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Whole-Cell Patch-Clamp Analysis of Recombinant NMDA Receptor Pharmacology Using Brief Glutamate Applications

Nathan G. Glasgow and **Jon W. Johnson**

Department of Neuroscience and Center for Neuroscience, University of Pittsburgh, Pittsburgh, Pennsylvania 15260.

Summary

NMDA receptors (NMDARs) are ionotropic glutamate receptors that are essential for synaptic plasticity, learning and memory. Dysfunction of NMDARs has been implicated in many nervous system disorders; therefore, pharmacological modulation of NMDAR activity has great therapeutic potential. However, given the broad physiological importance of NMDARs, modulating their activity often has detrimental side effects precluding pharmaceutical use of many NMDAR modulators. One approach to possibly improve the therapeutic potential of NMDAR modulators is to identify compounds that modulate subsets of NMDARs. An obvious target for modulating NMDAR subsets are the many NMDAR subtypes produced through different combinations of NMDAR subunits. With seven identified genes that encode NMDAR subunits, there are many neuronal NMDAR subtypes with distinct properties and potentially differential pharmacological sensitivities. Study of NMDAR subtype-specific pharmacology is complicated in neurons, however, because most neurons express at least three NMDAR subtypes. Thus, use of an approach that permits study in isolation of a single receptor subtype is preferred. Additionally, the effects of drugs on agonist-activated responses typically depend on duration of agonist exposure. To evaluate drug effects on synaptic transmission, an approach should be used that allows activation of receptor responses as brief as those observed during synaptic transmission, both in the absence and presence of drug. To address these issues, we designed a fast perfusion system capable of (1) delivering brief (~5 ms) and consistent applications of glutamate to recombinant NMDARs of known subunit composition, and (2) easily and quickly (~5 seconds) changing between glutamate applications in the absence and presence of drug.

Keywords

NMDA; NMDA receptor subtype; memantine; ketamine; open channel blocker; brief agonist application

1. Introduction

There is great interest in pharmacologically modulating ligand-gated ion channels to augment nervous system function or alleviate aberrant activity potentially underlying nervous system disorders. The whole-cell patch-clamp technique is essential in

understanding how drugs affect ligand-gated ion channel function, cell physiology, and the nervous system under normal and pathological conditions. Due to the great diversity of subtypes within each ligand-gated ion channel family, pharmacological analysis of a particular ligand-gated ion channel using native cells is complicated. Furthermore, the mechanisms underlying drug actions on ligand-gated ion channels may depend upon the concentration and duration of agonist exposure to receptors. Therefore, expression of recombinant ligand-gated ion channels in mammalian cell lines in conjunction with a fast perfusion system designed to deliver brief agonist applications is very useful in understanding how drugs affect ligand-gated ion channel function. Here we describe a method for whole-cell patch-clamp analysis of ligand-gated ion channel pharmacology that allows precise control of (1) the receptor subunit composition, (2) the agonist concentration, and (3) the duration of agonist exposure to receptors. Our method also allows brief application of agonist in the absence and presence of drug to the same cell. Here, we demonstrate use of the system to investigate inhibition of recombinant NMDARs during brief glutamate applications.

NMDARs are ionotropic glutamate receptors that exhibit voltage-dependent Mg^{2+} block, are highly Ca^{2+} permeable, and deactivate slowly. These properties contribute to the importance of NMDARs to cell survival, synaptic plasticity, and many forms of learning and memory (1). Aberrant activation of NMDARs is implicated in neurodegenerative diseases, ischemia, depression, and neuropathic pain (2-7). Pharmacological inhibition of NMDARs is considered to have great therapeutic potential in treating these disorders (1), although broad inhibition of NMDARs often results in undesirable side effects (8,9). Thus, identification of NMDAR antagonists selective for NMDARs that may be involved in a pathological state while preserving the function of NMDARs underlying normal function may be vital for successful pharmacological therapy (9-11).

NMDARs are heterotetramers composed of two GluN1 subunits either with two GluN2 subunits or with one GluN2 and one GluN3 subunit (1). There is a single gene that encodes eight splice variants of the GluN1 subunit, four genes that encode four GluN2 subunits (GluN2A, GluN2B, GluN2C and GluN2D), and two genes that encode two GluN3 subunits (GluN3a and GluN3B). Different combinations of GluN1, GluN2, and GluN3 subunits give rise to NMDAR subtypes with distinct properties. Combinations that include two identical GluN2 subunits form diheteromeric NMDARs (e.g. GluN1/2A) and combinations that include either two different GluN2 subunits, or mixtures of GluN2 and GluN3 subunits, form triheteromeric NMDARs (e.g. GluN1/2A/2B) (1,10). In principal cells in the cortex, at least 3 NMDAR subtypes, including GluN1/2A, GluN1/2B, and GluN1/2A/2B, are expressed and can be found postsynaptically (10,12). Consequently, it is difficult to study synaptic NMDAR subtype-specific pharmacology in neurons. Given this difficulty, we emulate synaptic release of glutamate using brief glutamate applications to tsA201 cells expressing recombinant GluN1/2A or GluN1/2B receptors. This approach allows pharmacological assessment of NMDARs with known, uniform subunit compositions. The methods described in this chapter provide a powerful approach to studying ligand-gated ion channel currents in response to brief agonist applications in the absence and presence of many types of drugs.

