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Transfection optimization of HEK-293 cells with NMDA receptor subunits NR1 and NR2A

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Transfection optimization of HEK-293 cells with NMDA receptor subunits NR1 and NR2A

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B.S. University of Richmond, 2016.

Advisor: Dennis C. Liotta, Ph. D.

An abstract of

A thesis submitted to the Faculty of the
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Abstract

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By Azmain Taz

Dysregulation of ionotropic N-methyl-D-aspartate receptors (NMDAR) is implicated in numerous brain disorders driving the need for the development of subunit-specific modulators. Cell-based assays are essential for the evaluation of new drugs and thus an optimized protocol for NMDAR expression in cells is important. Here, I investigated the optimal conditions for the transient transfection of HEK-293 cells with Lipofectmine 3000. The effect of different Lipofectamine concentrations, transfection media and cell confluency on transfection efficiency were studied using fluorescence microscopy and western blot. It was found out that DNA:Lipofectamine ratio of 1:2 and 80-90% cell confluency at the time of transfection are needed for high transfection efficiency.

Transfection optimization of HEK-293 cells with NMDA receptor subunits NR1 and NR2A

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1. Introduction

N-methyl-D-aspartate (NMDA) receptors are ligand-gated ion channels in the central nervous system that mediate slow excitatory synaptic transmission in response to glutamate neurotransmitter. NMDA receptors play a key role in synapse formation and synaptic plasticity[1]. As a result, dysfunction of the NMDA receptors is associated with several disorders of the central nervous system making them favorable targets for new therapeutic agents[1, 2].

NMDA receptors are heterotetramers composed of two glycine binding GluN1 subunits and two glutamate binding GluN2 subunits that form a central ion channel pore. The binding of glycine (or D-serine) to GluN1 and glutamate to GluN2 subunits leads to NMDA receptor activation[3]. Each subunit is a modular structure containing four discrete domains: the extracellular amino-terminal domain (ATD), the extracellular ligand-binding domain (LBD), the transmembrane domain (TMD) and an intracellular carboxyl-terminal domain (CTD) (Fig. 1). The GluN2 subunits have spatiotemporal expression in the brain[5, 6] and endow NMDA receptors with distinct functional and pharmacological properties[7]. Thus, ligands which are specific to different GluN2 subunits are useful tools for probing the function of different NMDA receptor subtypes and are important therapeutic targets.

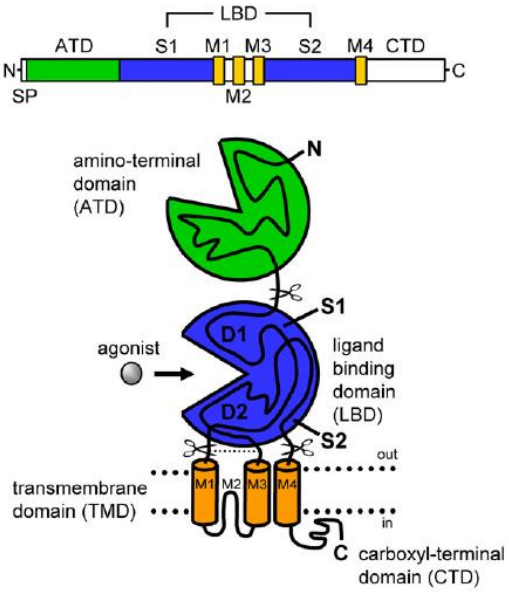

Fig 1. Linear representation of the subunit polypeptide chain and schematic illustration of the subunit topology (reprinted from ref 1).

The GluN1 subunit is encoded by a single gene which yields eight splice variants whereas each of the four GluN2 subunits (GluN2A-GluN2D) are encoded by separate genes[1, 4]. Recent genetic analyses have demonstrated that variations in the gene encoding GluN2A (NR2A) resulted in

childhood epilepsy/aphasia syndromes[8]. Moreover, a larger subset of NR2A mutations cause hyperactivity of GluN2A containing NMDA receptors leading to neurological disorders including early-onset epileptic encephalopathy and refractory seizures[9].

Numerous subunit-specific antagonists have been developed which help regulate the activity of NMDA receptors[10]. However, the search for subunit-specific modulators of NMDA receptors is an ongoing process and cell-based assays are key to determining the potency of these molecules. As a result, it is imperative to develop an optimized protocol for the expression of NMDA receptors in mammalian cells. Here, I have summarized my efforts to optimize the transient transfection of HEK-293 cells with NR1 and NR2A for the expression of NMDA receptor subunits GluN1 and GluN2A.

Transfection is the method of introducing foreign nucleic acids into cells to generate genetically modified cells. The foreign DNA or RNA can exist in cells stably or transiently depending on the nature of the genetic materials. Stable transfection is used to integrate foreign gene into the host genome and is passed onto future generation of the cell. Alternatively, transiently transfected cells are only expressed for a limited period of time and are lost by environmental factors and cell division[11].

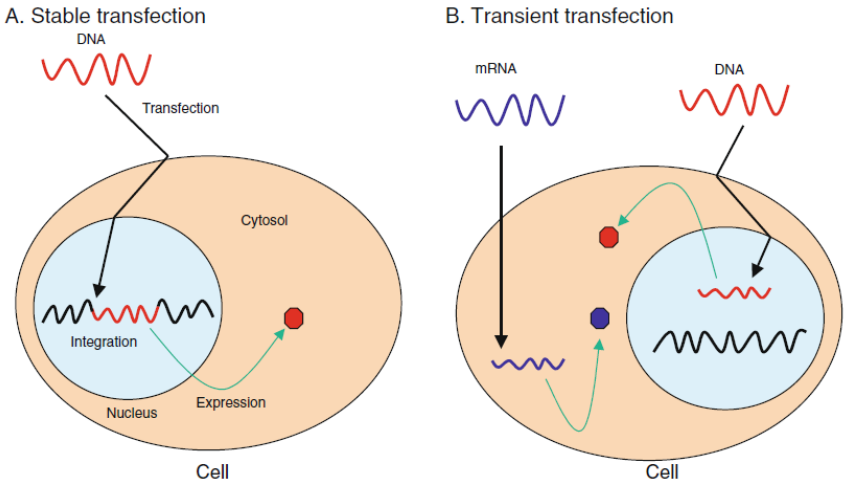

Fig 2. Schematic diagram of two different transfections. *Hexagons* are expressed proteins from transfected nucleic acids. *Black arrows* indicate delivery of foreign nucleic acids. (reprinted from ref 11).

