Video Article

Deriving the Time Course of Glutamate Clearance with a Deconvolution Analysis of Astrocytic Transporter Currents

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Abstract

The highest density of glutamate transporters in the brain is found in astrocytes. Glutamate transporters couple the movement of glutamate across the membrane with the co-transport of 3 Na^+ and 1 H^+ and the counter-transport of 1 K^+. The stoichiometric current generated by the transport process can be monitored with whole-cell patch-clamp recordings from astrocytes. The time course of the recorded current is shaped by the time course of the glutamate concentration profile to which astrocytes are exposed, the kinetics of glutamate transporters, and the passive electrotonic properties of astrocytic membranes. Here we describe the experimental and analytical methods that can be used to record glutamate transporter currents in astrocytes and isolate the time course of glutamate clearance from all other factors that shape the waveform of astrocytic transporter currents. The methods described here can be used to estimate the lifetime of flash-uncaged and synaptically-released glutamate at astrocytic membranes in any region of the central nervous system during health and disease.

Video Link

The video component of this article can be found at https://www.jove.com/video/50708/

Introduction

Astrocytes are one of the most abundant cell types in the brain with star-shaped morphology and fine membrane protrusions that extend throughout the neuropil and reach neighboring synaptic contacts [1,2]. The astrocytes' cell membrane is densely packed with glutamate transporter molecules [3]. Under physiological conditions, glutamate transporters rapidly bind glutamate at the extracellular side of the membrane and transfer it to the cell cytoplasm. By doing so, the transporters maintain low the basal concentration of glutamate in the extracellular space [4]. Glutamate transporters in fine astrocytic processes adjacent to excitatory synapses are ideally positioned to bind glutamate released during synaptic events as it diffuses away from the synaptic cleft. By doing so, the transporters also limit glutamate spillover towards peri- and extra-synaptic regions and onto neighboring synapses, reducing the spatial spread of excitatory signals in the brain [5-7].

Glutamate transport is an electrogenic process stoichiometrically coupled to the movement of 3 Na^+ and 1 H^+ along their electrochemical gradient and to the counter-transport of 1 K^+ [8]. Glutamate transport is associated with (but not stoichiometrically coupled to) an anionic conductance permeable to SCN^- (thiocyanate) > NO_3^- (nitrate) ≈ ClO_4^- (perchlorate) > I^- > Br^- > Cl^- > F^-, not to $CH_3SO_3^-$ (methane sulfonate) and $C_6H_{11}O_7^-$ (gluconate) [9-11]. Both currents (stoichiometric and non-stoichiometric) can be recorded by obtaining whole-cell patch-clamp recordings from astrocytes, visually identified under Dodt illumination or infra-red differential interference contrast (IR-DIC) in acute brain slices [12]. The stoichiometric component of the current associated with glutamate transport across the membrane can be isolated by using $CH_3SO_3^-$, or $C_6H_{11}O_7^-$ based intracellular solutions and can be evoked by flash-uncaging glutamate on astrocytes [13,14], or by activating glutamate release from neighboring synapses, either electrically [12] or with a targeted optogenetic control.

The time course of the stoichiometric component of the transporter current is shaped by the lifetime of the glutamate concentration profile at astrocytic membranes (*i.e.* glutamate clearance), the kinetics of glutamate transporters, the passive membrane properties of astrocytes, and during synaptic stimulations, by the synchronicity of glutamate release across the activated synapses [13]. Here we describe in full detail: *(1)* an experimental approach to isolate the stoichiometric component of glutamate transporter currents from whole-cell patch-clamp recordings from astrocytes using acute mouse hippocampal slices as an example experimental preparation; *(2)* an analytical approach to derive the time course of glutamate clearance from these recordings [13,14]. These methods can be used to record and analyze glutamate transporter currents from astrocytes in any region of the central nervous system.

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Protocol

1. Slice Preparation

1. Prepare 500 ml slicing/storage solution containing (in mM): 119 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 1.3 $MgSO_4 \cdot 7H_2O$, 4 $MgCl_2$, 26.2 $NaHCO_3$, 1 NaH_2PO_4, and 22 glucose, 320 mOsm, pH 7.4
2. Use a 250 ml beaker to prepare a submersion chamber for the slices, fill it with 200 ml slicing/storage solution, warm it in a water bath at 34 °C and bubble it with 95% O_2, 5% CO_2.
3. Keep the remaining slicing/storage solution in a glass bottle at 4 °C.
4. Use a cyanoacrylate adhesive to attach a small agar block (6%, prepared in ACSF) on the vibratome sample holder and store it at 4 °C.
5. 30 min before starting slicing, place the glass bottle containing the slicing/storage solution in a bucket filled with ice and bubble it with 95% O_2, 5% CO_2.

Note: *speed and precision are paramount for the dissection steps described below.*

6. Anesthetize the mouse (P14-21, C57BL/6) with halothane/isoflurane (isoflurane has been reported to enhance astrocytic glutamate uptake[15]), decapitate it, and dip the head in a 50 ml beaker containing oxygenated, cold slicing/storage solution.
7. Perform the dissection of the brain on an ice pack wrapped with paper towels and stored at -20 °C.
8. Use a scalpel to make a mid-sagittal skin incision on the dorsal side of the head, from the frontal to the caudal end, and expose the skull.
9. Place the lower shear blade of a small pair of surgical scissors in the occipital hole and make two cuts, towards the left and on the right hand side (45° angle).
10. Cut the skull along the mid-sagittal line, from the caudal to the frontal end.
11. Remove the brain with a spatula and dip it in oxygenated, cold slicing/storage solution.
12. With a scalpel, make five cuts to: *(1)* remove the olfactory bulbs and the frontal cortex; *(2)* remove the cerebellum; *(3)* remove the left and *(4)* right temporal lobes; *(5)* separate the two brain hemispheres with a mid-sagittal cut.
13. Dab the two parts of the brain with paper towels to remove any excess solution.
14. Glue the lateral surface of each brain section to the cold vibratome base plate: the dorsal side of the brain should be facing the vibratome blade; the ventral side of the brain should be in contact with the agar block, away from the vibratome blade.
    Note: *to obtain the highest-quality slices, it is important that the two parts of the brain are firmly glued to the vibratome base plate. To do this, use a cyanoacrylate adhesive that is not too liquid and that does not dry out too quickly.*
15. Secure the vibratome base plate to the dissecting chamber, set the slice thickness to 250 µm and adjust the width of the blade run. Proceed with slicing.
    Note: *if everything is oriented correctly, the vibratome should be cutting parasagittal slices from the dorsal towards the ventral and from the medial towards the lateral side of the brain.*
16. Once the blade has passed through the cortex and hippocampus, use a scalpel to cut the cortex/hippocampus from the midbrain and place each slice in the submersion chamber at 34 °C.
17. Discard the first couple of slices. Typically, 12 slices (250 µm thick) can be obtained from a P14-21 mouse brain.
18. Keep the slices at 34 °C for 30 min and let them cool down to room temperature for 30 min before using them for the electrophysiology recordings.