2. Materials

2.1 Cell Culture and Transfection

1. tsA201 cell culture medium: DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) and 1% Glutamax (Life Technologies).
2. Serum-free tsA201 cell culture medium: DMEM supplemented with 1% Glutamax.
3. tsA201 cells (The European Coalition of Cell Cultures, ECACC) are plated on 15 mm glass coverslips (Carolina Biological) in 35 mm petri dishes (BD Falcon).
4. cDNAs encoding the rat GluN1-1a (GenBank X63255 in pCDM8 vector), GluN2A (GenBank M91561 in PCDM8 vector), and GluN2B (GenBank M91562 in pCDNA1 vector) subunits are cotransfected with cDNA for enhanced green fluorescent protein (eGFP) to identify successfully transfected cells.
5. FuGene 6 Transfection Reagent (Promega).
6. D,L-2-amino-5-phosphonopentanoate (AP5) and 7-chlorokynurenic acid (7-CKA), competitive NMDAR antagonists (Tocris).

2.2 Fast Perfusion System (see Fig. 1)

1. Solution reservoirs are 30 ml syringes (BD Biosciences) attached to an in-house fabricated height adjustable bracket.
2. Solution flow from reservoirs is controlled by clamping silicone tubing (A-M Systems, Inc.) in solenoid pinch valves (NResearch Inc.).
3. Polyethylene tubing (PE 160, Becton Dickinson) is used to connect silicone pinch valve tubing to 2 to 1 Y connectors (Value Plastics, Inc., Y210-6) (see **Note 1**).
4. Polyethylene tubing (PE 50) connects Y connectors (see **Note 1**) to silicone tubing (outside diameter 1.2 mm and inside diameter 0.64 mm) that is attached to the back ends of individual square capillary glass (barrels) (Warner Instruments, SG800-5) with outside diameter 0.84 mm and inside diameter 0.6 mm.
5. Four barrels are aligned and glued (Krazy Glue) into an in-house fabricated barrel holder made from a single piece of aluminum, precisely shaped to cup four barrels (see **Note 2**).
6. The barrel holder is attached through an in-house fabricated barrel holder arm to the shaft of a stepper motor (Pacific Scientific, Powermax II SIGMAX M21). The barrel holder arm should give the barrels a 1" radius from the center of the stepper

¹Silicone tubing of appropriate size is used to connect PE tubing to Y connectors.

²Barrels were first cut to length (5 mm) by scoring with a diamond tipped scribe and then both ends were lightly fire-polished. To allow silicone tubing connections to go over the back ends of adjacent barrels, carefully heat individual barrels over a Bunsen burner and bend to a 30 - 60° angle. Only bend two barrels and align them in an alternating pattern of bent then straight barrels to ensure that silicone tubing will attach to the back ends of all the barrels. Glass should be cleaned in 95% ethanol and dried before gluing to the barrel holder. Make sure to align the open edge of the barrels with each other, and ensure that there is no space between barrels.

motor shaft so that stepper motor rotation translates to a nearly linear barrel movement.

7. Stepper motor rotation is controlled by a microstepping power supply (Precision Motor Control, LNII Series) set to 50,000 microsteps/revolution (*see Note 3*). Barrel movements are accomplished by smoothly accelerating and decelerating the frequency of brief voltage pulses sent out from a computer parallel port using software (barrel movement software) written in Basic and running in FreeDOS (www.freedos.org) (*see Note 4*).
8. Although the fast perfusion system is depicted and described with only two separate solutions flowing through barrel 1, 2, and 3, it is possible to have as many solutions as is experimentally necessary by using an appropriate manifold.

2.3 Whole-Cell Recordings

1. The external, control solution contains: 140 mM NaCl, 2.8 mM KCl, 1 mM CaCl₂, 10 mM HEPES, 10 μ M EDTA, and 100 μ M glycine (*see Note 5*). Adjust pH to 7.2 $\pm$.05 with NaOH, and adjust osmolality to 290 $\pm$ 10 mOsmol/kg with sucrose.
2. The pipette solution contains: 130 mM CsCl, 10 mM BAPTA, 10 mM HEPES. Adjust pH to 7.2 $\pm$ 0.05 with CsOH. Osmolality should be 275 $\pm$ 10 mOsmol/kg.
3. Recording pipettes are fabricated using borosilicate glass (with filament) with an outer diameter of 1.5 mm and an inner diameter of 0.86 mm (Sutter Instrument Company) pulled on a P-97 Flaming/Brown micropipette puller (Sutter Instrument Company) and lightly fire-polished.
4. Cells are imaged with an inverted fluorescence microscope with an eGFP filter set (Zeiss). Patch-clamp recordings are made while imaging cells and the recording pipette using a Retiga EXi Fast 1394 digital camera (QImaging).
5. Voltage-clamp current recordings are made with an Axopatch 200B amplifier (Molecular Devices) with a CV 203BU headstage (Molecular Devices) attached to a PatchStar micromanipulator (Scientifica) and digitized with a Digidata 1440A A/D converter (Molecular Devices).

