Video Article

Fast Micro-iontophoresis of Glutamate and GABA: A Useful Tool to Investigate Synaptic Integration

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URL: <http://www.jove.com/video/50701>

DOI: [doi:10.3791/50701](https://doi.org/10.3791/50701)

Keywords: Neuroscience, Issue 77, Neurobiology, Molecular Biology, Cellular Biology, Physiology, Biomedical Engineering, Biophysics, Biochemistry, biology (general), animal biology, Nervous System, Life Sciences (General), Neurosciences, brain slices, dendrites, inhibition, excitation, glutamate, GABA, micro-iontophoresis, iontophoresis, neurons, patch clamp, whole cell recordings

Date Published: 7/31/2013

Citation: Müller, C., Remy, S. Fast Micro-iontophoresis of Glutamate and GABA: A Useful Tool to Investigate Synaptic Integration. *J. Vis. Exp.* (77), e50701, doi:10.3791/50701 (2013).

Abstract

One of the fundamental interests in neuroscience is to understand the integration of excitatory and inhibitory inputs along the very complex structure of the dendritic tree, which eventually leads to neuronal output of action potentials at the axon. The influence of diverse spatial and temporal parameters of specific synaptic input on neuronal output is currently under investigation, e.g. the distance-dependent attenuation of dendritic inputs, the location-dependent interaction of spatially segregated inputs, the influence of GABAergic inhibition on excitatory integration, linear and non-linear integration modes, and many more.

With fast micro-iontophoresis of glutamate and GABA it is possible to precisely investigate the spatial and temporal integration of glutamatergic excitation and GABAergic inhibition. Critical technical requirements are either a triggered fluorescent lamp, light-emitting diode (LED), or a two-photon scanning microscope to visualize dendritic branches without introducing significant photo-damage of the tissue. Furthermore, it is very important to have a micro-iontophoresis amplifier that allows for fast capacitance compensation of high resistance pipettes. Another crucial point is that no transmitter is involuntarily released by the pipette during the experiment.

Once established, this technique will give reliable and reproducible signals with a high neurotransmitter and location specificity. Compared to glutamate and GABA uncaging, fast iontophoresis allows using both transmitters at the same time but at very distant locations without limitation to the field of view. There are also advantages compared to focal electrical stimulation of axons: with micro-iontophoresis the location of the input site is definitely known and it is sure that only the neurotransmitter of interest is released. However it has to be considered that with micro-iontophoresis only the postsynapse is activated and presynaptic aspects of neurotransmitter release are not resolved. In this article we demonstrate how to set up micro-iontophoresis in brain slice experiments.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50701>

Introduction

Neurons in the central nervous system receive a variety of synaptic inputs on their thin and ramified dendritic processes¹. There, the majority of the excitatory dendritic inputs are mediated by glutamatergic synapses. These synapses can be activated in a spatially distributed way, resulting in postsynaptic linear integration of excitatory postsynaptic potentials (EPSPs). If the synapses are activated simultaneously and in spatial proximity on the dendrite, these excitatory inputs can be integrated supra-linearly and generate dendritic spikes²⁻⁵.

Furthermore, the integration of excitatory inputs depends on the location of the input on the dendritic tree. Signals that arrive at the distal tuft region are much more attenuated than proximal inputs due to cable filtering⁶. In the hippocampus, distant inputs to the apical tuft dendrites are generated by a different brain region than those on proximal dendrites⁷. An exciting question is therefore, how synaptic input is processed by different dendritic compartments and if dendritic integration regulates the influence of these layered inputs on neuronal firing in different ways.

Not only the functional properties, morphological features of the dendrite, the location and clustering of the inputs are affecting the dendritic integration of excitatory inputs, also the additional inhibitory inputs from GABAergic terminals crucially determine the efficacy of the glutamatergic synapses^{8,9}. These different aspects of synaptic integration can be ideally investigated using neurotransmitter micro-iontophoresis, which allows spatially defined application of different neurotransmitters to dendritic domains. We demonstrate here how to successfully establish micro-iontophoresis of glutamate and GABA to investigate signal integration in neurons.