Since I would like to have the flexibility to express any combination of NMDA receptor subunit in cells depending on the modulator to be tested and type of assay to be performed, transient transfection is more appropriate for my purpose.

Several transection methods have been developed which use different approaches depending on the cell type and purpose. Biological methods such as virus-mediated transfection generate high transfection efficiency but can also cause immunogenicity and cytotoxicity. Physical methods such as microinjection, electroporation and laser-mediated transfection are simple and reliable but require specialized instrument and high levels of skill. Chemical methods include cationic polymer, calcium phosphate and cationic lipid produce high efficiency transfections with plenty of commercially available products however transfection efficiency varies over cell type and conditions[11]. Due to the wide availability of commercial products for chemical transfection, and the ease of use of the cationic lipid method, Lipofectamine 3000 (Invitrogen) has been used in this study.

Lipofectamine transfection reagents are specially designed lipids consisting of positively charged head group and one or two hydrocarbon chains (Fig. 3). The cationic head group interact with the negatively charged phosphate backbone of nucleic acid to form DNA-Lipid complex which enters the cells by endocytosis. Once inside the cell, the DNA needs to diffuse through the cytoplasm and enter the nucleus for gene expression[12].

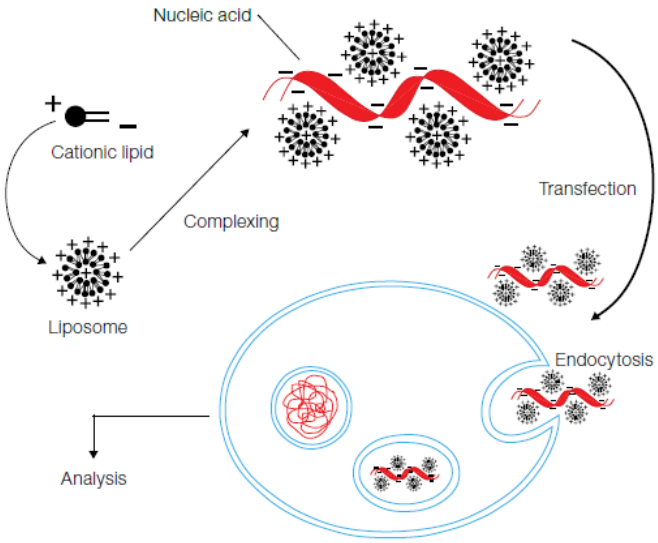

Fig 3. Lipid-mediated gene delivery (reprinted from ref 13).

The cell line of choice used in this study is HEK-293. This cell line was derived in 1973 by exposing the human primary embryonic kidney cell culture of an aborted embryo to the sheared DNA of adenovirus type 5 (AD5). It is assumed the 293 cell line might have been derived from neuronal lineage cell and more than 60 neuron-specific genes were revealed in 293 cells. In fact, HEK-293 cells have been extensively used for analysis of the neuronal synapse formation, in electrophysiology and neuropharmacology[13]. This makes 293 cells perfect candidates for the expression of NMDA receptors.

In this study, I will discuss how transfection efficiency depends on the concentration of Lipofectamine 3000, cell confluency and transfection medium.

2. Materials and Methods

NR1 and NR2A constructs were in pcDNA3.1 and gift from Dr. Vasanthi Jayaraman, University of Texas Health Science Center. HEK-293 cells and Green Fluorescent Protein DNA were generously provided by Dr. Hanjoong Jo, Emory University. Transfection reagent Lipofectamine 3000 was purchased from Invitrogen. NMDAR glycine-site antagonist DCKA (ab120255) and glutamate-site antagonist DL-AP5 (ab 120271). For western blot, the following antibodies were used: Anti-NMDAR1 Antibody, rabbit monoclonal (AB9864), Anti-NMDAR2A Antibody, rabbit polyclonal (AB1555), Goat Anti-Rabbit IgG HRP (10004301).

DNA constructs

Cloning of the NR1 and NR2A constructs using restriction enzymes was performed at Emory Integrated Genomics Core (EIGC) and showed the following mutations: C15S (NR1), LVPRGS insert after C22 (NR1), HHHHHHLVPRGS insert at H30 (NR2A). These mutations had been previously introduced for Luminescence Resonance Energy Transfer (LRET) experiments[14].

Bacterial Transformation

NR1 and NR2A constructs were transformed and amplified in bacteria DH5α (Invitrogen). 20 µL of diluted supercoiled DNA (100ng/10µL) was added to 100 µL of DH5α and mixed gently. The mixture was incubated on ice for 30 minutes followed by 45 seconds heat shock in a 42°C water bath and then 2 minutes incubation on ice. Invitrogen S.O.C Media (0.9 mL) was added to the cells and shaken for 1 hour at 37°C. The bacterial cells (20 µL) were then spread on LB agar plate with ampicillin and incubated overnight at 37°C. The next day one isolated colony was selected and amplified in LB Broth with Ampicillin by shaking overnight at 37°C. The LB Broth with bacteria was centrifuged at 7000 rpms for 30 minutes at 4°C. The supernatant was discarded

and the pellet was used to isolate the plasmid with ZymoPURE Plasmid Midiprep kit according to manufacturer's protocol. The absorbance spectrum of the isolated plasmid was measured using NanoDrop to obtain the concentration and purity of the DNA.

Electrophysiology

Whole-cell voltage-clamp recordings were performed by Jing Zhang from Dr. Traynelis' lab, Department of Pharmacology, Emory University. Briefly, HEK-293 cells were transfected with NR1, NR2A and GFP in 1:1:1 ratio using calcium phosphate. 24 hours after transfection, whole-cell voltage-clamp recordings were performed using transfected HEK 293 cells at −60 mV using an Axopatch 200B amplifier (Molecular Devices, Union City, CA) at room temperature (23°C)[14].

Cell growth and subculture

HEK-293 cells were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM, Corning) supplemented with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals), 1 mM L-glutamine (Invitrogen) and 5 mL penicillin/streptomycin (pen/strep) (Gibco). Cells were incubated at 37°C in 5% CO_2 and have a doubling time of 24 hours. The cells were passaged every two days and subcultured in 10 cm dishes. Cells were typically split in 1:3 and were always passaged in their log phase. For passaging cells, the growth medium was aspirated and the cells were washed twice with Hank's Balanced Salt Solution (HBSS) without calcium, magnesium (Corning). 2 mL of Trypsin-EDTA 1X (Corning) was added to 10 cm dish and was gently swirled for no more than 2 minutes. 3 mL growth media was added, pipetted up and down on the plate to lift cells from the dish and collected in a tube. The cell suspension was centrifuged at 1700 g for 3 minutes. The supernatant was discarded and the cell pellet was

resuspended in growth media. The number of cells were counted with a hemocytometer and the cells were seeded at 30% confluency.