2. Astrocyte Identification and Recordings

1. Prepare an internal solution containing (in mM): 120 KCH_3SO_3, 10 EGTA, 20 HEPES, 2 MgATP, 0.2 NaGTP, 5 QX-314Br, and 5 NaCl, 290 mOsm, pH 7.2.
2. Prepare an extracellular recording solution containing (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4 \cdot 7H_2O$, 1 $MgCl_2$, 26.2 $NaHCO_3$, 1 $NaHPO_4$, and 22 glucose, 300 mOsm, pH 7.4, saturated with 95% O_2, 5% CO_2.
3. Add the following drugs to the extracellular recording solution, to block activation of GluA, GluN, mGluRII, mGluRIII, $GABA_A$, $GABA_B$, and A1 adenosine receptors (in µM): 10 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide disodium salt (NBQX), 10 (RS)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), (2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid disodium salt (LY341495), 100 (R,S)-α-methylserine-O-phosphate (MSOP), 100 picrotoxin, 5 3-[[(3,4-Dichlorophenyl)methyl]amino]propyl] diethoxymethyl)phosphinic acid (CGP52432), 1 8-cyclopentyl-1,3-dipropylxanthine (DPCPX).
4. Set the temperature of the extracellular solution in the recording chamber; typical temperatures are between 34 - 36 °C.
5. Prepare patch-clamp electrodes from borosilicate glass capillaries (R ≈ 2.5 MΩ) using a dual-stage, glass micro-pipette puller.
6. Take one of the slices and place it in the recording chamber. Hold it down with a metal harp made with platinum wire and nylon strings.
7. Visually inspect the slices under Dodt illumination or IR-DIC. Astrocytes can be identified by their small cell body (Ø = 10 µm) and prominent nucleus (**Figure 1**).
8. For synaptic stimulations, place a bipolar stainless steel electrode, ~100 µm away from the astrocyte that you plan to patch.
9. Patch the astrocyte and break in the whole-cell configuration by applying a very gentle suction.
    Note: *Astrocytes typically have low input resistance (~10 MΩ), hyperpolarized resting membrane potential (~-90 mV), and no firing activity. The astrocytes are kept at its resting membrane potential throughout the experiments in voltage-clamp mode (i.e. the holding current should read 0 pA). Before each stimulation, a 10 ms hyperpolarizing voltage step (-3 mV) is used to monitor the series and input resistance of the astrocyte.*
10. Discard the recordings if the series resistance changes >20% or if the membrane potential of the astrocyte becomes depolarized. The membrane potential of the astrocyte can be directly measured by switching from voltage-clamp to current-clamp mode. Alternatively, while in voltage-clamp mode, the membrane potential can be monitored by reading the value of the holding potential that results in 0 pA holding current.
11. If synaptic stimulations are employed, alternate single and paired stimuli (*e.g.* 100 msec apart), every 10 - 20 sec.

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12. For UV photolysis experiments, connect an uncaging Xe lamp to the epifluorescence port of the microscope and add the caged compound to the extracellular solution. Transporter currents of ~100 pA amplitude can be obtained when flash-uncaging 100 µM MNI-L-glutamate in the whole field of view (Ø = 662.5 µm when using a 40X objective [14]). Alternate stimulations with the light path open and blocked, every 10 - 20 sec.
13. Evoke glutamate transients and record transporter currents in control conditions and in the presence of a sub-saturating concentration of the broad-spectrum glutamate transporter antagonist D,L-*threo*-β-Benzyloxyaspartic acid (TBOA; 10 µM) to reduce the transporter current amplitude to at least 30% of its control value (see sections 4, 5) or in the presence of a high concentration of TBOA (50 - 100 µM) to block transporter currents completely and isolate the sustained K^+-current (see section 3).

3. Pharmacological Isolation of the Sustained K^+-current

1. Record astrocytic currents in control conditions and in the presence of a high, saturating concentration of TBOA (50 - 100 µM).
2. Average at least 20 sweeps in TBOA (50 - 100 µM).
3. Fit the averaged traces obtained in 3.2 with the function:

$$F(t) = A\left(1 - e^{-\frac{t}{\tau_{rise}}}\right)$$

Note: *the TBOA-insensitive component (i.e. the one that is recorded in the presence of TBOA (50 - 100 µM)) represents the sustained K^+-current. Its time course is approximated by the mono-exponential function described above.*

4. Repeat this fit across different astrocytes and derive an average value of T_{rise} (*i.e.* the average rise time of the sustained K^+-current (see section 5)).

