Video Article

A High-throughput Calcium-flux Assay to Study NMDA-receptors with Sensitivity to Glycine/D-serine and Glutamate

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Abstract

N-methyl-D-aspartate (NMDA) receptors (NMDAR) are classified as ionotropic glutamate receptors and have critical roles in learning and memory. NMDAR malfunction, expressed as either over- or under-activity caused by mutations, altered expression, trafficking, or localization, can contribute to numerous diseases, especially in the central nervous system. Therefore, understanding the receptor's biology as well as facilitating the discovery of compounds and small molecules is crucial in ongoing efforts to combat neurological diseases. Current approaches to studying the receptor have limitations including low throughput, high cost, and the inability to study its functional abilities due to the necessary presence of channel blockers to prevent NMDAR-mediated excitotoxicity. Additionally, the existing assay systems are sensitive to stimulation by glutamate only and lack sensitivity to stimulation by glycine, the other co-ligand of the NMDAR. Here, we present the first plate-based assay with high-throughput power to study an NMDA receptor with sensitivity to both co-ligands, glutamate and D-serine/glycine. This approach allows the study of different NMDAR subunit compositions and allows functional studies of the receptor in glycine- and/or glutamate-sensitive modes. Additionally, the method does not require the presence of inhibitors during measurements. The effects of positive and negative allosteric modulators can be detected with this assay and the known pharmacology of NMDAR has been replicated in our system. This technique overcomes the limitations of existing methods and is cost-effective. We believe that this novel technique will accelerate the discovery of therapies for NMDAR-mediated pathologies.

Video Link

The video component of this article can be found at https://www.jove.com/video/58160/

Introduction

With current advances in medicine, life expectancy has increased significantly; however, so has the prevalence of age-related diseases. Diseases of the central nervous system (CNS) such as schizophrenia, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, and Parkinson's disease, among others, are no exception and have been projected to increase over the next decade[1,2,3]. The malfunction of ionotropic glutamate receptors known as N-methyl-D-aspartate receptors (NMDAR) has been linked to Alzheimer's disease, schizophrenia, traumatic brain injury, stroke, diabetes, and glaucoma among others, which warrants the need to study their biology for the development of effective, disease-modifying therapies[4,5,6,7].

NMDARs are composed of four monomers or subunits[4,8,9]. The structural composition of the NMDAR shows developmental and regional variability within the brain[7,10]. NMDARs are involved in synaptic plasticity, cognition, and the generation of rhythms for breathing and locomotion[11,12,13]. As a voltage-gated channel, it is largely non-conducting at resting membrane potential (-70 mV) and is blocked by magnesium to prevent further permeation of ions. The channel is activated by the binding of two ligands, glutamate and glycine/D-serine, and a simultaneous depolarization at the synaptic membrane mediated by AMPA receptors, another subclass of ionotropic glutamate receptors. The depolarization removes the magnesium blockage of the NMDAR, enabling the influx of cations, particularly calcium[14,15,16]. Even though the activation of NMDAR is essential for cell survival, excessive activation can lead to cell death[17,18,19] through excitotoxicity. This, in addition to the complex nature of the receptor, makes it challenging to perform studies necessary for developing effective therapies.

Different methods have been developed to study the NMDAR. However, each one has accompanying caveats. For example, one widely-used technique is a fluorescence-based assay that measures NMDAR-mediated changes in intracellular calcium in a stable cell line under the control of a tetracycline-inducible promoter (Tet-On)[20]. However, in this system, the supramaximal concentrations of ligands that are needed and the requirement that NMDAR inhibitors are present during the measurement makes it nearly impossible to detect activity of the competitive antagonist. In other similar systems, the expression of the functional receptor causes toxicity, requiring channel blockers such as ketamine[21,22] to preserve the cell cultures. These channel blockers sit at the core of the receptor and are difficult to wash out, especially in a plate-based format, so they interfere with functional studies of the receptor. Finally, in electrophysiological measurements such as patch clamping, there is limited throughput, and large-scale studies are very expensive[23]. Notwithstanding, the above systems are insensitive to glycine stimulation; hence, studying glycine-dependent activity of the NMDAR becomes a challenge.