Lipofectamine 3000 Transfection

Transfections were carried out in 6-well and 12-well plates. HEK-293 cells were co-transfected with NR1, NR2A and GFP for florescence microscopy imaging. The controls included were untreated cells (UTC) and cells transfected with GFP only. The total DNA mass used for each well in 6-well and 12-well plates were 2.5 µg and 1.25 µg respectively and NR1:NR2A:GFP was 1:2.25:3.

6 well plate (for each well): Desired volume of Lipofectamine 3000 was added to Opti-MEM reduced serum (Gibco) in a tube to make 100 µL and vortexed briefly. NR1, NR2A and GFP were added to a separate tube followed by P3000 reagent (5 µL) from the kit and Opti-MEM to make 100 µL and vortexed briefly. 100 µL of diluted Lipofectamine 3000 was added to the tube containing the DNA mixture and was vortexed briefly. The Lipofectamine-DNA mixture was incubated at room temperature for 20 minutes. The cells were taken out of the incubator, the media was aspirated and 800 µL of Opti-MEM or growth media was added by gently pipetting down the wall of the well. 200 µL of the Lipofectamine-DNA mixture was added to the cells dropwise. Before putting the cells into the incubator, the plate was slowly tilted in the North-South, East-West direction to distribute the transfection mixture evenly. After 4 hours, the transfection media was aspirated. 2 mL of growth media along with 300 nM DCKA and 30 nM DL-AP5 were added to the cells gently along the walls of the well.

12 well plate (each well): The procedure is the same as above with changes in reagent volume. Lipofectamine 3000 was diluted in Opti-MEM to a volume of 50 µL. 2 µL of P3000 was added

to the DNA mixture in Opti-MEM to a volume of 50 µL. After aspirating the growth media from the cells, 400 µL of Opti-MEM or growth media was added to the well followed by 100 µL of Lipofectamine-DNA mixture. After 4 hours, the transfection media was aspirated and 1 mL growth media was added to the cells.

Fluorescence Microscopy Imaging

24 hours after transfection, the cells were imaged with fluorescence microscope as quickly as possible to minimize disturbance to the cells. Brightfield channel was used to look for an area where the cell density if the highest. GFP channel was used to see how many cells was expressing GFP in that area. 48 hours after transfection the cells were imaged again.

Cell lysis and Protein Extraction

48 hours after transfection the cells were taken out of the incubator and placed on ice. The growth media was aspirated, washed once with HBSS with calcium, magnesium and 100 µL RIPA lysis buffer 1X (Boston BioProducts) with protease inhibitor (Roche) was added to each well in 6 well plate and 50 µL was added to each well in 12 well plate. The cells were scraped off the wells with cell scraper, the lysis buffer was collected in a tube and rotated for 30 minutes at 4°C. This was followed by centrifuge at 13000 g at 4°C for 12 minutes. The supernatant was collected and used to determine protein concentration using BCA assay.

GFP Fluorescence Intensity

The fluorescence intensity of GFP was determined using a plate reader. In a white opaque 96-well plate 90 µL of HBSS (with calcium, magnesium) and 10 µL of cell lysate were added. The fluorescence intensity was recorded at 475 nm excitation and 508 nm emission.

Western Blot

Samples for western blot were prepared by adding 23 µg protein to 4.67 µL of 6x Reducing buffer (SDS-Sample Buffer, Boston BioProducts) and RIPA buffer with protease inhibitor to make a final volume of 28 µL. The sample were heated to 95°C for 5 minutes and centrifuged and loaded in 8% SDS-PAGE gel and run at 75 V for 20 minutes and then at 130 V. The gel was transferred to PVDF membrane, blocked with 5% non-fat milk in TBS-T for 1 hour. After 3 washes with TBST-T the membrane was incubated overnight at 4°C with primary NR1 antibody in 5% milk, washed with TBS-T and incubated for 1 hour with secondary antibody at room temperature. After another wash, weak ECL solution was added, exposed to film and developed. If the signal was weak, strong ECL solution was added, exposed to film and developed. The membrane was incubated with stripping buffer for 15 minutes before adding the primary antibody for NR2A and repeating the process.

3. Results and Discussion

DNA construct

Bacterial transformation of NR1 and NR2A and plasmid isolation generated plasmids of high purity as determined by the absorbance spectrum from NanoDrop as shown in Table 1. Both the plasmids have high concentration and purity as indicated by the A_{260}/A_{280} and A_{260}/A_{230} values which fall in the range of 1.7-2.0 and greater than 1.5 respectively. No peak was observed at 320 nm in either case indicating that there were no contaminating organic compounds present.

DNA	Concentration (ng/µL)	A_{260}/A_{280}	A_{260}/A_{230}
NR1	5594.5	1.88	2.19
NR2A	1506.0	1.88	2.15

Table 1. Concentration and purity of NR1 and NR2A plasmids.

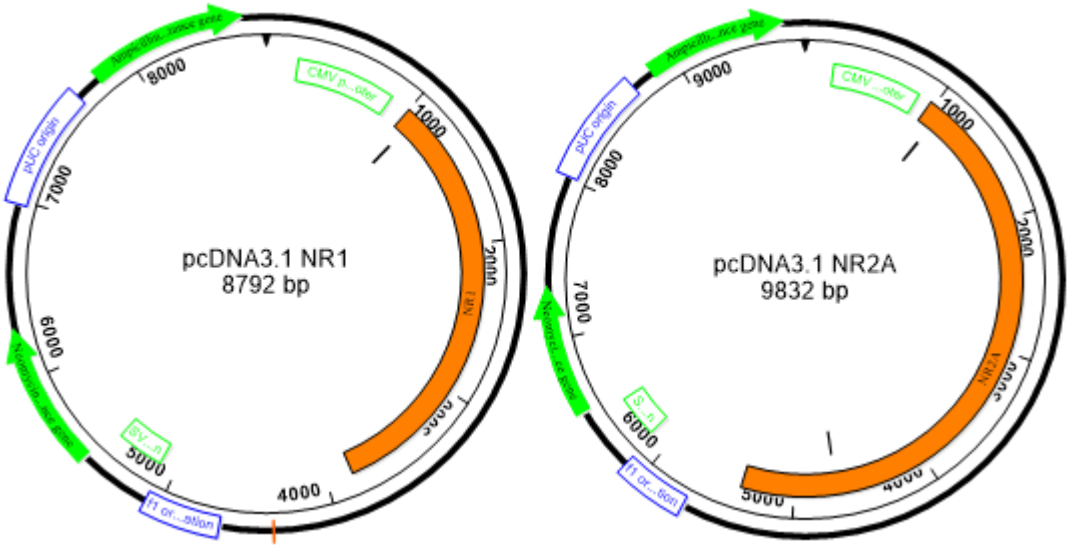

Fig 4. pcDNA3.1 constructs of NR1 and NR2A.