4. Isolation of the Facilitated Portion of Synaptically-Activated Transporter Currents (fSTCs)

1. Average at least 20 sweeps obtained with paired stimulations, in control conditions and in TBOA (10 µM) (**Figure 2a** left).
2. Average an identical number of sweeps obtained with single stimulations, in control conditions and in TBOA (10 µM) (**Figure 2a** middle).
3. Compare the amplitude of the average current response to the -3 mV voltage step in the four averaged traces (control single pulse, control paired-pulses, TBOA single pulse, TBOA paired-pulses).
4. If the amplitude of the current response is the same in all four traces, proceed to step 4.7.
5. If the amplitude of the average current response is different in any of the four traces, check that all individual traces were appropriate to be included in the average.
6. If the amplitude of the average current is different in any of the four traces, but all individual traces are appropriate to be included in the average, verify that in each trace the size of the fSTC scales linearly with the size of the current response to the test voltage step. If this is the case, scale all traces with respect to each other so that the current responses to the test voltage step are all equal in amplitude.
Note: *In the recording conditions described here (i.e. intracellular $CH_3SO_3^-$ or $C_6H_{11}O_7^-$), synaptic stimulations of excitatory axons generate astrocytic currents with complex waveforms consisting of a fast-rising transient inward stoichiometric current and a slow-rising, sustained inward K^+-current reflecting K^+ re-equilibration in the extracellular space following action potential propagation along neighboring axons. It is critical to remove this sustained K^+-current, as any residual current would lead to an overestimate of glutamate lifetime.*
7. Subtract the average trace obtained with single stimulations from the average trace obtained with paired stimulations (**Figure 2a** right). This step allows isolating the stoichiometric and the sustained K^+-current evoked by the second stimulus.
8. Shift the average trace obtained with single stimulations by a time interval that matches the inter-pulse interval used to deliver paired-pulses (*i.e.* 100 msec) (**Figure 2b**) .
9. Subtract the time-shifted average response obtained with single stimulations from the average response to the second stimulus obtained in 4.7 (**Figure 2c**). This step isolates the facilitated portion of the transporter current evoked by the second stimulus (the fSTC).
10. In most cases, the previous step removes entirely the sustained K^+-current. If this is the case, the isolation of the fSTC is complete and you can proceed with the deconvolution analysis and derive the time course of glutamate clearance from astrocytes. In some cases, however, a small sustained K^+-current is still present (**Figure 2d**) and further analysis is required (see section 5).
Note: remember that the analysis described in 4.7-4.10 must be performed on the average traces obtained in control conditions and in TBOA (10 µM).

5. Subtraction of the Residual zdustained K^+-current from fSTCs

1. Measure the amplitude of the sustained K^+-current remaining after performing the analysis described in section 4.
2. Scale the amplitude of the mono-exponential function described in 3.3 to the amplitude of the residual sustained K^+-current measured in 5.1 (**Figure 2d** left and middle). To do this, in equation 3.3, set the term *A* equals to the amplitude of the sustained K^+-current measured in 5.1 and the term T_{rise} equals to the average value of T_{rise} estimated in 3.4.
3. Subtract the resulting mono-exponential function from the fSTC and sustained K^+-current obtained in 4.10 (**Figure 2d** right). This step completes the isolation of the fSTC.

6. Isolation of Flash-activated Transporter Currents (FTCs)

1. Average at least 20 sweeps obtained with the light path open, in control conditions and in TBOA (10 µM).
2. Average an identical number of sweeps obtained with the light path closed, in control conditions and in TBOA (10 µM).
3. Compare the amplitude of the current response to the -3 mV voltage step in the four averaged traces (control single pulse, control paired-pulses, TBOA single pulse, TBOA paired-pulses).
4. If the amplitude of the current response is the same in all four traces, proceed to step 6.7.

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5. If the amplitude of the average current response is different in any of the four traces, check that all individual traces were appropriate to be included in the average.
6. If the amplitude of the average current is different in any of the four traces, but all individual traces are appropriate to be included in the average, verify that in each trace with the light path open the size of the FTC scales linearly with the size of the current response to the test voltage step. If this is the case, scale all traces with respect to each other so that the current responses to the test voltage step are all equal in amplitude.
Note: in the recording conditions described here (i.e. intracellular $CH_3SO_3^-$ or $C_6H_{11}O_7^-$), flash stimuli generate astrocytic currents consisting only of the fast-rising transient stoichiometric inward current.
7. Subtract the average trace obtained with the light path blocked from the average trace obtained with the light path open. This step allows removing the stimulus artifact and isolating the FTCs.
Note: *the analysis described in 6.7 must be performed on the average traces obtained in control conditions and in TBOA (10 µM).*