For this application, fine-tipped high resistance pipettes filled with concentrated neurotransmitter solutions are used. These pipettes are positioned close to the outer membrane of the cell, where the neurotransmitter receptors are located. A good visualization of the dendritic branches is required. This is best achieved using fluorescent dyes, which are introduced via the patch pipette. Then a very short (<1 msec) current pulse (in the range of 10 - 100 nA) is used to eject the charged neurotransmitter molecules. With these short pulses and effective capacitance compensation, postsynaptic potentials or currents can be evoked with high temporal and spatial precision, which means the location of the excitatory input is precisely known. Glutamate micro-iontophoresis can activate synapses in a defined radius, which is smaller than 6 μm as shown here (**Figure 1**⁹), but it is also possible to reach single synapse resolution¹⁰⁻¹².

Heine, M., *et al* showed that the spatial resolution of fast micro-iontophoresis can even be adjusted to spot sizes below 0.5 μm , which is smaller than spot sizes regularly achieved with two-photon uncaging of glutamate¹³. With fast micro-iontophoresis it is easily possible to use two or more iontophoretic pipettes and place them at different, even distant spots on the dendritic tree. In this way, integration of excitatory events, including those from different pathways, can be investigated. It is also possible to use a glutamate and a GABA filled iontophoretic pipette at the same time. In this way the effect of GABAergic inhibition at different locations relative to the excitatory input (on-path, off-path inhibition) can be studied. Also, the impact of inhibition by interneurons targeting specific neuronal domains, like distal dendrites, soma or axons¹⁴, can be investigated using GABA iontophoresis. In cultured neurons, fast micro-iontophoresis offers the opportunity to investigate single synapse distribution and the elementary aspects of synaptic communication in neurons in much more detail^{10,11}.

In this article we demonstrate in detail how to establish glutamate and GABA iontophoresis for the use in acute brain slices, which allows investigating synaptic integration of excitatory and inhibitory inputs in dependence of input location, input strengths, and timing, alone or in interplay. We will point out the advantages and limitations of this technique and how to troubleshoot successfully.

Protocol

1. System Requirements

1. Microscope system: Good visualization of the dendrite is crucial. If available, use a two-photon laser scanning microscope system. In our experiments we used a TRIM Scope II, LaVision Biotec, Bielefeld, Germany or an Ultima IV system, Prairie Technologies, Middleton, Wisconsin equipped with a Ti:Sapphire ultrafast-pulsed laser (Chameleon Ultra II, Coherent) and a high NA objective (60X, 0.9 NA, Olympus) to visualize the dendrites which we had filled with a fluorescent dye via the patch pipette. Although photo-damage is thought to be less severe using 2-photon scanning, reduce the laser power (below 8 mW at the tissue) and dwell times (below 1 μsec) or as much as possible.
2. A second possibility is to trigger a wide-field fluorescence light source (LED or a fluorescent lamp) synchronously with acquisition to reduce exposure time as much as possible. We have used a monochromator with an integrated light source (TILLPhotonics, Gräfelfing, Germany) on a Zeiss Axioskop 2 FS upright microscope which was equipped with Dodt-contrast infrared illumination (TILLPhotonics, Gräfelfing, Germany). In our experiments exposure times ranged from usually 10 msec to max. 30 msec.
3. Use a fast micro-iontophoresis amplifier, e.g. a two-channel micro-iontophoresis system MVCS-C-02 (npi electronic, Tamm, Germany) with fast capacitance compensation. The fine-tipped iontophoresis microelectrodes have resistances of 25 - 100 M Ω (crucially depending on the tip size and the pipette shape), and fast rise times can only be achieved if the capacitance compensation of the iontophoretic amplifier is optimally tuned. This fast compensation is necessary to apply current pulses with a brief and rapid onset in the sub-millisecond range to the iontophoretic pipette and thereby to eject the transmitter with high spatial resolution in spot sizes below 1 μm ¹³. Iontophoresis amplifiers are also available from several other companies, which we have not tested in our laboratory. These devices are to our knowledge not equipped with capacitance compensation circuitry.