3. Methods

Brief synaptic-like agonist applications to recombinant ligand-gated ion channels expressed in tsA201 cells during whole-cell recording can be achieved using the fast perfusion system described in **Subheading 2.2** (*see Fig. 1*). To emulate synaptic neurotransmitter release, the fast perfusion system must achieve brief agonist applications. Brief agonist applications to the entire cell under study are facilitated by “lifting” cells from the coverslip on which they

³With barrels at a ~1” (~25 mm) radius from the center of the stepper motor shaft, each microstep of stepper motor rotation is translated to 3 μ m of barrel movement. Because the total range of barrel movement is about 2500 μ m, less than 1000 microsteps (less than 1/50th revolution) are needed for total barrel movement. This translates to nearly linear barrel movement.

⁴A compiled version of the stepper motor program is available from the authors by email request.

⁵10 μ M EDTA is used to chelate contaminating free Zn²⁺, which inhibits GluN1/2A receptors in the nM range. The NMDAR coagonist glycine is present in all solutions to saturate the glycine coagonist-sites on NMDARs.

are cultured. The fast perfusion system must also allow easy changes of the solutions flowing through barrels to allow responses to brief agonist applications in the absence and presence of drug. As an example of fast perfusion system operation we focus on how NMDAR open channel blockers inhibit recombinant NMDAR responses to brief synaptic-like glutamate applications.

NMDAR open channel blockers are a class of use-dependent NMDAR antagonists. One NMDAR open channel blocker, memantine, is currently being used to treat Alzheimer's disease (13). Memantine along with another NMDAR open channel blocker, ketamine, have shown promise in the treatment of several other debilitating nervous system disorders (4-7,14,15). Memantine and ketamine share the same basic mechanism of action and have similar IC_{50} values and kinetics of inhibition at NMDARs (16,17). However, there are subtle kinetic differences in inhibition of NMDARs by memantine and ketamine. These differences demonstrate important considerations when designing experiments to evaluate how drugs affect ligand-gated ion channel currents in response to brief agonist applications. The methods described below explain the steps used to record recombinant NMDAR currents in response to brief glutamate applications in the absence and presence of open channel blockers.

3.1 Fast Perfusion System Design

3.1.1 Brief Application Strategy

1. Rapid and continuous barrel movement from barrel 1 to barrel 3 (*see Fig. 2A*), sweeping quickly by barrel 2, delivers brief synaptic-like glutamate applications (~5 ms) to lifted transfected cells.
2. Similar barrel movement from barrel 3 to barrel 1 (*see Fig 2A*) delivers another brief synaptic-like glutamate application to lifted transfected cells.
3. With careful calibration, the fast perfusion system can consistently deliver brief, repeated synaptic-like agonist applications to lifted transfected cells.
4. Lifting cells is crucial to ensure complete and rapid exchange of solution during brief agonist applications. Although the duration of agonist application is identical for recordings from attached cells and from lifted cells, the diffusionally-restricted space between the bottom of an attached cell and the coverslip slows solution exchange.

3.1.2 Changing Solutions Flowing Through Barrels

1. The Y-connectors described in **Subheading 2.2** (*see Fig. 1*) allow one of two solutions to flow through barrels 1, 2, and 3. Importantly, pinch valves 1a, 2a, or 3a are never open concurrently with pinch valves 1b, 2b, or 3b, respectively.
2. Change the solutions flowing through each barrel by closing pinch valves 1a, 2a, and 3a and immediately opening pinch valves 1b, 2b, and 3b.
3. During changes of solution flowing through barrels 1, 2, and 3, it is advisable to move to barrel position 4 to perfuse the cell with control solution (make sure pinch

valve 4 is always open). Perfusing the cell with control solution during changes of solutions flowing through barrels helps to avoid (1) releasing gas bubbles onto the cell as a result of opening and closing pinch valves, and (2) contact of the cell with glutamate + drug-containing solution due to temporary disruptions in solution flow during pinch valve opening and closing.

4. One benefit of this method is that the number of solutions that can be applied to the same cell is limited only by the number of inlets on a manifold that can replace the Y connector.

3.2 Transient Transfection of tsA201 Cells

1. tsA201 cells are maintained in culture and plated prior to transfection using standard cell culture procedures (18).
2. 12--24 hours before transfection, 1×10^5 tsA201 cells are plated in 1.5 ml of tsA201 cell culture medium on uncoated 15 mm glass coverslips in 35 mm petri dishes (3 coverslips/dish).
3. Warm serum-free tsA201 cell culture medium and FuGene 6 Transfection Reagent to room temperature.
4. The following steps refer to transfection of a single dish of plated cells. If transfecting multiple dishes of plated cells, increase the volume of solutions accordingly.
5. Transfer 95 μ l of serum-free tsA201 cell culture medium into a sterilized microcentrifuge tube.
6. Add 3 μ l of FuGene 6 Transfection Reagent to the tube, avoiding contact with the tube wall.
7. Vortex the tube for 1 second and incubate at room temperature for 5 minutes.
8. Add 1 μ g of cDNA total (2 μ l of cDNA at a density of 0.5 μ g/ μ l) to the tube in a ratio of 1:1:2 (eGFP:GluN1:GluN2x) (*see Note 6*).
9. Vortex the tube for 1 second and incubate at room temperature for 15 minutes.
10. Transfer 100 μ l of medium/FuGene 6 Transfection Reagent/cDNA mixture from the microcentrifuge tube to a petri dish of plated cells (*see Note 7*).
11. Add D,L-AP5 (GluN2A or GluN2B, 200 μ M; GluN2C or GluN2D, 400 μ M) and 7-CKA (200 μ M) to the petri dish (*see Note 8*).