Electrophysiology

Whole-cell patch-clamp recordings of HEK-293 co-transfected with NR1, NR2A and GFP showed the presence of whole-cell currents (Fig. 5). This proved that the receptors expressed from the NR1 and NR2A DNA constructs were functionally active.

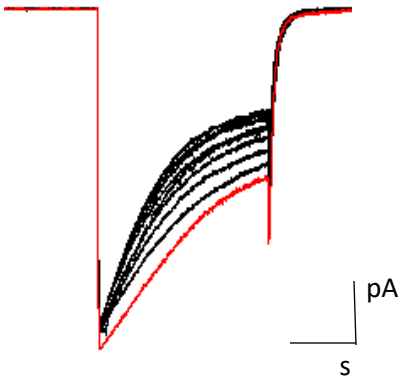

Fig 5. Whole-cell recordings of cells.

Lipofectamine 3000 transfections

Several transfection conditions were applied to determine the effect each one has on transfection efficiency.

DNA : Lipofectamine ratio

Two 6-well plates were transfected with DNA:Lipofectamine ratios of 1:1, 1:1.5, 1:2 and 1:3 in DMEM (10% FBS, 1mM L-glutamine, 5 mL pen/strep) (growth media). This means that in case of 1:1, if 2.5 µg of DNA is used then 2.5 µL of Lipofectamine will be used. The cells were 60% confluent at the time transfection. GFP images taken 24 hours post-transfection showed very little to no expression. 48 hours post-transfection images showed some cells expressing GFP (Fig 6). The cells transfected with 1:3 DNA:Lipofectamine had all died proving that it is too high concentration for cell survival. The images for 1:1, 1:1.5 and 1:2 do not show a significant difference in GFP expression. However, the manufacturer's protocol does not recommend a DNA:Lipofectamine ratio lower than 1:1.5. As a result, 1:1.5 and 1:2 were used for follow-up experiments.

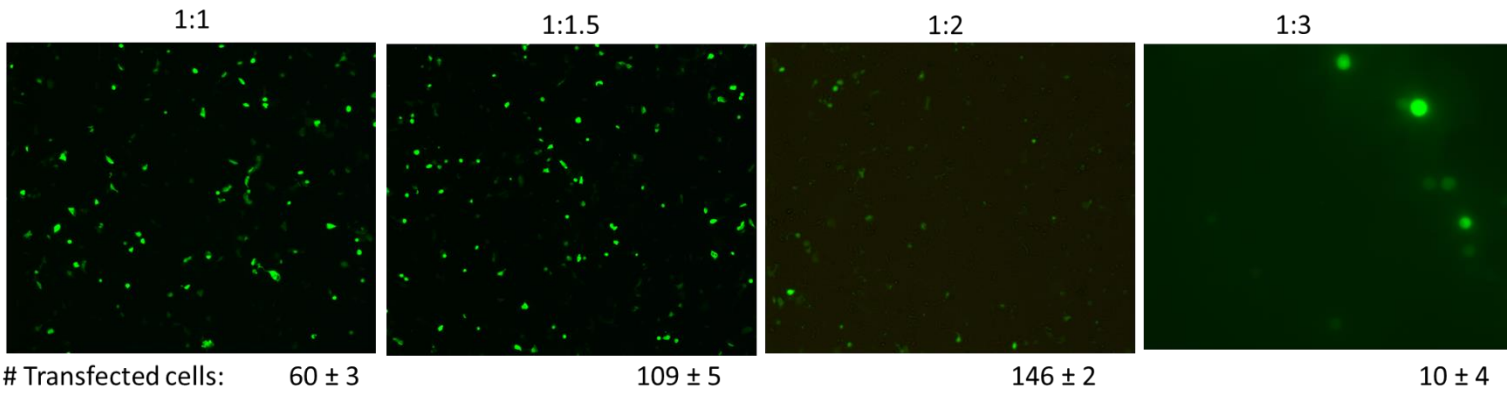

Fig 6. GFP images of cells 48 h post-transfection with DNA:Lipofectamine ratio 1:1, 1:1.5, 1:2 and 1:3.

Transfection medium

Three different transfection media were used for transfection using DNA:Lipofectamine ratios 1:1.5 and 1:2. Transfection media refers to the media in which the cells were present when DNA-Lipofectamine mixture was added to the cells. The three different media were Opti-MEM, growth media and growth media without pen/strep. The GFP images taken 48 h post-transfection are shown in Fig. 7. From the images, there seemed to be no apparent difference in GFP expression in different transfection media conditions. The GFP fluorescence intensity was recorded from equal volume of cell lysates as shown in Fig. 8 and Fig. 9. However, the fluorescence intensity values were indicative of neither a trend in GFP expression in cells nor any preference for a specific transfection media.