2. Prepare Solutions

1. Prepare artificial cerebrospinal fluid (ACSF) and internal solution as it is required for the experimental design. The only addition to the internal solution, which is required, is a red or green fluorescent dye (e.g. 50 to 200 μM Alexa Fluor 488 or 594 hydrazide, Invitrogen), depending on the optical equipment. Here an example for ACSF_{sucrose} which can be used for dissection, in mM: 60 NaCl, 100 sucrose, 2.5 KCl, 1.25 NaH_2PO_4 , 26 NaHCO_3 , 1 CaCl_2 , 5 MgCl_2 , 20 glucose; and for normal ACSF solution for patch-clamp experiments in mM: 125 NaCl, 3 KCl, 1.25 NaH_2PO_4 , 26 NaHCO_3 , 2.6 CaCl_2 , 1.3 MgCl_2 , 15 glucose.
2. Carbogenize (95% O_2 , 5% CO_2) all extracellular solutions constantly.
3. Prepare internal solution, for example, in mM: 140 K-gluconate, 7 KCl, 5 HEPES-acid, 0.5 MgCl_2 , 5 Phosphocreatine, 0.16 EGTA; with 50 - 200 μM Alexa 488.
4. For glutamate micro-iontophoresis prepare a solution with 150 mM glutamic acid and adjust the pH to 7.0 with NaOH. Add 50 -200 μM Alexa Fluor 488 or 594 hydrazide (Invitrogen) for visualization.
5. For iontophoresis of GABA prepare a 1 M GABA solution and adjust the pH to 5 with HCl ¹⁵. At this pH GABA is charged, only then it can be applied using iontophoresis. Please note that the low pH in the solution ejected to the extracellular space might effect GABA transmission itself^{16,17}.

Protect the GABA solution from light and frequently prepare fresh GABA stock solution, since older solution can lose its effectiveness.

6. If it is difficult to see GABAergic events, a high Cl^- driving force internal solution, for example by leaving out KCl, might help to visualize the GABAergic events to see if the GABA iontophoresis is working; however to investigate synaptic integration a physiological driving force might be recommended. For general detection of small GABAergic events, a protocol shown in **Figure 8C** can help.

3. Pull and Test the Iontophoresis Pipettes

1. In general, pulling the right pipettes is maybe the most critical step to achieve controlled neurotransmitter iontophoresis. When using iontophoresis in cell culture, it is possible to pull very fine electrodes similar to sharp microelectrodes¹⁰. In patch-clamp experiments in acute slices, however, these very thin pipettes bend on the slice surface when they are lowered into the tissue with an angle, making it impossible to reach deeper dendrites.
2. Therefore, pull a pipette with a very small tip, so that no neurotransmitter can leak out, but the tip has to be still rigid enough to penetrate into the tissue (**Figure 2**). For example, use 150 GB F 8P class pipettes (Science Products, Hofheim, Germany) and a horizontal puller (for example a DMZ-Universal Puller, Zeitz-Instruments GmbH, Martinsried, Germany; or a P-97 Puller, Sutter Instrument Company, Novato, CA) with several pulling steps to achieve a small opening, but also a short taper with a steep angle (**Figure 2** and **Table 2**).
3. It is also possible to use quartz glass pipettes to pull iontophoretic pipettes¹⁰. These are supposed to have better mechanical properties and to be more reliable; however special laser pullers are required. But it is also possible to achieve good results with normal glass pipettes, which are usually used for pulling patch pipettes.
4. Test the pipette performance and resistance in a chamber without tissue, before using them for the first time, since leakage of glutamate could harm the tissue.
5. Set up the iontophoresis amplifier correctly. Then fill the pipette with the neurotransmitter and dye containing solution and place it into the bath (ACSF).
6. Compensate the capacitance (**Figure 3**). Usually very sharp pipettes will have a higher capacitance than blunt ones.
7. Check the resistance of the pipette: The micro-iontophoresis amplifier used here has a build-in feature for measuring the pipette resistance. It evokes brief rectangular test pulses, which can be monitored with a standard oscilloscope or an A/D board connected to a computer with acquisition software. Depending on the shape and the tip size, the tip should have a resistance between 25 - 90 MΩ.
8. Focus on the tip with a 60X or 40X water immersion objective and switch to fluorescent imaging and if possible, zoom in. If fluorescent dye leaks out of the pipette tip, apply a small positive (in the case of glutamate) or negative (in the case of GABA, **Figure 4**) retain current (<0.02 μA). If that doesn't help to cancel the leakage, change the pipette.
9. Apply a strong step current or use the manual trigger and monitor the tip in the fluorescent image to see if the solution can be ejected out of the pipette. As mentioned above, the polarity of the current pulse is depending on the charge of the molecule that is supposed to be ejected. To eject glutamate it is a negative current and for GABA a positive current (**Figure 4**).
10. Air bubbles in the pipette that block ejection of dye and transmitter, can be cleared by applying a high ejection current several times.
11. Taken together, if there is no visible leakage and test ejection was successful, compensate the capacitance and start the experiment.
12. Caution: Since capacitance compensation is achieved by a feedback circuit, this circuit can overshoot or oscillate if it is overcompensated. Gently use the dial setting for capacitance compensation.
13. Depending on the puller stability it is sometimes necessary to adjust the puller settings from time to time, since the filament may have changed properties. However, if once a good pipette is designed, it can be used for several days. Therefore, after finishing the experiment store the iontophoretic pipette in a closed container without damaging the tip.
14. For the next experiment fill the pipette with the neurotransmitter solution, apply several strong eject pulses to clear the tip and then check if the properties (e.g. resistance) change significantly. Then compensate the capacitance and use the pipette again. Do not use a single pipette with different neurotransmitter solutions.