⁶The cDNA transfection ratio of 1:1:2 for eGFP:GluN1:GluN2x may vary depending on transfection efficiency with given vectors and subunits.

⁷The volume of serum-free tsA201 medium used for transfections depends upon the cDNA solution density. The medium/FuGene 6 Transfection Reagent/cDNA mixture should be at a final volume of 100 μ l for transfection of a single dish of plated cells. If the cDNA solution density differs from 0.5 μ g/ μ l, a different volume of cDNA solution should be added to the mixture to reach 1 μ g of cDNA; the amount of medium added should be adjusted to reflect this change.

⁸NMDARs tonically activated by ambient glutamate present in the culture medium are excitotoxic. Therefore, we add competitive antagonists to the culture medium after transfection of tsA201 cells. Other antagonists, including elevation of the Mg^{2+} concentration of the tsA201 cell culture medium to > 10 mM also may be used.

12. Wait at least 18 hours before recording from transfected cells (*see Note 9*)

3.3 Performing Brief Glutamate Applications in Control Solution

3.3.1 Estimating Duration of Brief Applications

1. Fill solution reservoirs 1a, 1b, 3a, and 3b with control solution and fill solution reservoirs 2a, and 2b with control solution diluted by 10% with deionized H₂O (diluted control solution).
2. Fill a recording pipette with pipette solution, attach to the pipette holder and then apply a small amount of positive pressure (~0.5 PSI) to the side port of the pipette holder (*see Note 10*). Lower the pipette into the recording chamber filled with control solution.
3. Position the barrels vertically so they do not touch the bottom of the dish during movement (*see Note 11*). Move the pipette into the optimal vertical position for solution application (*see Note 12*). Position the pipette in the horizontal plane so that the tip of the pipette is about 50 μ m in front of the front edge of the barrels and the tip of the pipette is aligned with the center of barrel 1. Use the barrel movement software to define that location as barrel position 1.
4. Sequentially for each of the remaining three barrels, use the barrel movement software to align the barrel with the tip of the open pipette. Use the barrel movement software to define barrel positions 2, 3, and 4.
5. Make brief solution applications to the open pipette by rapid continuous movements from barrel position 1 to 3 or barrel position 3 to 1, sweeping by the solution in barrel 2 (*see Subheading 3.1.1*). With pinch valves 1a, 2a, and 3a open, perform movements from barrel position 1 to 3 and back from barrel position 3 to 1. Measure the duration of solution application with the open pipette by measuring the current in response to the diluted control solution in barrel 2 (*see Fig. 2B*). Current changes reflect the differing solution osmolality flowing onto the open pipette tip and are used to measure the duration of barrel 2 solution application. We measured the half-width duration of solution application as 4.5 ± 0.6 ms and the solution exchange 10--90% current rise time as < 0.5 ms (*see Fig. 2B*).
6. Change the solutions flowing through the barrels by closing pinch valves 1a, 2a, 3a and opening pinch valves 1b, 2b, and 3b (*see Subheading 3.1.2*). Repeat and

⁹We find that 24--48 hours after transfection offers optimal current amplitudes, cell health, and cell confluency. Depending on current amplitudes, successful recordings from transfected cells can be made up to at least 72 hours after transfection. Vary the time between transfection and recording to optimize protein expression and cell health.

¹⁰We use a 1 ml syringe connected with PE tubing to the side port of the pipette holder and connected in parallel to a pressure gauge. Pressure and suction can be applied by using the plunger of the syringe or by mouth. A stopcock on the end of the syringe can be closed to hold positive and negative pressure in the pipette.

¹¹The distance between the upper surface of the coverslip and the lowest point on any of the barrels changes slightly when the stepper motor rotates to cause barrel movement (*see Note 3*). After positioning the barrels axially so they are near the cell, position the barrels vertically so they are close to the coverslip, but do not touch the coverslip during movements to each barrel position. The barrels could break if they contact the coverslip during fast movements.

¹²Choose the vertical position of the pipette relative to the barrel openings to optimize speed of solution changes. It is best to position the pipette so that it is near the vertical center of the barrel openings. However, note that the barrels should be angled so that they point ~30° below the horizontal plane. The pipette should be positioned vertically so that it sits near the middle of the solution streams flowing from the barrels.

evaluate current measurements described in the previous point with pinch valves 1b, 2b, and 3b open.

3.3.2 Whole-Cell Recording from Lifted Cells—Patch-clamp recording from lifted cells is similar to patch-clamp recording from attached cells. For more detailed information on standard application of the patch-clamp technique see Hamill et al., 1981 (19).