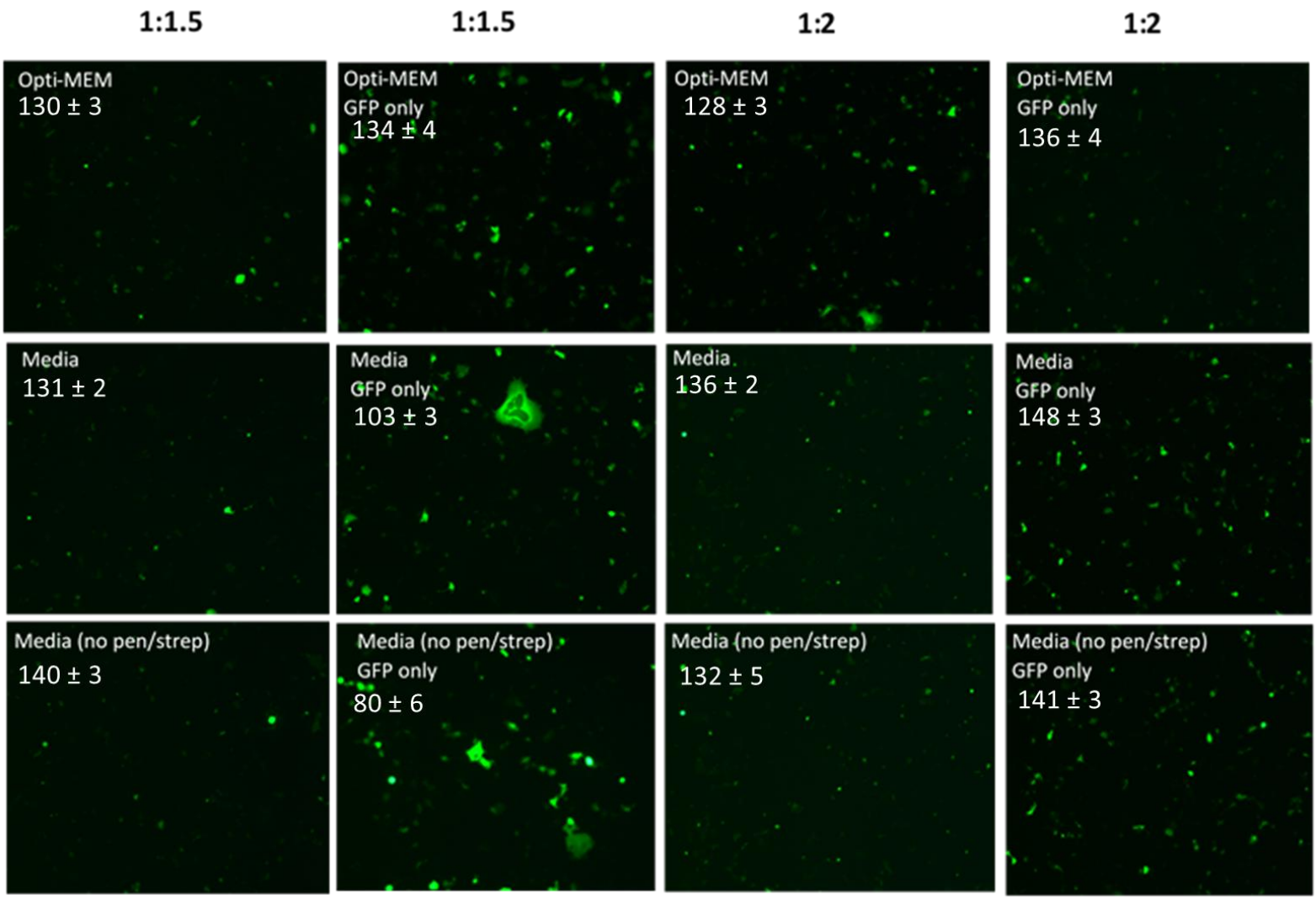

Fig 7. GFP images of cells 48 h post-transfection with DNA:Lipofectamine ratio 1:1.5 and 1:2. The numbers on each image indicate the mean number of cells counted from 3 samples under same transfection condition.

Western blot of cells transfected in Opti-MEM and growth media showed bands at around 120 kDa for NR1 and 170 kDa for NR2A (Fig. 10 and Fig. 11). Interestingly, cells transfected with DNA:Lipofectamine ratio of 1:2 showed a stronger band for NR1 compared to those with 1:1.5. This suggests greater expression of NR1 when 2.5 µg of DNA and 5 µL of Lipofectamine were used for transfection in either Opti-MEM or growth media in 6-well plate compared to using 2.5 µg of DNA and 3.75 µL of Lipofectamine. The high level of non-specific binding of the

secondary antibody could be due to the presence of unwanted proteins on the membrane due to insufficient washing of the PVDF membrane.

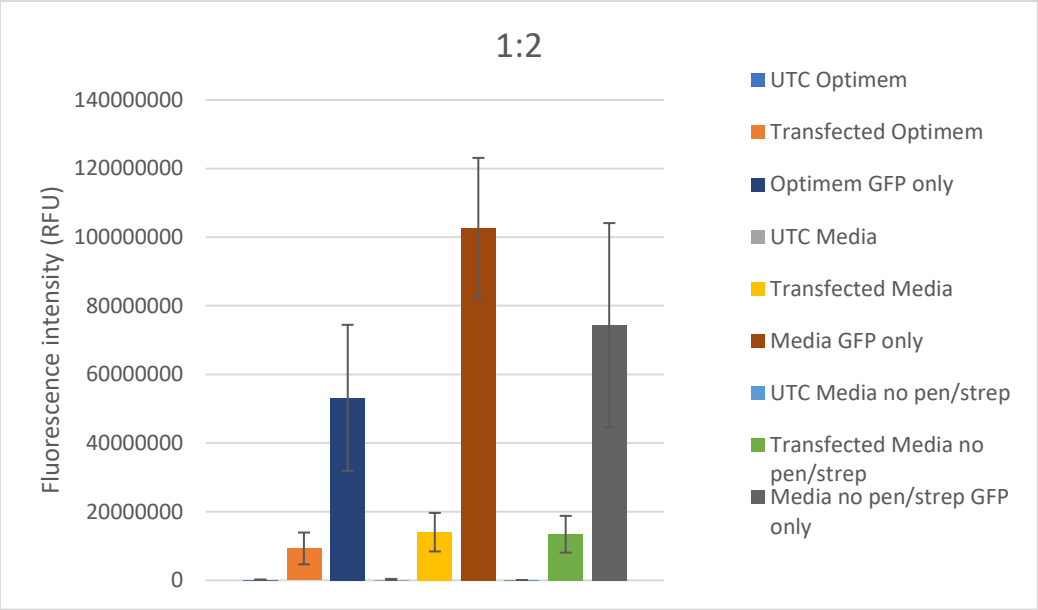

Fig 8. Fluorescence intensity of GFP from cell lysates on DNA:Lipofectamine ratio 1:2 transfections. *UTC:* Untreated cells. *Transfected:* Cells transfected with NR1, NR2A, GFP. *GFP only:* Cells transfected with GFP only.