4. Prepare the Brain Slices

1. If micro-iontophoresis is used for the first time definitely prepare the slices after establishing a reliably working puller program.
2. Perform anaesthesia and decapitation procedures in accordance to the animal care guidelines of your institution or local authority.
3. After the removal of the brain, transfer it to ice cold ACSF_{sucrose} (see Protocol 2.1).
4. Cut the region of interest into slices of appropriate thickness (for example 300 μm).
5. Incubate the slices in ACSF_{sucrose} at 35 °C for 30 min. Subsequently, transfer them to a submerged holding chamber containing normal ACSF at room temperature.
6. Throughout the preparation and experiment carbogenize the ACSF surrounding the slices with 95% O₂, 5% CO₂.

5. Establish a Whole-cell Recording

1. Position the iontophoresis pipette(s) already near the slice surface before patching a cell, to avoid long lasting positioning, after establishing the whole-cell mode.
2. Pull a low resistance patch pipette (3 - 5 mΩ), fill it with the dye containing internal solution and apply positive pressure to the pipette (30 - 60 mbar).
3. Enter the bath and approach the cell under visual guidance (infrared dot contrast or two photon gradient contrast image).
4. Monitor the pipette resistance with a test pulse (e.g. -10 mV, 20 msec) in voltage clamp mode. When touching the tissue correct the offset potential.
5. Approach the cell and gently push the pipette tip into it until a "dimple" can be seen clearly. Immediately release the pressure of the pipette, apply 40 - 60 mbar negative pressure and switch the membrane potential to -65 mV.
6. When the holding current reaches values below 100 pA, release the negative pressure.
7. After establishing a giga seal (resistance >1 GΩ), rupture the membrane with a short, strong suction to the pipette or brief overcompensation of the capacitance compensation circuit.
8. Depending on which mode (voltage or current clamp) is required for the experimental design, compensate appropriately.
9. Start to bring the iontophoretic pipette into its final position. If a more detailed description of how to successfully perform patch clamp recordings is needed, there are several excellent guidelines available^{18,19}.

