Chapter 3

Genetic and Functional Analysis of GRIN2A in Tumor Samples

Todd D. Prickett, Jared J. Gartner, and Yardena Samuels

Abstract

Ionotropic glutamate receptors (iGluRs) are large integral membrane multi-protein complexes that create ion channels in plasma membranes. Upon binding of receptor specific ligands (e.g., glutamate), increased efflux or influx of mono- or divalent cations (e.g., Ca^{2+}) promotes synaptic transmission, cellular migration, and survival. Three classes of iGluRs were originally defined after their respective agonists: AMPA, kainate, and NMDA receptors (NMDARs). Recently, we examined iGluR families at the genetic level using Next-Generation Sequencing (NGS) (whole-exome sequencing (WES)) and discovered a high prevalence of somatic mutations within the gene for one of the NMDAR subunits, GRIN2A, specifically in malignant melanoma. Following confirmation of the somatic mutations, we focused on functional characterization of a subset of the GRIN2A mutants that demonstrated a loss of NMDAR functionality. We used gene expression and protein biochemistry to examine complex formation between GluN1 subunit (encoded by GRIN1) and GluN2A subunit (encoded by GRIN2A), anchorage-independent growth in soft agar and cellular migration. Furthermore, we used shRNA depletion of endogenous GRIN2A in melanoma cells expressing either wild-type GRIN2A or mutant GRIN2A and measured cellular proliferation compared to negative controls. Our data show that somatic mutation of certain residues in GluN2A results in increased survival and is the first such report to demonstrate the functional importance of GRIN2A mutations in melanoma and the significance ionotropic glutamate receptor signaling plays in malignant melanoma.

Key words NMDA receptor, Melanoma, Sequencing, Somatic mutation, Ion channel

1 Introduction

N-methyl-D-aspartate receptors (NMDAR) play major roles in many neuronal processes and disease etiologies such as learning, memory, neurodegeneration (i.e., Schizophrenia), epilepsy, and cancer [1–6]. The NMDARs are heterotetrameric complexes consisting of two NR1 (GRIN1) subunits and two NR2 (GRIN2 (A–D)), or a mixture of GRIN2 and GRIN3 subunits predominantly expressed in neuronal cells. Upon binding of its cognate ligands (e.g., glutamate), NMDAR permits Ca^{2+} influx resulting in increased intracellular calcium levels leading to activation of calcium-dependent signal transduction [7]. To understand how
NMDA receptors might be connected to the etiology of melanoma, whole-exome sequencing and functional biochemical and cell biology assays can be applied.

To study the mechanisms that NMDARs complexes employ in the functioning of cells multiple genomic and proteomic approaches can be used. Utilizing whole-exome sequencing, a high prevalence of somatic mutations in GRIN2A in malignant melanoma was discovered [3] revealing the unexpected discovery that GRIN2A was somatically mutated in ~25% of melanoma cases. Employing techniques such as coexpression of both NMDAR subunits in transformed human cells allows for biochemical analysis of complex formation, ion channel influx, as well as functional studies allow for detection of mutation effect on gene [2]. To examine the roles that the normal versus mutated GRIN2A plays in melanoma cells, anchorage-independent (soft agar colony formation) and migration assays were performed to help delineate whether the wild-type GRIN2A gene product might be a proto-oncogene or a tumor suppressor. Mutated GRIN2A versions of proto-oncogenes would have magnitudes of growth (colony formation) and increased migratory potential over the wild-type gene products. Conversely, wild-type GRIN2A versions of tumor suppressors would show little to no soft agar colonies nor migratory cells compared to mutated versions of tumor suppressors. Furthermore, using cellular proliferation assays and shRNA-mediated knockdown of GRIN2A (wild-type vs. mutants) should help in determining what the tumorigenic potential is for mutated versions of GRIN2A in melanoma. Taken together, the genomic and proteomic analyses will show how these mutations affect NMDAR function, giving a better understanding of how NMDA receptors work in melanomagenesis.

2 Materials

2.1 Sequencing Materials

1. DEPC H₂O.
2. Hot start, high fidelity Taq polymerase 5 units/μL.
3. Exonuclease 20 units/μL.
4. Shrimp Alkaline Phosphatase 5 units/μL.
5. Sephadex G50 fine.
6. Filter plate millipore (0.45 μm, clear, nonsterile).
7. 5× sequencing buffer.
8. M13 forward primer.
9. dNTP mix. Stock of each dNTP at an initial concentration of 100 mM. Combine 1 mL of each dNTP + 6 mL DEPC H₂O
for a final concentration of 10 mM. Mix well, aliquot in DNase/RNase-free tubes, and store at −20 °C.