Here, we describe a novel approach for studying NMDAR that overcomes the discussed limitations. Our technique capitalizes on the baculovirus expression system to express the receptor at functional levels with an optimal ratio of the subunits in as little as 16 hours. Furthermore, the use of baculovirus allows for a simple and combinatorial approach, which provides a broad characterization of the distinct recombinant NMDAR subtypes. Unlike other assays, this protocol does not require channel blockers because of the use of weak antagonists. The method's strongest advantage is that after washout of the weak antagonist, the receptor is sensitive to modulation of the individual glycine- and glutamate-binding sites in addition to dual modulation of both glycine/D-serine and glutamate ligand-binding sites. The assay recapitulates known pharmacology of the NMDAR receptor and the effects of its known positive and negative modulators. Finally, generation of this *in vitro* cellular assay overcomes the cellular toxicity caused by excessive calcium influx and allows for functional studies of the receptor in a high-throughput fashion, which can accelerate discoveries of NMDAR modulators in disease states.

Protocol

1. Preparation of Cells

NOTE: This protocol, including data generation, uses HEK293 cells transduced with a baculovirus encoding NR1 and NR2A cells.

1. Seed the appropriate number of HEK293 cells and add the NR1 and/or NR2A virus at the appropriate final concentrations (1.00 µL each). For a 384-well plate, use 10,000 cells/well in a final volume of 30 µL.
2. Alternatively, seed HEK293-NR1-NR2A cells at the appropriate cell number and volume (for a 384-well plate, use 10,000 cells/well in a final volume of 30 µL). Add tetracycline at a concentration of 2 µg/mL to induce NR2A expression and add the protection compound (100 µM MDL105,519 or 5 µM CGP070667[24]).
3. NOTE: The baculovirus encoding NR1 and NR2 should have an approximate titer between 5 x 10^9 - 10 x 10^9 infectious unit per milliliter[24].

2. Measurement of NMDA-receptor Activity

1. Prepare a 384-well plate with HEK293 cells transduced with NR1/NR2 or HEK293-NR1-NR2A cells in the presence of either protective compound. Use a clear bottom, black frame, poly-D-lysine coated plate.
   NOTE: Protection with 100 µM MDL105,519 is used to confer sensitivity to glycine, while 5 µM CGP070667 is used to confer sensitivity to glutamate.
2. Incubate the plate at 37 °C and 5% CO_2 for 16 hours (overnight).
3. Remove the plate from the incubator and gently discard the cell media by slowing flipping the plate over a compatible biowaste container. Gently tap the plate on a paper towel to clean the media off edges of the plate and drain any remaining media.
4. Prepare the calcium6-dye by solubilizing one vial of calcium6 dye (1 plate size) in 10 mL of incubation buffer (HBSS pH 7.5, 20 mM HEPES, 1 mM $MgCl_2$, 1 mM probenecid). Incubate the plate for 2 hours at 37 °C and 5% CO_2.
5. Remove the plate from the incubator and let the cells adjust to room temperature for 10 minutes.
6. Gently pour out the calcium6-dye as described in step 2.3 and add 30 µL of assay buffer (HBSS pH 7.5, 20 mM HEPES, 1.8 mM $CaCl_2$, 1 mM probenecid).
7. Repeat step 2.6 twice for a total of 3 washes. Leave 30 µL of assay buffer on the cells after the last wash.
8. Let the cells rest for 5 minutes at room temperature.
9. Prepare the ligand plate by making a 4-fold stock of 400 µM glycine and 400 µM glutamate (final concentration of 100 µM; optimal concentration of ligands to be determined as described below). Transfer a minimum of 25 µL of ligand stock into a V-bottom 384-well plate.
10. Load the ligand plate and cell plate into the FDSS (Functional Drug Screening System).
11. Measure the baseline fluorescence (F_o) of the cell plate for 30 seconds. Transfer 10 µL of the ligand stock into the cell plate using the FDSS dispensing function and measure the calcium flux by recording calcium6-dye fluorescence for 5 minutes.
12. Calculate the maximal fluorescence ratio by dividing the maximal fluorescence obtained by the baseline fluorescence (F_{max}/F_o).

3. Optimization of NMDAR Expression Levels

1. To determine the optimal amount of baculovirus to use, perform a titration of both NR1 and NR2A viruses. Prepare a 384-well plate with HEK293 cells (recommendation of 10,000 cells/well, 30 µL of media) and include the protection compounds as described in step 2.1.
2. Add varying amounts of the NR1 virus in different rows of the plate (for example: add 0 µL, 2 µL, 1 µL, 0.5 µL, 0.25 µL in rows A-E respectively). Add duplicates for data accuracy.
3. Add varying amounts of the NR2A virus in different columns of the plate (for example: add 0 µL, 2 µL, 1 µL, 0.5 µL, 0.25 µL in rows A-E respectively). Add duplicates for data accuracy.
4. Incubate the plate at 37 °C and 5% CO_2 for 16 hours (overnight).
5. Determine the optimal ratio and amount of the NR1 and NR2A viruses by performing a calcium flux measurement on the FDSS (see above).