1. Transfer a coverslip with transfected tsA201 cells to the recording chamber containing room temperature bath solution. Place the recording chamber onto the microscope stage, and then place an efflux tube and reference electrode into the chamber (*see Note 13*).
2. Using the fluorescence microscope, identify an isolated eGFP-positive cell (*see Note 14*).
3. Position the barrels vertically to ensure that they do not make contact with the coverslip through the full range of barrel movement (*see Note 11*). Position the barrels in the horizontal plane so that the front edge of barrel 1 is near the cell, and the center of barrel 1 is aligned with the cell (*see Fig. 2A*). Then move the barrels axially away from the cell, without changing the alignment of barrel 1 with the cell, to avoid crashing the recording pipette into the barrels (*see Note 15*).
4. Position the recording pipette just above the cell. Before forming a gigaohm seal, move the barrels axially towards the cell until about 50 μm from the cell.
5. Lower the recording pipette and form a gigaohm seal. Adjust electrode capacitance, and then achieve a whole-cell configuration.
6. Set whole-cell parameters (cell capacitance and series resistance) and adjust series resistance compensation to ~80%.
7. To lift the cell, apply a constant negative pressure of 1--1.5 PSI to the side port of the pipette holder. Slowly begin to move the pipette straight up from the coverslip. You should see the cell lift from the coverslip. Continue lifting the cell slowly until it is completely free from the coverslip. Move the pipette with the lifted cell into the optimal position for solution application (*see Note 12*).
8. Once the lifted cell is positioned, reduce the constant negative pressure to the side port of the pipette holder to 0.3--0.6 PSI. Readjust the whole-cell parameters, as capacitance should have decreased from lifting the cell. Also, the membrane capacitance of lifted cells often decreases throughout experiments, which may require further adjustments to whole-cell parameters.

¹³To maintain fluid levels in the recording chamber, we siphon solution through a glass efflux tube. The height of solution in the recording chamber is determined by the height of the waste end of the efflux tube.

¹⁴It is important to record only from isolated eGFP-positive tsA201 cells. When recording from lifted cells, it is often difficult to tell if there are thin attachments to other cells, which could drastically alter the recordings.

¹⁵When using lifted cells, it is possible to move the lifted cell to the barrels, even if they are placed relatively far from the starting location of the cell, to simplify barrel positioning. However, aligning the barrels as described minimizes the need to move the cell after lifting, increasing success rate.

9. Making an initial glutamate application of about 30 s is recommended to reduce response variability during the rest of the experiment.

3.3.3 Quantification of Receptor Response Time Course

1. Fill solution reservoirs 1a, 1b, 3a, and 3b with control solution, and fill solution reservoirs 2a and 2b with control solution containing 1 mM glutamate.
2. Make brief glutamate applications to the lifted cell by rapid continuous movements from barrel position 1 to 3 or barrel position 3 to 1, sweeping by the solution in barrel 2 (*see Subheading 3.1.1*).
3. Gauge the similarity to NMDAR-EPSCs of recombinant NMDAR responses by measuring the kinetics of recombinant receptor currents in response to brief glutamate applications.
4. Quantify the activation time course of recombinant NMDAR currents as the 10--90% rise time. We measured a mean 10--90% current rise time in response to brief glutamate applications to GluN1/2A receptors of 4.8 ± 0.6 ms (*see Fig. 2C*) and in response to brief glutamate applications to GluN1/2B receptors of 12.7 ± 5.6 ms (*see Fig. 2D*).
5. Quantify the decay time course of recombinant NMDAR currents by fitting the current decay with a double exponential function and determining the weighted time constant of decay ($\tau_w = (\tau_{fast})(fraction_{fast}) + (\tau_{slow})(fraction_{slow})$). We measured a mean τ_w in response to brief glutamate applications to GluN1/2A receptors of 27.5 ± 4.1 ms (*see Fig. 2C*) and in response to brief glutamate applications to GluN1/2B receptors of 420 ± 34 ms (*see Fig. 2D*).
6. Compare results to expected EPSC kinetics. The recombinant NMDAR response kinetics we measured are similar to previous measurements of NMDAR-EPSC kinetics and also to results of previous studies using brief glutamate applications to recombinant NMDARs in transfected cells (20-23).
7. Change the solutions flowing through the barrels by closing pinch valves 1a, 2a, 3a and opening pinch valves 1b, 2b, and 3b (*see Subheading 3.1.2*). Repeat and evaluate the kinetic measurements of recombinant NMDAR currents in response to brief glutamate applications (*see Note 16*).
8. After finishing an experiment, measure the duration of glutamate application to that specific cell to control for variations in solution flow rate and other potential sources of error, which may lead to exclusion of that experiment from analysis. Turn off series resistance compensation and whole-cell parameters. Return holding potential to 0 mV. Deliver > 2 PSI of positive pressure to the side port of the pipette holder to remove the cell and membrane debris from the tip of the pipette. Dilute the glutamate-containing solutions in reservoirs 2a and 2b (*see Fig. 1*) with

¹⁶Make sure that peak current amplitudes and response kinetics in response to brief glutamate applications are similar when pinch valves 1a, 2a, and 3a are open and when pinch valves 1b, 2b, and 3b are open. If significant differences are observed, further optimize the system as described in **Subheading 3.3.4**.

deionized H₂O by at least 10%. Measure changes in pipette current in response to barrel movements with the open pipette (*see Subheading 3.3.1*). Make sure to measure solution applications with pinch valves 1a, 2a, and 3a open and also with pinch valves 1b, 2b, and 3b open.

