if we do not know if it is sufficient. The embedding is automatically done by commercially available machines and protocols are very similar between the brands; we use 48 h of 10% formalin.

50. line 682 - Please ensure that all figures and tables have title for the figure

We added the missing titles (lines 718, 731). Moreover, we added 2 new figures and respective legends: Figure 1, as you suggested, showing how to assemble the dialysis-EEG device (lines 684-695). Figure 3 and respective legend should bring more clarity to entire manuscript (lines 706-716). Previous Figure 1 became Figure 2, and previous Figures 2-6 became numbers 4-8 (lines 697, 718, 726, 731, 738, 749).

51. line 767 - Please do not make subsections. I have removed it.

Thank you, we did not know.

Figure 1

Figure 2

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Supplemental Coding Files

Figure 2 Soukupova et al.svg

Figure 3

Figure 4

Figure 5

Figure 8