4. Ligand Titration

1. Prepare the cells as described in step 2.3.
2. Prepare dilutions of each ligand in the presence of saturating concentrations (100 µM) of the other ligand as a 4-fold stock solution. For example: for a 10 µM final test concentration of glycine, prepare a 40 µM stock solution of glycine including 400 µM glutamate.
3. Proceed with the FDSS measurement as described in step 2.11.

5. Compound Testing

1. Prepare the cells as described in step 1.
2. Prepare the ligand plate with a 5-fold stock of final ligand concentration in the assay buffer.
3. Prepare the compounds plate with the 4-fold stock of desired final concentration of compounds in the assay buffer. For a final compound concentration of 10 µM in the assay buffer, prepare a 40 µM stock solution.
    1. Keep the concentration of the dimethyl sulfoxide (DMSO) constant across all samples by preparing a pre-dilution of the compound in DMSO before further dilution into the assay buffer.
    2. For a dose response of the compound in the final assay ranging between 3 and 30 µM, prepare a pre-dilution of the compound in DMSO ranging from 1 to 10 mM. Dilute those in the assay buffer to the 4-fold stock concentration. The final concentration of the DMSO should not exceed 0.5%.
    NOTE: It is recommended to test each sample in triplicates.
4. Load the ligand, compound, and cell plate into FDSS.
5. Measure the baseline fluorescence for 30 seconds.
6. Add 10 µL of compound stock and measure the fluorescence for 5 minutes.
7. Add 10 µL of ligand stock and measure the fluorescence for 5 minutes.
8. Determine the integral of the fluorescence ratio by calculating the area under the curve of the fluorescent ratios during the last 5 minutes of the measurement.
    NOTE: Alternatively, the maximal ratio of fluorescence after ligand addition can be used for analysis.

Representative Results

Before testing the effects of small molecules, one must determine the optimal expression levels of NMDARs as well as the optimal ligand concentrations. As described, HEK293 cells were seeded at 10,000 cells per well in a 384-well plate, in the presence of 5 µM CGP060667, then transduced with varying amounts of NR1 and NR2A viruses. After incubation overnight, ligand-induced calcium flux was measured (**Figure 1**). The results of these experiments reveal the best quantity and ratio of virus to use for each subunit (**Figure 1**, yellow box).

Once the optimal quantity and ratio for transduction with the NR1 and NR2A were determined, a ligand titration was performed to determine the optimal ligand concentration for activation of NMDARs. To accomplish this, one of the co-ligands was added in varying concentrations at fixed, saturating concentrations of the other co-ligand (**Figure 2A-2B**). Similar results were obtained for other subunit combinations of the NMDAR such as NR1/NR2B and NR1/NR2D (**Figure 2C**), demonstrating the assay's applicability to other NMDAR family members[24]. After determining optimal expression levels and ligand concentrations, one can proceed with testing the effects of NMDAR-modulators in the assay.

Consistent with published reports, this first plate-based assay to study NMDARs with sensitivity to both glycine and glutamate on a large scale reproduces data on the known properties of the NMDAR[14,25,26]. Here, we use the activity of two compounds, [(R)-[(S)-1-(4-bromophenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077), a glutamate site antagonist, and [7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(H)quinolone] (L701,324), a glycine site antagonist, to exemplify the predicted results of the assay[27,28] (**Figure 3**). Finally, each compound was tested at the EC80 of the respective natural ligand of the binding site. Reduction of the ligand concentrations is expected to increase the potency of antagonists, while it is expected to have no effect on the potency of non-competitive inhibitors or pore-blockers.