3.3.4 Fast Perfusion System Optimization

1. Stepper motor controller power output. Depending on the stepper motor controller, the output power may be adjustable. If so, modifying the output power can change stepper motor operation, either introducing or eliminating oscillations that may result from rapid acceleration and deceleration of the stepper motor. With some power settings, we observed oscillations when monitoring system performance using an open pipette that could have an undesirable impact on brief agonist applications to transfected cells.
2. Weight of barrel holder arm and barrel holder. Due to rapid acceleration and deceleration of the stepper motor, the stepper motor can overshoot desired positions or oscillate. The rotational inertia imposed by the weight of the barrel holder arm and barrel holder can strongly impact stepper motor overshoot and oscillations. The weight of the barrel holder arm and barrel holder should be minimized to reduce overshoot and oscillations if present.
3. Acceleration of stepper motor. The acceleration and deceleration of the stepper motor should be optimized for system stability and to minimize the duration of agonist application. At more rapid accelerations and decelerations, the stepper motor may overshoot desired positions or oscillate. At slower accelerations and decelerations, the duration of agonist application may be too long.
4. Rate of solution flow. Careful adjustment of the solution flow rate is essential to achieving consistent and brief agonist applications. It is important to maintain similar solution flow rates so that inconsistencies in application duration do not arise (*see Note 17*). Also, lifted cells are attached only to the tip of the recording pipette, making them vulnerable to being blown away if the solution flow rate is too fast.
5. Degassing solutions prior to use. Removing gas from solutions prior to starting experiments can help to (1) keep bubbles from destroying cells and (2) keep bubbles from blocking barrels, slowing or stopping solution flow. Gas bubbles can form unpredictably in tubing during experiments, and it can be difficult to determine if solution has stopped flowing from a particular barrel during an experiment. To degas solutions, pour solutions into a vacuum flask and apply negative pressure. Stop negative pressure when few gas bubbles form in solution.
6. Mixing of barrel solutions. It is important to ensure that a cell is exposed almost exclusively to the desired solution at each barrel position. Solution mixing could

¹⁷Differences in the rate of solution flow from barrel 1 and 3 can increase the agonist application duration while moving from barrel position 1 to 3 relative to the agonist application duration while moving from barrel position 3 to 1. Also, differences in solution flow rate from reservoirs 1a and 1b, etc. can have a significant impact on the duration of agonist application in the presence or absence of drug. Such differences could lead to complications in interpreting the effect of a drug.

occur, for example, within the Y connectors, or after solutions leave the barrels if the cell is not properly positioned relative to the barrels. One way to test for mixing is to fill solution reservoirs 1a, 2a, 3a, and 4 with control solution and solution reservoirs 1b, 2b, and 3b with control solution containing agonist at a concentration orders of magnitude above its EC₅₀ for the receptors under study; we use 10 mM glutamate. While whole-cell recording from a lifted cell expressing recombinant receptors, start recording at barrel position 4 with all other pinch valves closed and determine control (in the absence of agonist) holding current. Open pinch valves 1b, 2b, and 3b and be sure that holding current does not change while the cell is in front of barrel 4. Move to barrel position 3 to observe the response to glutamate, and after current has reached steady-state, be sure that there is no further change in current when moving to barrel positions 2 and 1. Move to barrel position 3, close pinch valve 3b, and open pinch valve 3a, and ensure that control holding current is observed. Repeat this procedure for the other barrels, and also change the solution flowing through adjacent barrels to be sure that the cell is exposed only to the solution flowing from the appropriate barrel. If evidence of mixing is observed, identify and correct the source of the problem (e.g., malfunctioning pinch valves or incorrect positioning of the cell relative to the barrels).

3.4 Performing Brief Glutamate Applications in Presence of Channel Blockers

1. Use whole-cell patch-clamp recordings from lifted cells expressing GluN1/2A or GluN1/2B receptors to record responses to brief glutamate applications as described in **Subheading 3.3**, with modifications described below.
2. Fill solution reservoirs 1a, 3a, and 4 with control solution and reservoir 2a with control solution containing 1 mM glutamate. Fill solution reservoirs 1b and 3b with control solution + drug and reservoir 2b with control solution containing 1 mM glutamate + drug (*see Fig. 1*).
3. Choose an appropriate frequency of brief glutamate applications to lifted cells expressing a particular NMDAR subtype. The frequency must be low enough to ensure complete current decay following glutamate application and allow recovery from desensitization before the subsequent glutamate application, yet fast enough to allow for experiments that may require many brief glutamate applications (potentially > 100 applications). We used a glutamate application frequency of 0.2 Hz for both GluN1/2A (*see Fig. 3A*) and GluN1/2B (*see Fig. 3B*) receptors.
4. Measure the baseline peak current value in response to brief glutamate applications in the absence of drug (baseline current). We required 10 consecutive, steady glutamate responses to establish that a stable baseline current had been reached, which were then averaged to give the baseline current mean value (*see Fig. 3A, B*).
5. Add drug to the solutions flowing through the barrels by closing pinch valves 1a, 2a, and 3a and opening pinch valves 1b, 2b, and 3b (*see Subheading 3.1.2*). Make sure to allow enough time for complete changes of solutions flowing through the barrels (*see Note 18*).