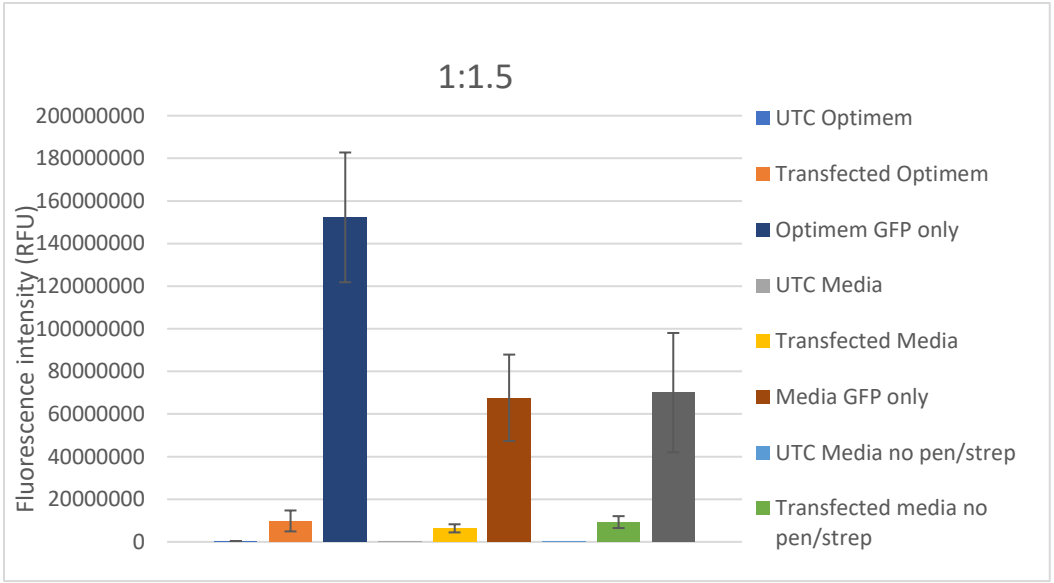

Fig 9. Fluorescence intensity of GFP from cell lysates on DNA:Lipofectamine ratio 1:1.5 transfections. *UTC:* Untreated cells. *Transfected:* Cells transfected with NR1, NR2A, GFP. *GFP only:* Cells transfected with GFP only.

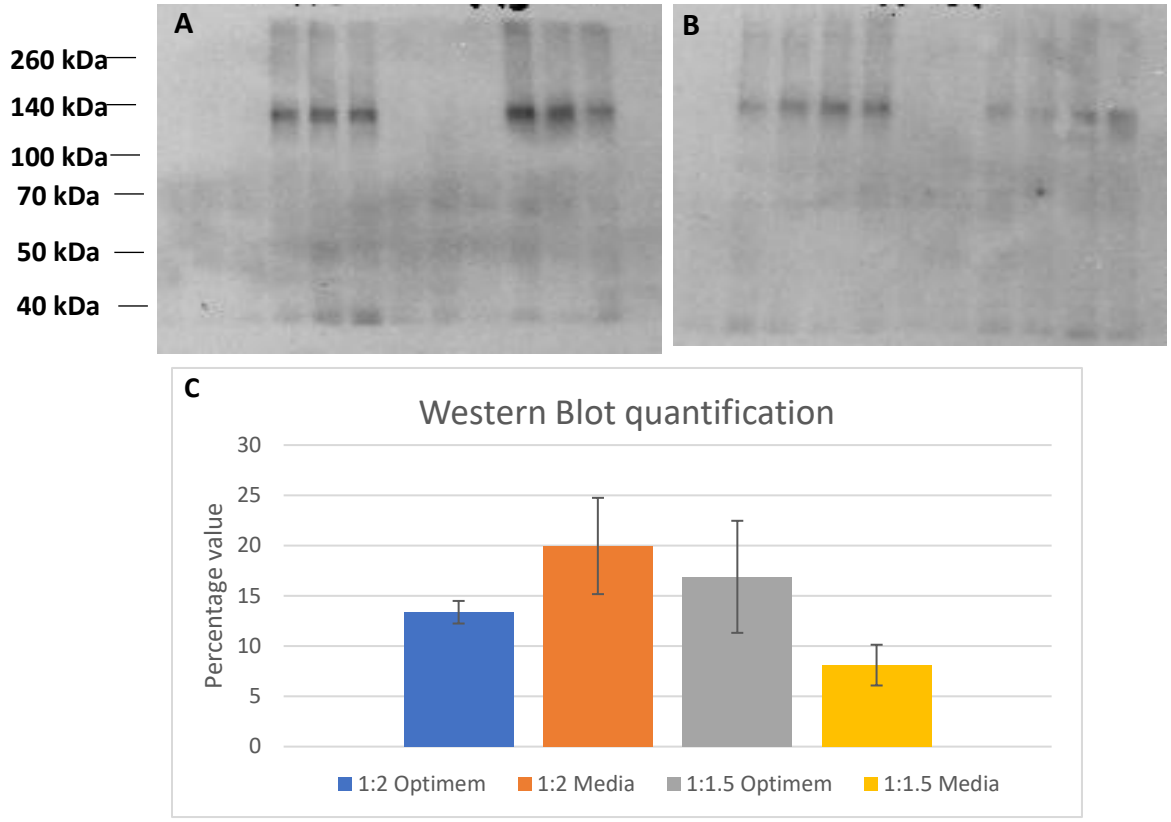

Fig 10. Western blot for NR1. 48 h post-transfection with DNA:Lipofectamine ratio (A) 1:2 (B) 1:1.5 (C) Quantification of NR1 bands using ImageJ.

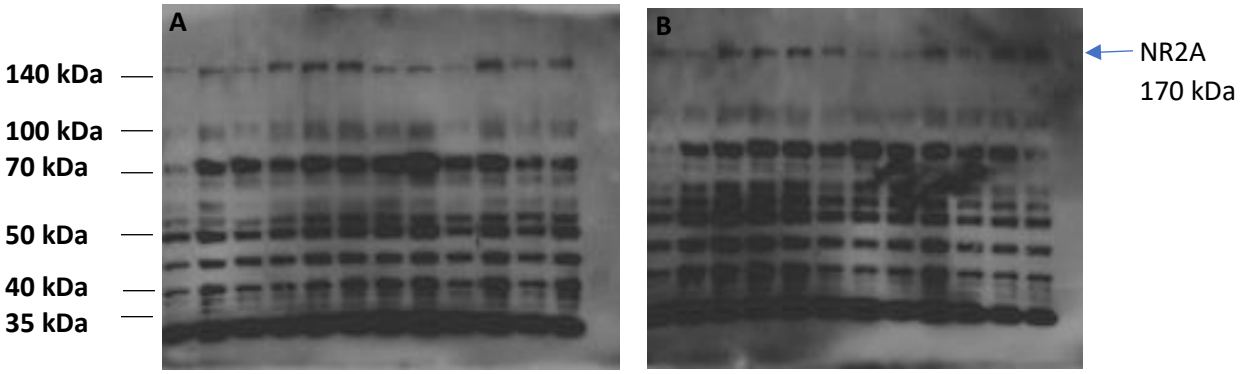

Fig 11. Western blot for NR2A. 48 h post-transfection with DNA:Lipofectamine ratio (A) 1:2 (B) 1:1.5

Confluency of cells

The previous two transfections were performed at 60% and 70% confluency of cells. In both cases, the GFP images recorded 48 hours post-transfection did not shown high transfection efficiency. For the third experiment, cells were plated the day before transfection such that they were 80-90% confluent at the time of transfection. Cells at 80-90% confluency were transfected with DNA:Lipofectamine ratio of 1:1.5 in Opti-MEM. 48 hours after transfection, GFP images were recorded as shown in Fig. 12. Comparison of the images taken after transfection at 80-90% confluency with the ones taken after transfection at 60-70% confluency clearly showed that the former condition increased transfection efficiency of the cells.