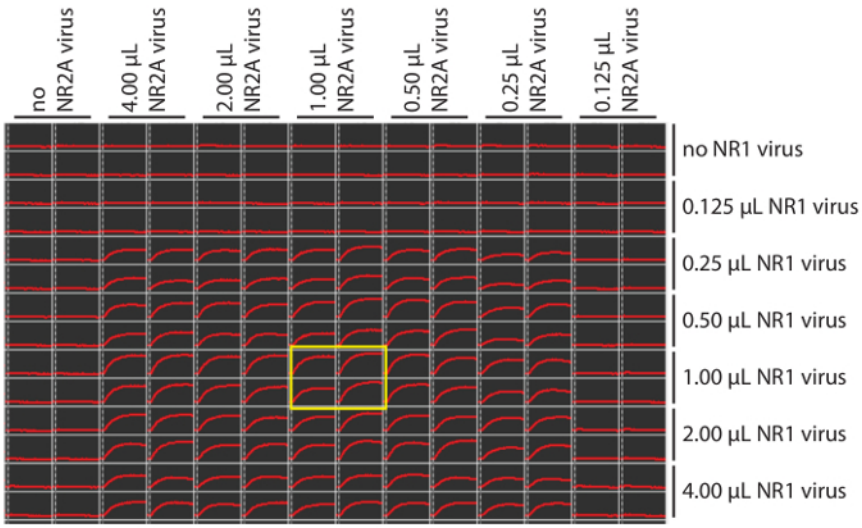

Figure 1: Titration of NR1 and NR2A subunits. Different volumes of virus that encode NMDAR subunits NR1 and NR2A were titrated to determine the amount needed for optimal functional activity of the receptor. A 1:1 (v/v) ratio NR1:NR2A was observed as the optimal ratio with 1 µL of each (yellow box). Please click here to view a larger version of this figure.

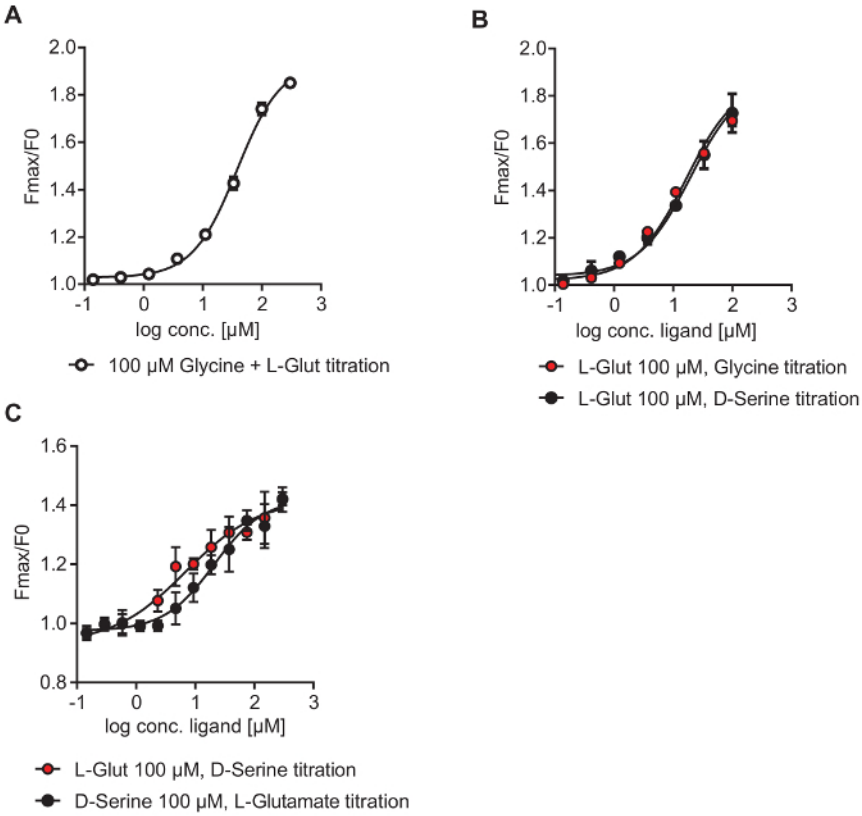

Figure 2: Ligand titration. A. In a fixed concentration of glycine (100 μM), varying glutamate concentrations were titrated and calcium flux was recorded. Error bars represent standard deviation. B. Glutamate remained at a concentration of 100 μM and was titrated against different concentrations of D-serine and glycine, and calcium flux was measured. There was no significant difference found between the calcium flux in glycine and in D-serine. Error bars represent standard deviation. C. Cells transduced with NR1 and NR2D baculovirus were stimulated with either 100 μM glutamate and varying concentrations of D-serine or 100 μM D-serine and varying concentrations of glutamate and calcium flux was measured. Error bars represent standard deviation. Please click here to view a larger version of this figure.