6. Open channel blockers require that the channel be activated to bind and inhibit the channel. The number of brief glutamate applications in the presence of drug needed to reach a steady level of NMDAR inhibition depends on the drug's kinetics and must be determined for each drug and NMDAR subtype. For each successive application of glutamate in the presence of drug, the peak current should be smaller than the previous peak current until reaching a steady level (inhibited current). We required 5 consecutive, steady glutamate responses to establish that a stable inhibited current had been reached, which were then averaged to give the inhibited current mean value (*see Fig. 4A, B*). We used memantine and ketamine, two NMDAR open channel blockers with slightly different kinetics, to illustrate differences in the number of glutamate applications in the presence of drug needed to reach steady NMDAR inhibition. We used 20 applications of glutamate in the presence of memantine and 40 applications of glutamate in the presence of ketamine to reach steady levels of NMDAR inhibition with GluN1/2A (data not shown) and GluN1/2B receptors (*see Fig. 4A, B*).
7. Remove drug from the solutions flowing through the barrels by closing pinch valves 1b, 2b, and 3b, and opening pinch valves 1a, 2a, and 3a (*see Subheading 3.1.2*). Make sure to allow enough time for complete changes of solutions flowing through the barrels (*see Note 18*).
8. Open channel blockers like memantine and ketamine require channel activation to unbind and allow recovery from inhibition (*see Note 19*). The number of brief glutamate applications in the absence of drug following NMDAR inhibition must be determined for each drug and NMDAR subtype. For each successive application of glutamate in the absence of drug following NMDAR inhibition, the peak current should be larger than the previous peak current until reaching a steady level after recovery from inhibition is complete (current after recovery). We required 10 consecutive, steady glutamate responses to establish that a stable current after recovery had been reached, which were then averaged to give the current after recovery mean value (*see Fig. 4A, B*). We used 20 applications of glutamate in the absence of memantine and 40 applications of glutamate in the absence of ketamine following NMDAR inhibition to reach steady levels of current after recovery with GluN1/2A (data not shown) and GluN1/2B receptors (*see Fig. 4A, B*).
9. Measure peak currents in response to brief glutamate applications in the absence and presence of drug as the mean current over a 3 ms window centered at the time of peak current.

¹⁸The time required for complete changes of solutions flowing through barrels can be estimated with the following experiment. Fill solution reservoirs 1a, 2a, and 3a with control solution and fill solution reservoirs 1b, 2b, and 3b with diluted control solution. With an open pipette positioned at barrel position 1, measure the time course of current change in response to closing pinch valve 1a and opening pinch valve 1b. The current should change approximately exponentially until reaching a steady level in the presence of the diluted control solution in reservoir 1b. Measure the 10–90% current rise time to estimate the time required for changing the solution flowing through barrel 1. We waited for 5x the 10–90% current rise time after closing pinch valve 1a and opening pinch valve 1b to consider the change of solution flowing through barrel 1 complete. Repeat measurements of current change in response to closing pinch valve a and opening pinch valve b for barrels 2 and 3.

¹⁹Measure recovery in all experiments to ensure that decreases in peak currents in response to brief glutamate applications in the presence of drug are due to the drug itself and not due to other changes in the cell that may decrease peak currents.

10. Calculate the percent inhibition by open channel blockers using the equation: % inhibition = $100 * (1 - (\text{inhibited current}) / (0.5 * (\text{baseline current} + \text{current after recovery})))$. We averaged the values for baseline current and current after recovery to account for changes in cell properties during experiments. For **Fig. 4**, we used concentrations of memantine and ketamine near their IC₅₀ values at NMDARs. We measured percent inhibition of responses to brief glutamate applications to GluN1/2B receptors in the presence of 1 μ M memantine as 49% (see **Fig. 4A**), and in the presence of 0.5 μ M ketamine as 56% (see **Fig. 4B**).

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References

1. Traynelis SF, Wollmuth LP, McBain CJ, et al. Glutamate Receptor Ion Channels: Structure, Regulation, and Function. *Pharmacological Reviews*. 2010; 62(3):405–496. [PubMed: 20716669]
2. Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. *Nat Rev Drug Discov*. 2004; 3(3):205–214. [PubMed: 15031734]
3. Lau A, Tymianski M. Glutamate receptors, neurotoxicity and neurodegeneration. *Pflügers Archiv - European Journal of Physiology*. 2010; 460(2):525–542. [PubMed: 20229265]
4. Duman RS, Aghajanian GK. Synaptic dysfunction in depression: potential therapeutic targets. *Science*. 2012; 338(6103):68–72. [PubMed: 23042884]
5. Autry AE, Adachi M, Nosyreva E, et al. NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature*. 2011; 475(7354):91–95. [PubMed: 21677641]
6. Pittenger C, Sanacora G, Krystal JH. The NMDA receptor as a therapeutic target in major depressive disorder. *CNS Neurol Disord Drug Targets*. 2007; 6(2):101–115. [PubMed: 17430148]
7. Collins S, Sigtermans MJ, Dahan A, et al. NMDA receptor antagonists for the treatment of neuropathic pain. *Pain Med*. 2010; 11(11):1726–1742. [PubMed: 21044263]
8. Palmer GC. Neuroprotection by NMDA receptor antagonists in a variety of neuropathologies. *Curr Drug Targets*. 2001; 2(3):241–271. [PubMed: 11554551]
9. Lipton SA. Failures and successes of NMDA receptor antagonists: molecular basis for the use of open-channel blockers like memantine in the treatment of acute and chronic neurologic insults. *NeuroRx*. 2004; 1(1):101–110. [PubMed: 15717010]
10. Paoletti P, Bellone C, Zhou Q. NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nat Rev Neurosci*. 2013; 14(6):383–400. [PubMed: 23686171]
11. Lipton SA. Paradigm shift in neuroprotection by NMDA receptor blockade: memantine and beyond. *Nat Rev Drug Discov*. 2006; 5(2):160–170. [PubMed: 16424917]
12. Gladding CM, Raymond LA. Mechanisms underlying NMDA receptor synaptic/extrasynaptic distribution and function. *Molecular and Cellular Neuroscience*. 2011; 48(4):308–320. [PubMed: 21600287]
13. Witt A, Macdonald N, Kirkpatrick P. Memantine hydrochloride. *Nat Rev Drug Discov*. 2004; 3(2):109–110. [PubMed: 15040575]
14. Prommer EE. Ketamine for pain: an update of uses in palliative care. *J Palliat Med*. 2012; 15(4):474–483. [PubMed: 22500483]
15. Anitha M, Nandhu MS, Anju TR, et al. Targeting glutamate mediated excitotoxicity in Huntington's disease: neural progenitors and partial glutamate antagonist--memantine. *Med Hypotheses*. 2011; 76(1):138–140. [PubMed: 20943326]