6. Place the Iontophoretic Pipette and Generate a Postsynaptic Iontophoretic Potential

1. In general, iontophoretic events can be evoked at defined locations depending on the desired experiment, for example, at a spiny dendrite for glutamate micro-iontophoresis, at the dendritic shaft, soma or axon initial segment for GABA micro-iontophoresis.
2. Approach the cell up to approximately 1 μm distance without touching it. After reaching the position of interest it is crucial that no neurotransmitter is leaking out and that the pipette capacitance is compensated correctly.
3. If approaching the cell with a glutamate filled iontophoretic pipette causes detectable depolarization of the membrane potential, adjust the retain current if possible, or change the pipette.
4. Apply short negative current pulses, starting from zero and increase the current systematically (e.g. 0.1 - 0.4 msec, 0.01 - 1 μA pulses). This helps to find out in which range the iontophoretic current evokes the desired responses in the specific experimental set-up.
5. If there is no response detectable, lift the pipette several hundred micrometers and apply a strong eject current ($>0.1 \mu\text{A}$) to clean the tip. Adjust the capacitance compensation, approach the cell, and try again.
6. If there is still no response, reduce the retain current. Be very careful with the dial settings since this procedure can cause uncontrolled neurotransmitter release. Therefore, constantly monitor the recording to detect respective changes in membrane potential.
7. If it is difficult to detect GABAergic events, it can be helpful to use an internal solution composition yielding in a high Cl^- driving force. To achieve this, reduce the Cl^- concentration in the pipette solution (see protocol section 2.6.). This will result in larger GABAergic events at resting membrane potential due to a higher driving force.
8. Alternatively, inject long current steps resulting in membrane potential changes from -100 mV to -48 mV (**Figure 8C**). With this protocol the Cl^- driving force is increased at hyperpolarized potentials causing a depolarizing GABA response.
9. It is also possible to use the step current injection protocol to determine the reversal potential of the evoked events, which helps to determine the GABAergic nature of the events. Leakage of GABA is harder to detect than leakage of glutamate. In this case, a constant monitoring of the input resistance can help, when the GABA filled pipette is approached. If the input resistance decreases, the retain current can be increased or a different pipette should be used.
10. As control experiments for glutamate iontophoresis, we suggest a Ca^{2+} imaging experiment, using 200 μM OGB-1 and no EGTA in the pipette solution to visualize local calcium influx that might be caused by leaking glutamate.
11. In general, to achieve a stable response it is very important to have a mechanically stable pipette to avoid drift over time. Drift can be caused by temperature changes, therefore it is recommended to switch on the equipment at least half an hour before the measurements to avoid thermal drift. Be sure to use good cartridge seals in the pipette holder and the tip is really fixed. The holder itself can be additionally fixed with Teflon tape; furthermore, be sure that there is no tension on the cables from the headstage or manipulator, which also is a potential source of drift.

Representative Results

A simple approach to determine the spatial spread of iontophoresis is to retract the iontophoretic pipette stepwise from the dendrite, while keeping the ejected glutamate constant. We found that the spatial extent of a micro-iontophoretic stimulation had a diameter of approximately 12 μm (**Figure 1** showing radius). How deep in the tissue the iontophoresis can be used depends on the rigidity of the pipette. However, the iontophoretic pipettes needed for experiments in slices (**Figure 2**), which are used here, are not limiting the depth of penetration. Rather the optical system and the decreasing resolution in the depth of the brain slice is the limiting factor. For a good quality recording it is crucial that no transmitter is leaking out of the pipette. In the case of glutamate, a leakage can be identified if there is a sudden depolarisation when the dendrite is approached with the pipette tip or if the baseline suddenly becomes unstable (**Figure 5**). After establishing a stable recording, it is possible to evoke EPSPs of defined amplitudes, dendritic spikes or action potentials with this technique on any location throughout the dendritic tree (**Figure 6**). By applying glutamate with fast micro-iontophoresis, the properties of EPSPs, their propagation and summation, simultaneously at different even distant locations, can be investigated (**Figure 7**). GABAergic events can be evoked alone or in addition to glutamate with a second pipette by filling the iontophoretic pipette with a highly concentrated GABA solution (see: Protocol section 2) and a positive eject current. There is a simple protocol to confirm the GABAergic nature of the events and to make GABAergic events easier to detect, if not using a high driving force internal solution: Inject negative currents (approx. 1 sec; the amplitude depends on the input resistance of the cell) resulting in hyperpolarising voltage steps, starting at around -100 mV and then increase in 5 mV steps (**Figure 8**). At very negative potentials the GABAergic events are easier to detect, due to the higher driving force. And if the signals reverse around the calculated Cl^- reversal potential for the solutions, it is very likely that they are GABAergic in nature (**Figure 8**). With an additional GABA micro-iontophoretic pipette, it is possible to investigate, for example, the effect of GABAergic inhibition on glutamatergic events, like dendritic sodium/calcium spikes, by varying the relative timing of dendritic spike and IPSP (**Figure 9**), the relative location of both events, or their amplitudes. (All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of the University of Bonn, the German Center for Neurodegenerative Diseases and the state Northrhine-Westfalia.)