7. Deconvolution analysis

1. Fit the transporter current (fSTC or FTC) recorded in control conditions (**Figure 3a** left) and in TBOA (10 µM) (**Figure 3a** right) and isolated as described in sections 4 - 6, with the multi-exponential function:
$$G(t) = A_1 \left(1 - e^{-\frac{t_0 - t}{\tau_{rise}}}\right)^n \left(e^{-\frac{t_0 - t}{\tau_{fast}}} + A_2 e^{-\frac{t_0 - t}{\tau_{slow}}}\right)$$
2. Create an instantaneously rising function that decays mono-exponentially according to the function:
$$H(t) = Ae^{-\frac{t}{\tau}}$$
and that best describes the decaying phase of the transporter current recorded in TBOA (10 µM) (**Figure 3b**).
Note: *the function described in 7.2 (i.e. H(t)) represents the time course of glutamate clearance from astrocytes in the presence of TBOA (10 µM).*
3. Deconvolve the mono-exponential function obtained in 7.2 (**Figure 3b** and **3c** middle) from the fit of the transporter current recorded in TBOA (10 µM) obtained in 7.1 (**Figure 3a** right, **c** left).
Note: *deconvolution is a mathematical operation included in many analysis software packages like (e.g. Matlab, Python). In IgorPro, the programming code that we typically use to perform this kind of analysis, the deconvolution operation can be efficiently computed as described below, by using discrete fast Fourier transforms. First, use the FFT operation to compute the discrete fast Fourier transform of the mono-exponential function obtained in 7.2 and of the fit of the transporter current recorded in TBOA obtained in 7.1. Next, use the IFFT operation to compute the inverse discrete fast Fourier transform of the ratio of the two FFT functions. The resulting function I(t) can be described as follows:*
$$I(t) = G(t) \times^{-1} H(t) = IFFT\left(\frac{FFT(G(t))}{FFT(H(t))}\right)$$
*The step described in 7.3 allows deriving the filter of the transporter current in TBOA (10 µM) (**Figure 3c** right). The filter represents the distortion factors that convert the lifetime of glutamate at astrocytic membranes into the transporter current isolated in sections 4 - 6 (i.e. the fSTC or FTC).*
4. Deconvolve the filter (**Figure 3c** right, **Figure 3d** middle) from the fit of the transporter current in control conditions (**Figure 3a** left, **Figure 3d** left).
Note: *this step allows deriving the time course of glutamate clearance from astrocytes in control conditions (**Figure 3d** right). The assumption underlying this deconvolution step is that the temporal profile of the filter remains unaltered in control conditions and in TBOA (10 µM).*
5. To obtain a quantitative estimate of the overall time course of glutamate clearance, calculate the centroid (<*t*>) of the waveform obtained in step 7.4. This is done by calculating <*t*> as:
$$< t >= \frac{\int G(t)tdt}{\int G(t)dt}$$
where *G(t)* is the waveform obtained in step 7.1. In the equation described above, the term *t* corresponds to the time window over which the integral is calculated.
Note: *when the method was first established, <t> was calculated over time windows that were left unconstrained [13]. This approach can be used as long as the integration window is set to be wider than the duration of glutamate clearance and the clearance waveform decays completely back to baseline. If this is not the case, however, this method of estimating <t> encounters some limitations. For example, if the clearance waveform does not decay exactly back to baseline, then <t> increases with the width of the integration window. To avoid any potential source of inaccuracy for the estimate of <t>, we now calculate it over a time window corresponding to 10% of the peak of the transporter current, before and after its onset [14]. The latter approach improves the consistency with which <t> is measured across cells. This comes in handy particularly when analyzing small effects of pharmacological treatments on the time course of clearance.*

Representative Results

The success of the analytical approach described here critically depends on obtaining high-quality electrophysiological recordings of transporter currents from astrocytes in any region of the central nervous system. In acute mouse hippocampal slices, astrocytes can be readily identified under Dodt illumination or IR-DIC because of their small cell body (Ø = 10 µm) and prominent nucleus (**Figure 1**). Their distinctive star-shaped morphology can be appreciated with epifluorescence, confocal, or two-photon laser scanning microscopy, when adding a fluorophore like Alexa 594 (50 µM) to the intracellular solution, (**Figure 1**). At the electrophysiology level, astrocytes are characterized by low input resistance,

hyperpolarized membrane potential (~-90 mV) and inability to generate action potentials. Astrocytes generate currents in response to electrical or optogenetic stimulation of neighboring neurons and UV photolysis of caged compounds like MNI-L-glutamate.

Astrocytic transporter currents can be recorded using Cs^+ or K^+ based internal solutions. Note that Cs^+, a popular K^+ substitute, supports glutamate transport but slows the glutamate transporter cycling rate [16,17] (because glutamate transporters bind and/or translocate Cs^+ across the membrane more slowly than K^+). If Cs^+ reduces significantly the number of transporters available for binding glutamate, it also leads to smaller/slower transporter currents than the ones recorded with K^+ based internal solutions [16,17].

When using $CH_3SO_3^-$ or $C_6H_{11}O_7^-$ as the main intracellular anion, the current generated by glutamate transporters in astrocytes is due to the stoichiometrically-coupled movement of 1 glutamate, 3 Na^+, 1 H^+ and 1 K^+ across the membrane. This stoichiometric current is the only one recorded from astrocytes in photolysis experiments and we refer to it as the FTC. When performing photolysis experiments, it is recommended to alternate UV stimulations obtained with the light path open and blocked. The traces obtained with the light path blocked are useful to isolate the stimulus artifact generated by the flash lamp and can be subtracted from the ones obtained with the light path open to perfectly isolate the FTC.

The current recorded in astrocytes when evoking synaptic release of glutamate has a more complex waveform. It is composed of a fast-rising, transient inward component due to the stoichiometrically-coupled glutamate transporter current described above. In addition, it comprises a slow-rising, sustained inward K^+-current due to influx of K^+ accumulated in the extracellular space after action potential propagation along neighboring axons. A pharmacological approach can be used to isolate the transporter current from the sustained K^+-current and requires the use of high concentrations of the broad-spectrum glutamate transporter antagonist TBOA (100 µM) at the end of the experiment [12]. The method described here uses a purely analytical approach to isolate the glutamate transporter current from the sustained K^+-current and therefore allows for shorter experiments (**Figure 2**).

As a first step, we interleave single and paired-pulse synaptic stimulations (100 msec apart), every 10 - 20 sec, and average an equal number of the sweeps obtained with single and paired-pulse stimuli. The amplitude of the first current evoked by the paired stimuli should match the amplitude of the current evoked by the single stimulus. We begin this analysis by subtracting the current evoked by the single stimulus from that evoked by the paired stimuli. This subtraction allows isolating the response to the second stimulus, which is also composed of a fast-rising, transient glutamate transporter current and a slow-rising, sustained K^+-current (**Figure 2a** right). Next, we shift the response to the single stimulus by a time interval corresponding to the inter-pulse interval of the paired stimuli, so that the time of onset of the single response matches the time of onset of the second response isolated above (**Figure 2b**). By subtracting these two currents, we isolate the facilitated portion of the transporter current, which here we call fSTC (**Figure 2c**). The kinetics of the fSTC are similar to the kinetics of the synaptically-activated transporter current (STCs) isolated pharmacologically with high concentrations of the glutamate transporter antagonist TBOA (100 µM) [13]. For this reason and for simplicity, in previous work, we referred to the fSTC as STC [13,14]. Despite being currents with similar properties, fSTCs and STCs are not the same current. For this reason and for accuracy, in this work, we will refer to them explicitly as fSTCs and STCs. The subtraction method described here allows for accurate isolation of fSTCs in most recordings. However, in some cases, a residual sustained K^+-current is still present. In this case, it is necessary to isolate the sustained K^+-current in separate experiments using high concentrations of TBOA (50 - 100 µM). The time course of the pharmacologically-isolated sustained K^+-current can be described by a mono-exponential function in the form:

$$F(t) = A\left(1 - e^{-\frac{t}{\tau_{rise}}}\right)$$

By recording astrocytic currents in different cells, we estimate the average value of T_{rise}. We next create a mono-exponential function like the one described above, in which T_{rise} corresponds to the average value of T_{rise} measured experimentally in different astrocytes and A corresponds to the amplitude of the residual sustained K^+-current that we want to remove (**Figure 2d**).

The method we just described to isolate fSTCs works nicely when evoking glutamate release from facilitating synapses and can also be used to study glutamate release from synapses that undergo paired-pulse depression. However, it is of little use when the glutamatergic synapses under study do not show any clear form of short-term facilitation or depression. In this case, the pharmacological isolation of transporter currents with high concentrations of TBOA (100 µM) remains the most feasible solution to isolate the stoichiometrically-coupled, synaptically-evoked glutamate transporter current.

To perform the deconvolution analysis and measure the lifetime of glutamate at astrocytic membranes it is necessary to isolate FTCs or fSTCs in control conditions and in the presence of a sub-saturating concentration of TBOA (10 µM), to reduce the transporter current amplitude to at least 30% of its value in control conditions (*i.e.* without blocking it entirely). The goal here is to slow down significantly the time course of the transporter current without losing the ability to distinguish it from the noise of the recordings. The underlying assumption is that in the presence of a small concentration of TBOA, when the uptake capacity of astrocytes is diminished, the main factor shaping the decay of the transporter current is the gradual decline of the glutamate concentration at the astrocytic membrane [12,18]. We fit the transporter current in control conditions and in TBOA (10 µM) with the multi-exponential function:

$$G(t) = A_1\left(1 - e^{-\frac{t_0 - t}{\tau_{rise}}}\right)^n \left(e^{-\frac{t_0 - t}{\tau_{fast}}} + A_2 e^{-\frac{t_0 - t}{\tau_{slow}}}\right)$$

(**Figure 3a**). We approximate the glutamate clearance in TBOA (10 µM) with an instantaneously-rising function with mono-exponential decay (**Figure 3b**) and we deconvolve it from the multi-exponential function describing the transporter current in TBOA (10 µM; **Figure 3c**). The resulting waveform is the "filter", and represents the combination of factors that distort (*i.e.* filter) the glutamate concentration profile into the transporter current (**Figure 3c**). Deconvolving the filter from the multi-exponential fit of the transporter current in control conditions allows estimating the time course of the glutamate concentration profile at the recorded astrocyte in control conditions (**Figure 3d**). For this deconvolution analysis, we assume that the kinetics of the filter remain unaltered in the absence and in the presence of TBOA (10 µM). This

is a reasonable assumption because all factors that contribute to the filter (*i.e.* transporter kinetics, electrotonic filtering, and during synaptic stimulations, asynchronous release of glutamate across synapses) are unlikely to be influenced by the presence of TBOA, a compound that reduces the number of transporters available for binding glutamate.

We use the centroid (<*t*>) as a measure of the glutamate lifetime at astrocytic membranes in different cells. <*t*> represents the "center of mass" of the clearance waveform, and is sensitive to changes in both rise time and decay of the glutamate clearance waveform. The centroid is expressed as:

$$< t >= \frac{\int G(t)tdt}{\int G(t)dt}$$

There are two ways to decide the time interval over which we can calculate the integrals at the numerator and denominator of this expression. Because the clearance waveform rises from and decays back to zero, when the method was first established, there was no specification made on how to set the integration window, and in fact this was done rather loosely [13]. One way around this issue is to limit the integration window to the time it takes to reach an arbitrary proportion of the FTC/fSTC peak, for example 10% [14]. This simple criterion allows being consistent when performing this analysis in different cells, improves the accuracy in the estimates of <*t*> when the clearance waveform does not decay perfectly to zero, and improves the sensitivity of the analysis to small differences in clearance waveforms that might occur in different groups of cells.