16. Kotermanski SE, Wood JT, Johnson JW. Memantine binding to a superficial site on NMDA receptors contributes to partial trapping. *J Physiol.* 2009; 587(Pt 19):4589–4604. [PubMed: 19687120]
17. Kotermanski SE, Johnson JW. Mg²⁺ imparts NMDA receptor subtype selectivity to the Alzheimer's drug memantine. *J Neurosci.* 2009; 29(9):2774–2779. [PubMed: 19261873]
18. Phelan MC. Techniques for mammalian cell tissue culture. *Curr Protoc Mol Biol.* 2006 Appendix 3:Appendix 3F.
19. Hamill OP, Marty A, Neher E, et al. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 1981; 391(2):85–100. [PubMed: 6270629]
20. Vicini S, Wang JF, Li JH, et al. Functional and pharmacological differences between recombinant N-methyl-D-aspartate receptors. *J Neurophysiol.* 1998; 79(2):555–566. [PubMed: 9463421]
21. Erreger K, Dravid SM, Banke TG, et al. Subunit-specific gating controls rat NR1/NR2A and NR1/NR2B NMDA channel kinetics and synaptic signalling profiles. *J Physiol.* 2005; 563(Pt 2):345–358. [PubMed: 15649985]
22. Cull-Candy, SG.; Leszkiewicz, DN. *Sci STKE.* Vol. 2004. 255: 2004. Role of distinct NMDA receptor subtypes at central synapses.; p. re16
23. Tovar KR, McGinley MJ, Westbrook GL. Triheteromeric NMDA Receptors at Hippocampal Synapses. *J Neurosci.* 2013; 33(21):9150–9160. [PubMed: 23699525]

Fig. 1. Schematic of fast perfusion system designed to allow brief applications of 1 mM glutamate (Glu) in control solution (Ctrl) in the absence and presence of a single drug concentration (Drug).

Fig. 2.

Measuring the duration of glutamate application. (A) Schematic of barrel movement in relation to an open recording pipette. Barrel movements are from barrel position 1 to 3 (and from barrel position 3 to 1), briefly sweeping by barrel 2. (B) Example of a current recording from an open recording pipette in response to moving from barrel position 1 to 3, sweeping by barrel 2, which has solution of different osmolality than barrels 1 or 3 (application half-width, 3.7 ms; solution exchange 10–90% current rise times, 1 to 2: 0.26 ms; 2 to 3: 0.22 ms). (C, D) Examples of whole-cell voltage-clamp recordings of lifted tsA201 cells expressing GluN1/2A receptors (C; 10–90% rise time, 4.0 ms; τ_w , 29.6 ms) or GluN1/2B receptors (D; 10–90% rise time, 9.0 ms; τ_w , 421 ms) in response to brief applications of 1 mM glutamate (Glu, black bar). Cells were held at -65 mV.

Fig. 3. Brief applications of glutamate to lifted cells expressing two different NMDAR subtypes. (**A, B**) Example whole-cell voltage-clamp recordings of lifted tsA201 cells expressing GluN1/2A receptors (**A**) or GluN1/2B receptors (**B**) in response to 5 brief applications of 1 mM glutamate (Glu, black bars) at a frequency of 0.2 Hz. Cells were held at -65 mV.

Fig. 4.

Antagonist kinetics affect the number of brief glutamate applications needed to reach a steady level of current inhibition and a steady level of currents after recovery from inhibition. (**A**, **B**) Examples of whole-cell voltage-clamp recordings from lifted tsA201 cells expressing GluN1/2B receptors in response to brief applications of 1 mM glutamate (Glu, short black bars) at 0.2 Hz in control solution or in the presence of 1 μ M memantine (**A**) or 0.5 μ M ketamine (**B**) (long black bars). The average of peak currents from the first 10 glutamate responses shown gives the baseline current mean value, the average the peak currents from the last 5 glutamate responses in the presence of memantine or ketamine gives the inhibited current mean value, and the average of the peak currents from the last 10 glutamate responses gives the current after recovery mean value. Cells were held at -65 mV.