10. 10× PCR buffer: 16.6 mL of 2 M Ammonium Sulfate, 89 mL of 1.5 M Tris–HCl (pH 8.8), 13.4 mL of 1 M MgCl₂, 1.4 mL of 14.4 M of 2-Mercaptoethanol, 79.6 mL of dH₂O. Combine ingredients mix well and store at −20 °C.

2.2 GRIN2A Functional Studies

All the solutions used in Subheading 3 use molecular biology-grade water (RNase-free, DNase-free, Protease-free, and Pyrogen-free) suitable for cloning, Western Blot (WB) and Immunoprecipitation (IP) analysis, stable expression, proliferation assays, migration, and soft agar assays, shRNA knockdown analysis and measurement of calcium uptake. A list of items used to determine, detect, and measure GRIN2A are shown below:

1. GRIN1 and GRIN2A open reading frame (ORF) constructs for expression and GRIN2A shRNA for knockdown.
2. PCR 2 × master mix.
3. HEK293T and 31T melanoma.
4. 1% lysis buffer: 1% NP-40, 50 mM Tris–HCl pH 7.5, 150 mM NaCl, Complete Protease Inhibitor tablet EDTA-free, 1 μM sodium orthovanadate, 1 mM sodium fluoride, and 0.1% β-mercaptoethanol.
5. Protein A/G beads.
7. Antibodies: anti-GluN1 (sc162902), anti-GluN2A (sc1468), or anti-GluN2A (sc-136004) (Santa Cruz Biotechnology), anti-P-p38 MAPK, anti-p38 (Cell Signaling), and anti-GAPDH (Calbiochem-EMD Biosciences).
8. SYBR Green I.
9. 8.0 μm of BD Biocoat.
10. TCN-213 (dissolved in DMSO).
13. Calf intestine alkaline phosphatase (CIP).
14. LB broth/Super Broth medium.
15. Enhanced chemiluminesences reagent (ECL).
16. NotI (20 U/mL) and XbaI (10 U/mL).
17. Bovine serum albumin (BSA).
18. DNA ligation kit.
19. Puromycin (4 μg/mL) and Neomycin (~400 μg/mL).
20. Gel Extraction Kit, Plasmid DNA kit, RNA purification kit.
22. 35 mm glass-bottomed culture dishes.
23. Flou-3 AM (4-(6-Acetoxymethoxy-2,7-dichloro-3-oxo-9-xanthenyl)-4’-methyl-2,2’(ethylene dioxy)dianiline-N,N,N’,N’-tetraacetic acid tetrakis(acetoxymethyl) ester).

3 Methods

3.1 Primer Design

Primers for targeted sequencing should be designed according to the capabilities of your sequencer. Recommendations below should be adapted if necessary:

1. Primers are designed to create amplicons in the size range of 300–600 bp covering all coding exons.
2. In event that exons are larger than amplicon size primers were tiles to overlap by 100 bases until entire exon was covered.
3. BLAST algorithm is used for all primers against the target genome to ensure amplification of only desired target with no other potential annealing locations.
4. An M13 sequence is added to all reverse primers so that all amplicons can be sequenced using the same sequencing primer.
5. There are several freely available online tools to help with primer design. PrimerTile (http://research.nhgri.nih.gov/tools/) and PCRTiler (http://pcrtiler.alaingervais.org:8080/PCRTiler/) both utilize Primer3 for primer design and perform searches for cross hybridization.
6. Example of primers designed to target GRIN2A is provided in Table 1.

3.2 Touchdown PCR

Touchdown PCR is used to generate the amplicons for sequencing. Touchdown uses higher annealing temperatures that are gradually lowered allowing for increased specificity as well as yield of desired target amplicon [8].

1. Run PCR reaction (10 μL total volume).
   - A master mix can be prepared without the primers or template DNA and used accordingly to perform multiple reactions in a high-throughput manner.
Table 1
Primers designed for Sanger sequencing of GRIN2A. An M13F sequence has been tagged onto the end of all reverse primers allowing for sequencing of all amplicons with same sequencing primer. Exons that are represented by multiple primers are to necessitate the full coverage of the coding exon while still keeping the amplicon at appropriate length for sequencing platform.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Gene</th>
<th>Exon</th>
<th>Oligo sequence</th>
<th>TM</th>
<th>Mol. weight</th>
</tr>
</thead>
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<td>GRIN2A_R1</td>
<td>GRIN2A</td>
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<td>Exon1_3’_reverse</td>
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<th>Exon</th>
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<td>GTAAAACGACGGCCAGTGTGAGCCAGCGAGCAGGATAGC</td>
<td>66.31 °C</td>
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Components | Reaction volume
--- | ---
ddH₂O | 3.3 μL
10× PCR buffer | 1 μL
10 μM dNTP | 1 μL
DMSO | 0.6 μL
50 μM forward primer | 1 μL
50 μM reverse primer | 1 μL
Hot start Taq polymerase | 0.6 μL
(3 ng/μL) template gDNA | 2 μL

- Cycling conditions.