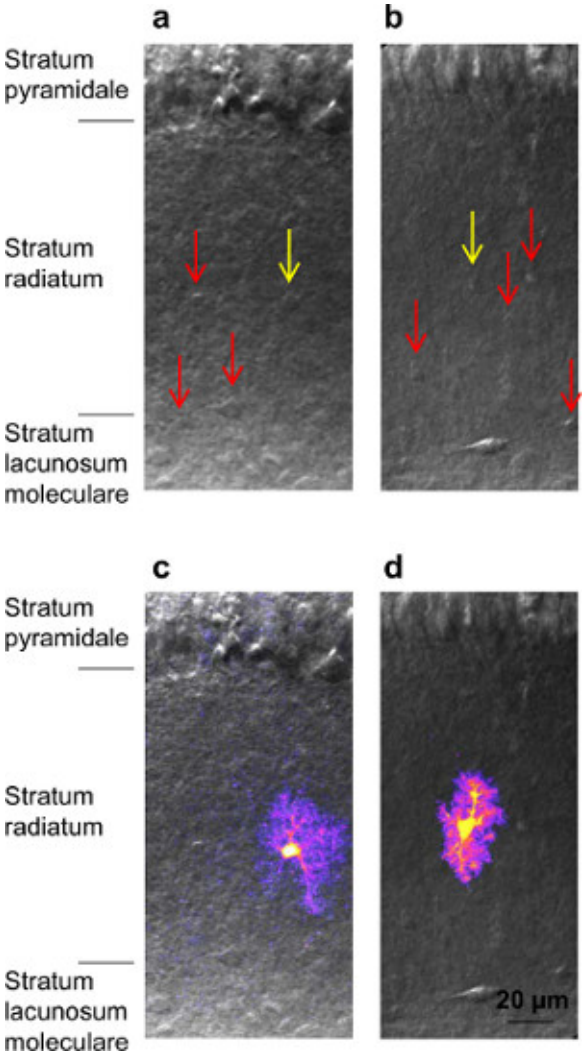

Figure 1. Identification of astrocytes in acute hippocampal slices. (**a-b**) Dodt images of hippocampal astrocytes in CA1 stratum radiatum. Under Dodt illumination and IR-DIC, astrocytes can be identified by their small cell body and prominent nucleus. The red arrows point to multiple astrocytes that can be identified in the slices preparation. The yellow arrow shows the astrocyte that was patched and filled with a fluorescent dye (see below). (**c-d**) Overlaid Dodt image of the hippocampal slices and two-photon of the patched astrocytes, highlighted with a yellow arrow in (**a-b**). For this experiment, astrocytes were patched and filled with a KCH_3SO_3 - based internal solution added with Alexa 594 (50 µM). The astrocytic processes extend a few tens of µm away from the cell body. The annotations of the left identify the three main cellular layers of the hippocampal CA1 formation that can be recognized in the slices.

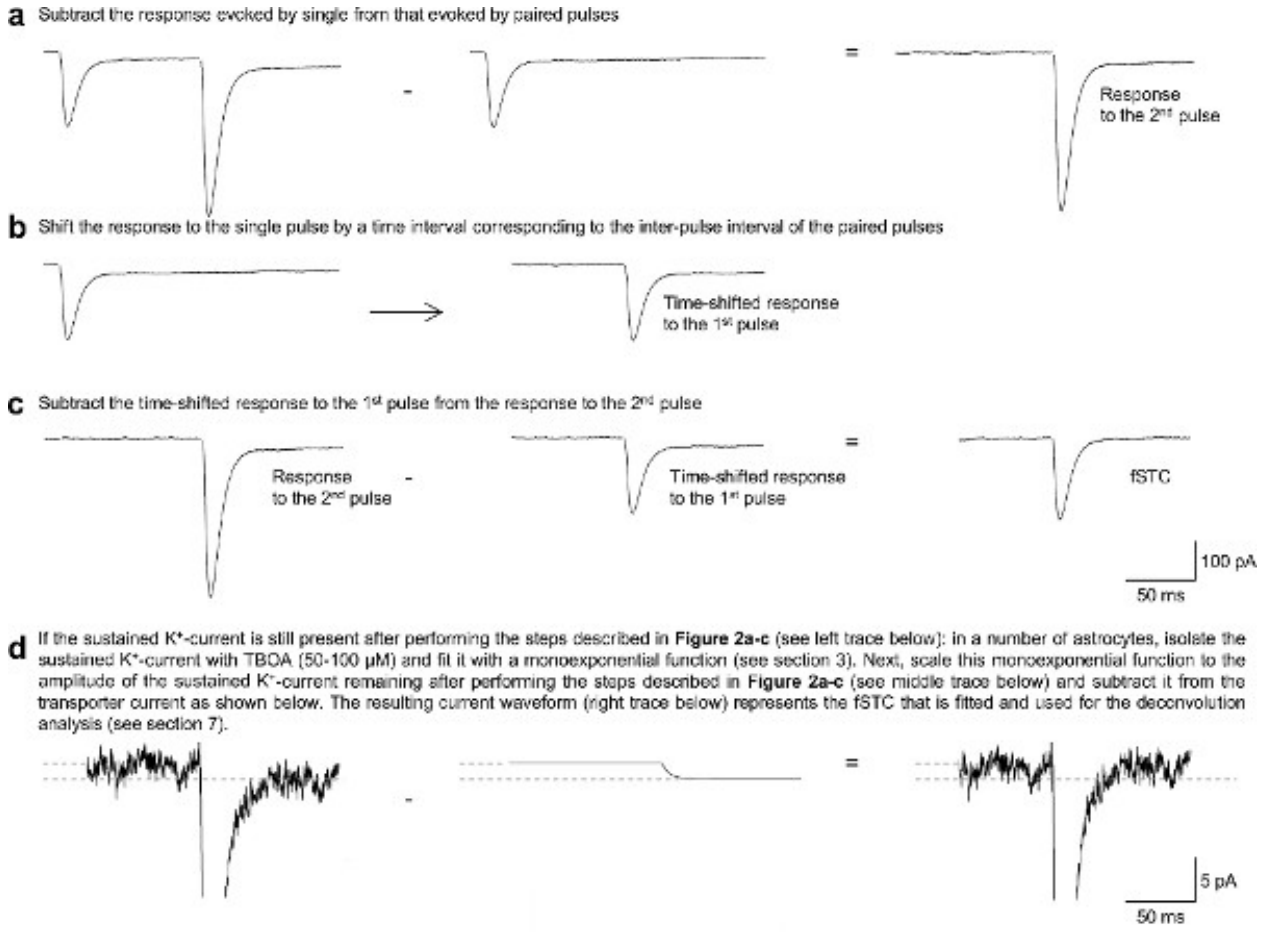

Figure 2. Isolation of fSTCs from synaptically-activated transporter currents recorded in astrocytes. (**a-c**) Scheme of the analytical steps required to isolate the fSTCs from synaptically-activated transporter currents recorded in astrocytes. (**d**) Analytical approach used to remove the residual sustained K^+-current from the fSTCs (see section 5). Click here to view larger figure.