Figure 1. Spatial extent of iontophoretically ejected glutamate determined by pipette retraction. **A)** Maximum intensity projection of a two-photon image of a CA1 pyramidal cell dendrite filled with 100 μM Alexa 594 and the iontophoretic pipette in the initial position (scale bar 8 μm). Insets show retraction of the iontophoretic pipette and the corresponding iontophoretic EPSP recorded at the soma. Arrow indicates the position of the iontophoretic pipette tip. **B)** Distance dependence of EPSP amplitudes relative to the initial position of the iontophoretic pipette tip ($\leq 1 \mu\text{m}$ away from dendrite, $n = 6$ branches). With this we could estimate the radius of the glutamate spread by systematically retracting the pipette. Inset shows representative example traces. Error bars represent mean $\pm$ SEM. (Adapted from Müller *et al.* 2012⁹, reprinted with permission from Elsevier).

Figure 2. How an iontophoretic pipette should look like. **A)** Infrared CCD image of an iontophoretic pipette compared to a small 5.5 $\text{M}\Omega$ patch pipette using a 60X objective. **B)** Iontophoretic pipette with scale using a 60X objective. Calibration: smallest = 10 μm .

Figure 3. Capacitance compensation of the iontophoretic pipette using the build in test-pulse (10 nA, 10 msec, npi electronic, Tamm, Germany). It is important to compensate the capacitance correctly to monitor the right pipette resistance and to ensure fast and accurate neurotransmitter application.

Figure 4. Principles of micro-iontophoresis. **A)** Schematic drawing of a CA1 pyramidal neuron in whole cell patch-clamp configuration and an iontophoretic pipette filled with glutamate. Glutamate is negatively charged, therefore a positive current applied to the pipette will keep it from leaking out of the pipette: The retain current is positive (left panel). To eject glutamate from the pipette, apply a negative current (right panel). In this way glutamate is forced out of the pipette and can evoke excitatory events in the postsynaptic cell. **B)** Schematic drawing of CA1 pyramidal neuron in whole cell patch-clamp configuration and an iontophoretic pipette filled with GABA. GABA is positively charged at a low pH. Therefore, a negative current will keep it from leaking out of the pipette (left panel). To eject GABA apply a positive current (right panel). In this way GABA comes out of the pipette and can evoke inhibitory events in the postsynaptic cell.

Figure 5. Good and bad iontophoretic pipettes. **A)** Representative example of an iontophoretic glutamatergic EPSP generated on a dendritic branch of a CA1 pyramidal neuron. **B)** Representative example of an iontophoretic dendritic spike generated a dendritic branch in the CA1 area of the hippocampus with ongoing mild glutamate leakage from the pipette tip.

Figure 6. Representative results for single glutamate micro-iontophoresis. **A)** Schematic drawing of a patched CA1 pyramidal neuron and an iontophoretic pipette filled with glutamate. **B)** EPSP evoked in a CA1 pyramidal neuron with glutamate iontophoresis. **C)** Dendritic Na^+ / Ca^{2+} spike evoked on a proximal dendrite of a CA1 pyramidal neuron, lower trace shows the slope of the voltage trace, peak indicates the peak slope of the dendritic spike. **D)** When increasing the current applied to the iontophoretic the amplitude of the EPSP will increase until it crosses the action potential threshold.

Figure 7. Representative results for double glutamate micro-iontophoresis at different locations on the dendritic tree. **A)** Schematic drawing of a patched CA1 pyramidal neuron and two iontophoretic pipettes filled with glutamate, which are positioned on a proximal, and a distal dendrite, respectively. **B)** Iontophoretic EPSP evoked on a proximal dendrite of a CA1 pyramidal neuron (1). **C)** Iontophoretic EPSP evoked at a distal dendrite (2).