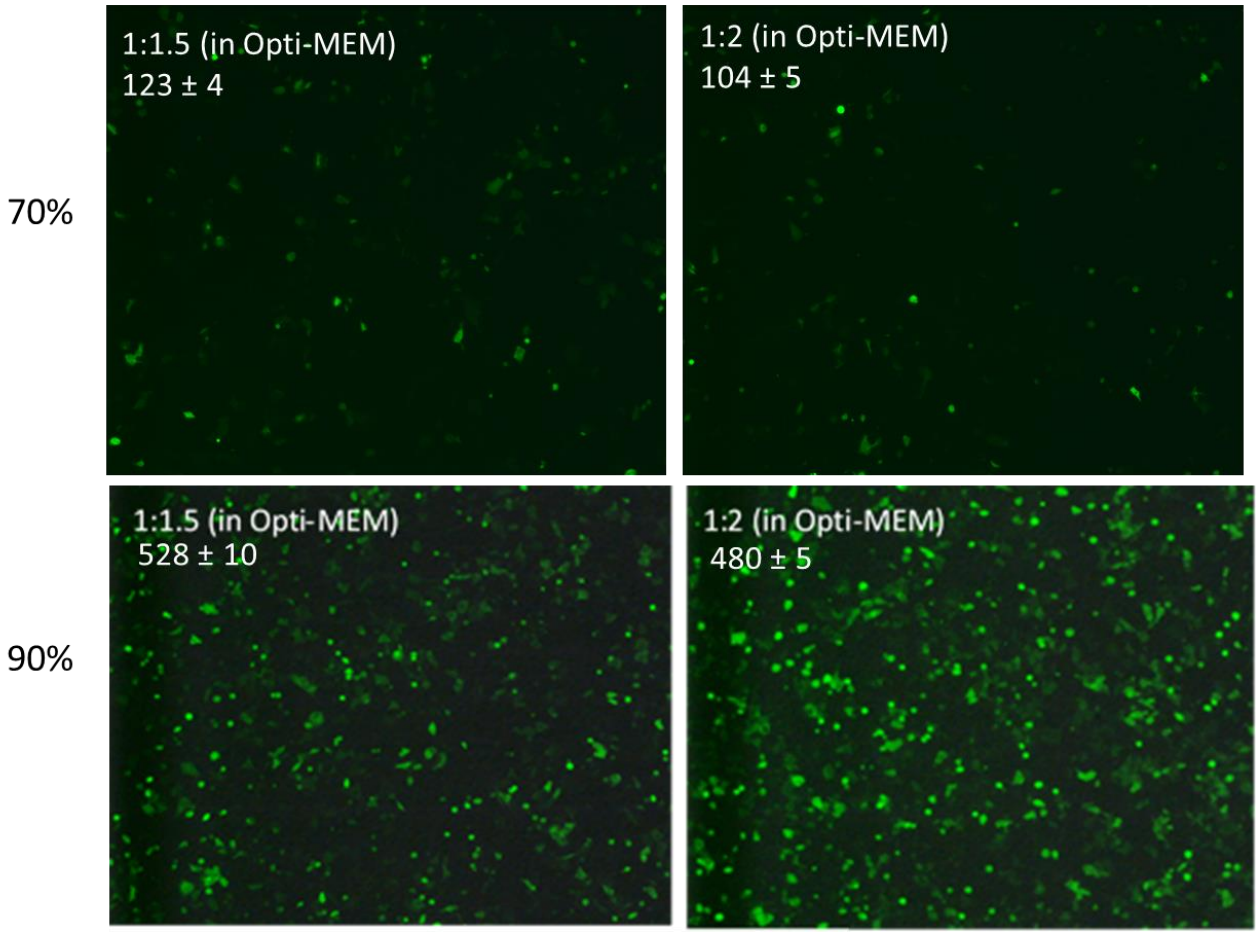

Fig 12. GFP images of cells 48 h post-transfection. The numbers on each image indicate the mean number of cells counted from 3 samples under same transfection condition.

Varying the ratio of NR1:NR2A

Previously published transfections protocols for expression on NMDA receptors in HEK-293 cells have used NR1:NR2A ratio of 1:3 and 1:1[15, 16]. In order to observe the effect of changing different NR1:NR2A ratio on transfection efficiency, HEK-293 cells were transfected at 80-90% confluency with NR1:NR2A ratio of 1:1 and 1:3, DNA:Lipofectamine ratio of 1:1.5 in Opti-MEM transfection media. Images of cells taken 48 hours after transfection are shown in Fig. 13. No significant difference in transfection efficiency could be observed from images. Western blot and farther experiment need to be carried out to determine the effect of varying proportion of NR1 and NR2A.

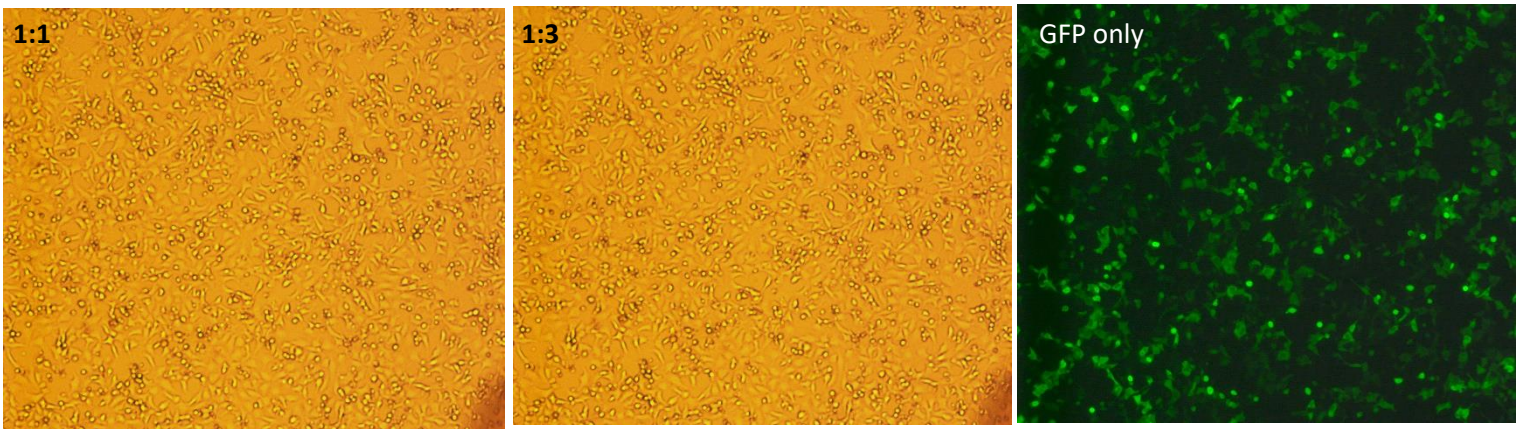

Fig 13. Brightfield and GFP images of HEK-293 cells 48 h post-transfection.