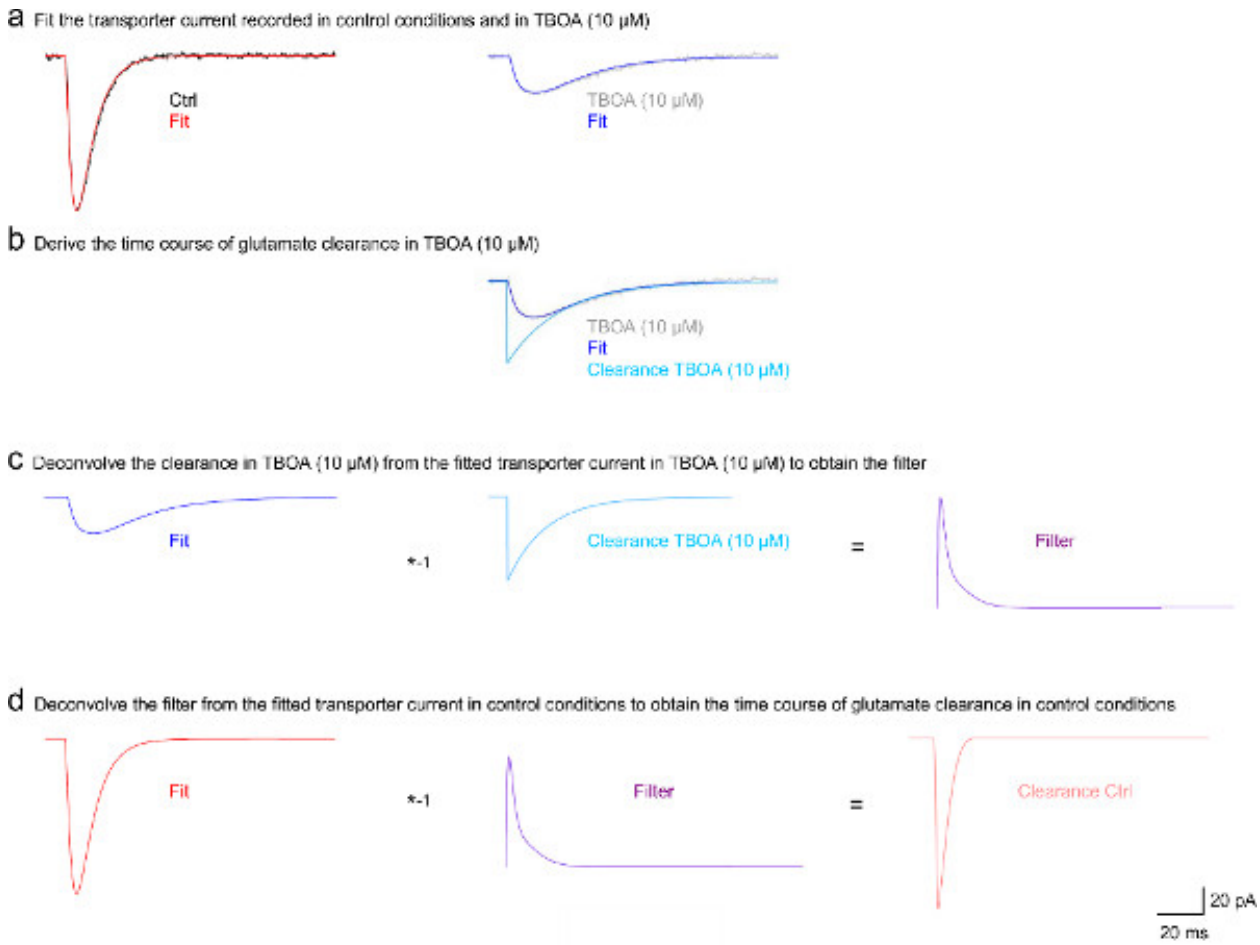

Figure 3. Deconvolution analysis used to derive the time course of glutamate clearance from astrocytes. (**a**) Mono-exponential fit of the fSTC/FTC isolated in control conditions (left) and in the presence of TBOA (10 µM; right). (**b**) The time course of glutamate clearance in TBOA (10 µM) is approximated by an instantaneously-rising function with mono-exponential decay. (**c**) Deconvolving the time course of glutamate clearance in TBOA (10 µM) from the multi-exponential fit of the transporter current in TBOA (10 µM) allows estimating the filter. (**d**) Deconvolving the filter from the multi-exponential fit of the transporter current in control conditions allows estimating the time course of glutamate clearance from astrocytes in control conditions. Click here to view larger figure.

Discussion

Here we describe an experimental approach to obtain electrophysiological recordings from astrocytes, an analytical protocol to isolate glutamate transporter currents in astrocytes and a mathematical method to derive the time course of glutamate clearance from astrocytic transporter currents.

The success of the analysis relies on the ability to obtain high-quality patch clamp recordings from astrocytes and on the accuracy of the fitting algorithms used to describe the transporter currents. The deconvolution analysis relies on the following two assumptions. *(1)* The multiple processes that distort the time course of glutamate clearance into the experimentally-recorded transporter current can be represented as a linear system. Although in absolute terms many of the factors that shape transporter currents are non-linear systems (in principle glutamate binding and transport can both undergo saturation), experimental evidence indicates that their linear regime is broad. Accordingly, the time course of transporter currents and of glutamate clearance remains the same over a broad range of stimulus strength, release probability, and glutamate concentrations [13,14,18]. Although this approximation seems to be legitimate for our experimental conditions, it should be validated before repeating this analysis in different experimental preparations. *(2)* The characteristics of the filter are unaffected by the presence of TBOA (10 µM). This assumption seems reasonable because all factors that contribute to the filter (*i.e.* transporter kinetics, electrotonic filtering, and during synaptic stimulations, asynchronous release of glutamate across synapses) are unlikely to be influenced by the presence of TBOA.

By deriving the glutamate clearance waveform from fSTCs and FTCs, we obtain two different types of information. By analyzing fSTCs, we measure the time that astrocytic membranes are exposed to glutamate released from synapses. By evoking FTCs with full field UV illumination (*i.e.* by uncaging glutamate over the whole astrocytic surface), we measure the time that astrocytic membranes are exposed to uncaged glutamate. In these uncaging experiments all glutamate molecules are released (*i.e.* uncaged) at the same time and all transporters are activated at the same time, by the same glutamate concentration. Therefore, by analyzing FTCs, we can obtain an indirect readout of the astrocytic glutamate uptake capacity.