Figure 8. Representative results for GABA micro-iontophoresis. **A)** Schematic drawing of a patched CA1 pyramidal neuron and an iontophoretic pipette filled with GABA. **B)** Iontophoretic IPSP evoked on a proximal dendrite of a CA1 pyramidal neuron. **C)** Long current injection of systematically changing amplitudes to a CA1 pyramidal neuron via the patch pipette, to determine the reversal potential of the evoked event (current injections from -400 pA to 0 pA). Artifact indicates time-point of GABA iontophoresis. The evoked event indicates a reversal potential of approximately -70 mV (arrow).

Figure 9. Representative results of simultaneous glutamate and GABA iontophoresis to investigate integration of inhibition and excitation. **A)** Schematic drawing of a patched pyramidal neuron and one pipette filled with glutamate (green) and on with GABA (orange). **B)** Iontophoretically evoked dendritic spikes alone, in subsequent sweeps, lower traces show dV/dt , peaks indicate dendritic spikes. **C)** Iontophoretically evoked IPSP alone. **D)** Both, glutamatergic and GABAergic events together.

	Location specificity	Transmitter specificity	Toxicity / side effects	Presynaptic stimulation	Long-term experiment	Costs/complexity
Micro-iontophoresis	++	+++	++	-	++	++
2-photon uncaging	+++	+++	+	-	+	+
Synaptic stimulation	-	-	+++	+++	+++	+++

Table 1. Comparison of different techniques. Advantages and disadvantages of techniques to stimulate neurons according to different criteria (- = bad, + = not optimal, ++ = good, +++ = best).

	Patch pipettes		Iontophoretic pipettes	
	Pre-pulls P (A) Single pull	Last Pull P(B)	Pre-pulls P(A) Single Pull	Last Pull P(B)
Heat H	700	480	510	600
Force Pre Pull F(TH)	018	035	018	008
Distance Threshold s(TH)	017	012	025	015
Delay Heatstop t(H)	050	030	050	030
Distance Heatstop s(H)	030	000	030	000
Delay F(F1)	000	136	000	050
Force Pull 1 F1	000	065	200	400
Distance Pull 2 s(F2)	000	005	000	001
Force Pull 2 F2	000	080	000	095
Adjust (AD)	121	000	121	000

Table 2. Exemplarily puller protocol. For a horizontal puller (DMZ-Universal Puller, Zeitz-Instruments GmbH, Martinsried, Germany), using GB150F-8P filaments (Science Products, Hofheim, Germany) for both patch and iontophoretic pipettes.

Discussion

Here we explain how to apply fast micro-iontophoresis of neurotransmitters to investigate synaptic integration on dendrites. This technique has been successfully used to investigate glutamatergic and GABAergic synaptic transmission in different brain regions *in vitro* and *in vivo*^{9,20-22}. Micro-iontophoresis has been used for more than 60 years, but in early years it was mostly used to either locally apply neurotransmitters and drugs on slow or intermediate timescales²³ or for microinjection of substances into cells²⁴.

Micro-iontophoresis became a particularly interesting tool for studying dendritic integration since the introduction of amplifiers, which are equipped with a fast capacitance compensation for high-resistance electrodes^{10,11}. With this improvement it became possible to apply brief rectangular iontophoretic currents resulting in postsynaptic responses, which resembled more realistically the time course of synaptic events^{10,25}.

What are the technical requirements and difficulties to deal with, when establishing micro-iontophoresis? An iontophoretic amplifier is needed that allows for compensation of the high capacitance of the fine iontophoretic pipettes. The correct tuning of the capacity compensation is very important if high speed operation in conjunction with high resistance microelectrodes is required. Uncompensated stray capacitances are charged from the iontophoretic current that is supplied by the instrument, and therefore slows application. Compensating the capacitance properly is crucial and explained in detail in the video. Additionally, it is critical that the optical system gives a good fluorescent image of the dendrite and the iontophoretic pipette without inducing photo-damage of the tissue. Optimally, use a two-photon system and reduce laser power and dwell times as much as compatible with a decent image quality. If a more conventional light source is used, make sure to reduce the exposure time as much as possible, for example, by using mechanical shutters or electrical triggering. Probably the most critical point is the pipette design, which will allow for a defined and specific application of the neurotransmitter used. This step does require some effort and time to find the optimal settings. Here, we present protocols for glutamate and GABA iontophoresis, if other neurotransmitters, blockers or modulators are used, it is necessary that the substance is highly concentrated and even more importantly that it is charged. For example, since GABA has a net charge of zero at a pH of 0, the pH has to be adjusted (see Protocol section 2), resulting in a net positive charge.