4. Conclusion

Lipofection is one of the most commonly used method of transfection that is simple to use as well as produce high transfection efficiency. In this study, Lipofectamine 3000 has been used to transiently transfect HEK-293 cells with NR1, NR2A and GFP. Different transfection conditions have been tried and the effect of each condition on transfection efficiency have been monitored using fluorescence microscopy, GFP fluorescence intensity measurements and Western blot.

Fluorescence microscopy used to take GFP images of transfected cells suggested that using DNA:Lipofectamine ratio of 1:1.5 and 1:2, highest transfection efficiency was obtained when cells were transfected when they are 80-90% confluent. Moreover, GFP images alone could not tell any difference between the transfection efficiency when NR1:NR2A ratio used was 1:1 as opposed to 1:3.

GFP fluorescence intensity were measured from cell lysates from cells transfected in three different transfection media namely Opti-MEM, growth media and growth media with no pen/strep. However, no trend nor preference for one transfection media over another was observed. This suggests that serum-starving the cells plays no role uptake of foreign DNA. Moreover, the presence of antibiotics does not affect the cell viability at this stage.

NR1 bands observed from the western blot of cells after transfection showed stronger signal for DNA:Lipofectamine ratio 1:2 compare to that of 1:1.5, irrespective of the transfection media. This means that at 1:2 ratio, the concentration is not too high to be toxic to cells but also sufficient for efficient delivery of DNA to cells.

As a result, it can be concluded the optimal conditions for transfecting HEK-293 cells with NR1 and NR2A are 80-90% confluency at the time of transfection, DNA:Lipofectamine ratio of 1:2 in

Opti-MEM or growth media. This optimized protocol can be used to express NMDA receptors in HEK-293 cells for numerous cell-based assays to evaluate the activity of new drugs.

5. References:

1. Traynelis, S. F.; Wollmuth, L. P.; McBain, C. J.; Menniti, F. S.; Vance, K. M.; Ogden, K. K.; Hansen, K. B. Glutamate Receptor Ion Channels: Structure, Regulation, and Function. *Pharmacol. Rev.* **2010**, 62, 405–496.

2. Paoletti, P.; Bellone, C.; Zhou, Q. NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nat. Rev. Neurosci.* **2013,** 6, 383-400.

3. Benveniste, M.; Mayer, M. L. Kinetic analysis of antagonist action at N-methyl-D-aspartic acid receptors. Two binding sites each for glutamate and glycine. *Biophys. J.* **1991,** 59, 560-573.

4. Karakas, E.; Furakawa, H. Crystal sstructure of a heterotetrameric NMDA receptor ion channel. *Science.* **2014,** 344, 992-997.

5. Akazawa, C.; Shigemoto, R.; Bessho, Y.; Nakanishi, S.; Mizuno, N. Diferential expression of five N-methyl-D-aspartate receptor subunot mRNAs in the cerebellum of developing and adult rats. *J. Comp. Neurol.* **1994**, 347, 150-160.

6. Monyer, H.; Burnashev, N.; Laurie, D. J.; Sakmann, B.; Seeburg, P. H. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron.* **1994**, 12, 529-540.

7. Gielen, M.; Siegler Retchless, B.; Mony, L.; Johnson, J. W.; Paoletti, P. Mechanism of differential control of NMDA receptor activity by NR2 subunits. *Nature.* **2009**, 459, 703-707.

8. Yuan, H.; Low, C. M.; Moody, O. A.; Jenkins, A.; Traynelis, S. F. Ionotropic GABA and glutamate receptor mutations and human neurologic diseases. *Mol. Pharmacol.* **2015**, 88, 203-217.

9. Yuan, H.; Hansen, K. B.; Zhang, J.; Pierson, T. M.; Markello, T. C.; Fajardo, K. V. Functional analysis of a de novo GRIN2A missense mutation associated with early-onset epileptic encephalopathy. *Nat. Commun.* **2014**, 5, 3251.

10. Strong, K. L; Jing, Yao; Prosser, A. R.; Traynelis, S. F.; Liotta, D. C. NMDA modulators: an updated patent review (2013-2014). *Expert. Opin. Ther. Pat.* **2014**, 12, 1349-1366.

11. Kim, T. K.; Eberwine, J. H. Mammalian cell transfection: the present and the future. *Anal. Bioanal. Chem.* **2010,** 397, 3173-3178.

12. Felgner, P. L.; Gadek, T. R.; Holm, M.; Roman, R.; Chan, H. W.; Wenz, M.; Noethrop, J. P.; Ringold, G. M.; Danielsen, M. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci.* **1987,** 84, 7413-7417.

13. Bio-rad. Transfection methods overview. www.bio-rad/transfection.

14. Stepanenko, A. A.; Dmitrenko, V. V. HEK293 in cell biology and cancer research: phenotype, karyotype, tumorigenicity, and stress-induced genome-phenotype evolution. *Gene.* **2015,** 569, 182-90.

15. Yuan, H.; Hansen, K. B.; Vance, K. M.; Ogden, K. K.; Traynelis, S. F. Control of *N*-methyl-D-aspartate Receptor Function by the NR2 Subunit Amino-Terminal Domain. *J. Neorosci.* **2009,** 29, 12045.

16. Sirrieh, R. E.; MaLean, D. M.; Jayaraman, V. Amino-terminal Domain Tetramer Organization and Structural Effects of Zinc Binding in the *N*-Methyl-D-aspartate (NMDA) Receptor. *J. Biol. Chem.* **2013,** 288, 22555.

17. Pierrio, C.; Nicole, O.; Buisson, A. GluN2B Subunit Labeling with Fluorescent Probes and High-Resolution Live Imaging. *Methods. Mol. Biol.* **2017,** 1677, 171-183.