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It is important to note that the methods described here allow estimating the *time course* of glutamate clearance, not the actual value of the *glutamate concentration* evoking the transporter currents. The estimated time course is an average measure of the time course of glutamate clearance at all areas of the astrocytic membrane to which we have electrophysiological access: it is not sensitive to heterogeneities in glutamate uptake at different sites within the astrocytic membrane [19]. This information is more likely to be obtained with high-resolution microscopy of glutamate-sensitive fluorescent reporters [20]. However, the currently available glutamate-sensitive reporters exhibit relatively narrow dynamic ranges. This can introduce non-linear distortions between the glutamate concentration profile and the fluorescent signal, limiting the use of these probes to extract temporal information on the glutamate concentration transient. Therefore, it is probably by combining all these different approaches that one can get the most comprehensive understanding of the dynamics of glutamatergic signals at astrocytic membranes.

In summary, the methods presented here can be used to estimate the clearance time course of synaptically-released or flash-uncaged glutamate at astrocytic membranes. They provide valuable tools that can be used to estimate the lifetime of glutamate in the extracellular space at different stages of development [13] in a variety of animal species [14] and brain regions under physiological and pathological conditions [21].

Disclosures

The authors declare no conflict of interest.

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References

1. Ventura, R. & Harris, K.M. Three-dimensional relationships between hippocampal synapses and astrocytes. *J. Neurosci.* **19**, 6897-6906 (1999).
2. Witcher, M.R., Kirov, S.A., & Harris, K.M. Plasticity of perisynaptic astroglia during synaptogenesis in the mature rat hippocampus. *Glia.* **55**, 13-23, doi:10.1002/glia.20415 (2007).
3. Danbolt, N.C. Glutamate uptake. *Prog. Neurobiol.* **65**, 1-105 (2001).
4. Herman, M.A. & Jahr, C.E. Extracellular glutamate concentration in hippocampal slice. *J. Neurosci.* **27**, 9736-9741, doi:10.1523/JNEUROSCI.3009-07.2007 (2007).
5. Arnth-Jensen, N., Jabaudon, D., & Scanziani, M. Cooperation between independent hippocampal synapses is controlled by glutamate uptake. *Nat. Neurosci.* **5**, 325-331, doi:10.1038/nn825 (2002).
6. Barbour, B. An evaluation of synapse independence. *J. Neurosci.* **21**, 7969-7984 (2001).
7. Rusakov, D.A. & Kullmann, D.M. Extrasynaptic glutamate diffusion in the hippocampus: ultrastructural constraints, uptake, and receptor activation. *J. Neurosci.* **18**, 3158-3170 (1998).
8. Zerangue, N. & Kavanaugh, M.P. Flux coupling in a neuronal glutamate transporter. *Nature.* **383**, 634-637, doi:10.1038/383634a0 (1996).
9. Eliasof, S. & Jahr, C.E. Retinal glial cell glutamate transporter is coupled to an anionic conductance. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 4153-4158 (1996).
10. Wadiche, J.I., Amara, S.G., & Kavanaugh, M.P. Ion fluxes associated with excitatory amino acid transport. *Neuron.* **15**, 721-728 (1995).
11. Wadiche, J.I. & Kavanaugh, M.P. Macroscopic and microscopic properties of a cloned glutamate transporter/chloride channel. *J. Neurosci.* **18**, 7650-7661 (1998).
12. Bergles, D.E. & Jahr, C.E. Synaptic activation of glutamate transporters in hippocampal astrocytes. *Neuron.* **19**, 1297-1308 (1997).
13. Diamond, J.S. Deriving the glutamate clearance time course from transporter currents in CA1 hippocampal astrocytes: transmitter uptake gets faster during development. *J. Neurosci.* **25**, 2906-2916, doi:10.1523/JNEUROSCI.5125-04.2005 (2005).
14. Scimemi, A., Tian, H,. & Diamond, J.S. Neuronal transporters regulate glutamate clearance, NMDA receptor activation, and synaptic plasticity in the hippocampus. *J. Neurosci.* **29**, 14581-14595, doi:10.1523/JNEUROSCI.4845-09.2009 (2009).
15. Zuo, Z. Isoflurane enhances glutamate uptake via glutamate transporters in rat glial cells. *Neuroreport.* **12**, 1077-1080 (2001).
16. Barbour, B., Brew, H., & Attwell, D. Electrogenic uptake of glutamate and aspartate into glial cells isolated from the salamander (Ambystoma) retina. *J. Physiol.* **436**, 169-193 (1991).
17. Bergles, D.E., Tzingounis, A.V., & Jahr, C.E. Comparison of coupled and uncoupled currents during glutamate uptake by GLT-1 transporters. *J. Neurosci.* **22**, 10153-10162 (2002).
18. Diamond, J.S. & Jahr, C.E. Synaptically released glutamate does not overwhelm transporters on hippocampal astrocytes during high-frequency stimulation. *J. Neurophysiol.* **83**, 2835-2843 (2000).
19. Benediktsson, A.M., *et al.* Neuronal activity regulates glutamate transporter dynamics in developing astrocytes. *Glia.* **60**, 175-188, doi:10.1002/glia.21249 (2012).
20. Hires, S.A., Zhu, Y., & Tsien, R.Y. Optical measurement of synaptic glutamate spillover and reuptake by linker optimized glutamate-sensitive fluorescent reporters. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 4411-4416, doi:10.1073/pnas.0712008105 (2008).
21. Scimemi, A., Meabon, J., Woltjer, R.L., Sullivan, J.M., Diamond, J.S., Cook, D.G. Amyloidβ1-42 slows clearance of synaptically-released glutamate by mislocalizing astrocytic GLT-1. *J. Neurosci.* **33**, 5312-5318 (2013).

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