When micro-iontophoresis is established, it will provide an extended toolset to study synaptic integration in neurons. It allows stimulating synapses of interest selectively with a particular neurotransmitter. Using a selected neurotransmitter on defined locations, postsynaptic currents and potentials and their propagation can be investigated. Furthermore, it is possible to use multiple iontophoretic electrodes simultaneously to investigate the integration of several glutamatergic inputs or systematically change the relative timing or the strength of events.

Some general critical points have to be considered when using micro-iontophoresis. The profile of neurotransmitter concentration after micro-iontophoresis is different from endogenous release. Murnick and others¹⁰ reported that the speed of release from the iontophoresis tip (about 1 msec) is likely to be significantly slower than synaptic release from vesicles, which occurs in fractions of a millisecond (0.2 msec)²⁶. Also, diffusion to and from the synaptic cleft is likely slowing concentration rise and decay of iontophoretically applied neurotransmitters. Furthermore, the volume of ejected neurotransmitter is likely to be higher than physiologically released volumes, however, with high-resistance electrodes, single glutamatergic and GABAergic postsynapses could be activated using micro-iontophoresis¹⁰⁻¹². A spatial selectivity in the range of a few micrometers has also recently been achieved by two-photon uncaging of neurotransmitters^{3,4,27}.

While considerably more cost intensive, two-photon uncaging has several advantages over micro-iontophoresis. In particular it can be used to release neurotransmitter simultaneously at multiple synaptic sites and does not require the precise positioning of a fine electrode tip. However, it also has some important disadvantages to be considered when selecting a method for a particular experiment. Many caged compounds have unwanted side effects such as the blockade of neurotransmitter receptors²⁸. For example, many glutamate and GABA cages have been reported to interfere with GABAergic inhibition. Also, the relatively high laser power required for two-photon uncaging may result in photo-damage of the postsynaptic neuron, particularly when the stimulation is applied repeatedly over several minutes. Furthermore, while micro-iontophoresis

is limited in the number of stimulated sites, these sites can be chosen freely on the whole dendritic tree of a neuron, for example on distal and basal dendrites, to simulate layered synaptic input from different brain regions. Two-photon uncaging, as it is currently used by most groups, will be limited to synaptic sites in a particular focal plane and in the field of view, which depends on the objective used (typically 40X or 60X water immersion). It has to be considered that both techniques always lead to activation of extrasynaptic receptors, which may make the interpretation of the results more difficult.

Another disadvantage of micro-iontophoresis is that only the postsynapse can be activated. If presynaptic function and the role of vesicle-released neurotransmitter need to be set into the experimental focus, local electrical synaptic stimulation may be a method of choice. However, the disadvantages of electrical synaptic stimulation compared to micro-iontophoresis are the unknown location of the activated synapses and the co-activation of axons from different neuronal populations, potentially belonging to other neurotransmitter systems (**Table 1**).

In summary the most important advantage of micro-iontophoresis is, in our view, that it is possible to use several different neurotransmitters and to study their interaction on neuronal compartments such as dendrites⁹.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

We thank Hans Reiner Polder, Martin Fuhrmann and Walker Jackson for carefully reading the manuscript. The authors received funding that was provided by the ministry of research MIWF of the state Northrhine-Westfalia (S.R.), the BMBF-Projekträger DLR US-German collaboration in computational neuroscience (CRCNS; S.R.), Centers of Excellence in Neurodegenerative Diseases (COEN; S.R.), and the University of Bonn intramural funding program (BONFOR; S.R.).

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