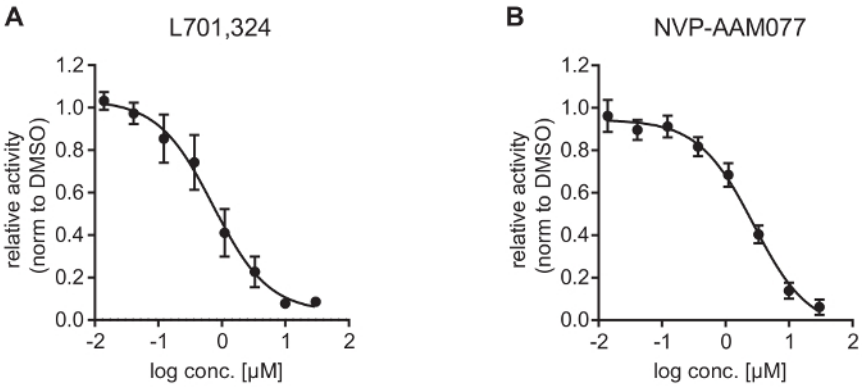

Figure 3: Characterizing activity of ligand binding site antagonist. Using the predetermined optimal ligand and subunit expressions, the effects of a glutamate-binding site antagonist, NVP-AAM077, and a glycine-binding site antagonist, L701,324 were characterized by the calcium flux assay for NR1/NR2A at the indicated concentrations. A concentration equal to the EC80 of glycine or glutamate in the presence of the saturating concentration (100 μM) of the other ligand was used after addition of NVP-AAM077 and L701,324, respectively. Error bars represent standard deviation. Please click here to view a larger version of this figure.

Discussion

The success of this assay depends largely on the health of the HEK cells used. Cells undergoing exponential growth and with low passage number should be used. This assay involves many transfers and additions of solutions, so using caution will ensure higher accuracy in its results. Concentrations of compounds and all other reagents should be also cross-checked to minimize errors. When replacing cell media with the assay

buffer for the calcium flux assay, the plate should be tilted gently so that the cells do not detach. Finally, the use of poly-D-lysine coated plates in this protocol increases attachment of the cells to the plate.

There may be differences in NMDAR expression and the amount of virus needed for subunit expression depending on the passage number and fitness of the HEK293 cells at the time of transduction. Also, the baculovirus has a short shelf life ranging from six months to a year. Therefore, the quality of the virus is an important parameter for this assay. A standard immunofluorescence assay can be used to detect and confirm the expression levels and localization of the NMDAR units before further experiments[24]. Other cell lines can be used to study the NMDA receptor using this protocol. However, the tolerance and expression of NMDARs using the baculovirus must be confirmed before proceeding with the protocol; otherwise, unexpected results may occur. Additionally, compounds such as topoisomerase II inhibitors have been reported to increase the efficacy of baculovirus expression[29]. These alternatives can be tested and optimized according to the user's preferences.

The study of biological processes in a cell type different than the original cell type raises questions within the scientific community. A limitation of this assay is the use of kidney cell lines (HEK 293) as a model system to study the ionotropic glutamate receptor, NMDAR. HEK cells were used because of their depolarized membrane potential similar to that of depolarized neurons. In addition, the similarity between NMDARs in the brain and in our model is confirmed by our supplementary studies. The activity of positive allosteric modulators such as GNE-8324, negative modulators such as ifenprodil, and channel blockers such as ketamine, as well as the voltage dependence of the NMDAR, were all confirmed in our model[22,23,24]. Also, our ability to adjust the amount of virus for the NR1 and NR2A subunits ensures optimal functional expression levels.

Because of the low efficiency, high cost, and minimal success outcome, the characterization of individual receptors and their unique pharmacology are currently conducted in low-throughput manual patch-clamp assays[22,23]. On the other hand, excitotoxicity remains a major obstacle when using stably transfected cells, because overexpression of functional NMDARs are harmful to cells due to the ligands (glutamate and glycine/D-serine) that are released into the media[18]. As a stronger alternative, our assay uses baculovirus-mediated expression of functional NMDARs, which overcomes excitotoxicity and yields rapid, titratable expression of the receptors. Rather than being limited by the sticky nature of channel blockers, this assay also takes advantage of antagonists of weak ligand-binding sites. These antagonists compete with endogenous ligands in the media and protect the ligand-binding sites. Hence, after washout of the antagonists, sensitivity to exogenously-added ligands of the glutamate- and glycine/D-serine- binding sites is restored.

This assay, combined with recent advances in molecular biology such as CRISPR, will help target and characterize novel regulators of NMDAR expression and function. Concurrently, this assay will help identify small molecules that may directly or indirectly modulate NMDAR activity without the need to generate stable cell lines, thereby providing compatibility with genetic- and small molecule- based screens.

Disclosures

The authors have no conflicts of interest and nothing to disclose.

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