<table>
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<th>Stage</th>
<th>Cycles</th>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (s)</th>
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</table>

2. Verify amplification of a single PCR product.

Run your PCR products on a gel to confirm amplification of only a single PCR product of the desired size can help to identify any problems that may have occurred. Multiple bands or a smear are usually the result of mispriming. These can sometimes be eliminated by raising the annealing temperature in step 2 of the touch-down PCR by a few degrees for all stages.

1. Once a PCR product has been verified perform an exonuclease (Exo) and SAP treatment to remove single-strand DNA and free dNTPs.

Single Exo/SAP reaction scale accordingly for multiple reactions:
Components | Reaction volume
--- | ---
d\textsubscript{H}_2\text{O} | 1 μL
SAP buffer | 0.3 μL
Exonuclease | 0.3 μL
SAP | 0.6 μL
PCR product | 4 μL

Reaction conditions:

<table>
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<tr>
<th>Temperature</th>
<th>Duration (Time)</th>
</tr>
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<tr>
<td>37 °C</td>
<td>30 min</td>
</tr>
<tr>
<td>80 °C</td>
<td>15 min</td>
</tr>
<tr>
<td>4 °C</td>
<td>∞</td>
</tr>
</tbody>
</table>

2. After the EXO/SAP reaction store plates overnight at 4 °C or at −20 °C until ready.

### 3.3.2 Sequencing Reaction

Use chain-terminating chemistry to label sample.

1. Dilute EXO/SAP reactions 1:2 (add 6.5 μL dH\textsubscript{2}O per well).
2. Place 2 μL DNA product into each well of a clean PCR plate or tube.
3. Master Mix recipe (1 rxn):

<table>
<thead>
<tr>
<th>Components</th>
<th>Reaction volume</th>
</tr>
</thead>
</table>
d\textsubscript{H}_2\text{O} | 1.7 μL
5× sequencing buffer | 1 μL
50 μM M13 seq primer | 0.04 μL
Big dye | 0.26 μL

4. Add 3 μL master mix and spin briefly.
5. Sequencing PCR conditions.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Cycles</th>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (s)</th>
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<td>94</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>50</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>Hold</td>
</tr>
</tbody>
</table>
3.3.3 Sephadex Purification

To remove free labeled nucleotides.

Single reactions can be diluted and spun through a G50 purification column. For more high-throughput screens it is often more useful to make your own Sephadex plates. These can be made ahead of time and stored (you will need to use a well for each reaction that is being performed, 96 reactions will use one 96-well plate).

1. Pour dry Sephadex over a column loader plate and scrape into all of the wells, returning excess to a container.
2. Place the filter plate upside down on a loader and flip so that the Sephadex falls into the wells.
3. Add 300 μL sterile water to each well.
4. Allow the plate to incubate at room temperature for at least 3 h, or overnight at 4 °C sealed in a plastic bag.
5. Before loading sequencing reactions, spin plates placed on the top of a collection container at 2100 × g for 5 min to remove H₂O and create columns.
6. Dilute sequencing reactions to a total volume of 20 μL with H₂O.
7. Load all 20 μL directly onto Sephadex columns in filter plate.
8. Spin sample through a Sephadex plate and into a collection plate at 2100 × g for 5 min.

3.3.4 Run Sample on Sequencer

Samples were run on an Applied Biosystems 3730 × L Sanger Sequencer. Samples in 96-well PCR plates were setup in plate assemblies making certain that the plate septas were correctly placed. Plates assemblies were then placed into the instrument, stacking up to 16 plate assemblies at a time. Samples were then sequenced using the run scheduler to insert each individual plates ID. Once samples were sequenced, the data was then taken and analyzed for somatic changes in GRIN2A.

3.4 Construction of Wild-Type and Mutant GRIN2A Expression Vectors

- Cloning of mouse GRIN1 and mouse GRIN2A was done using PCR primers ((forward (5'-tctagagccaccatgagcacca tgcgcctgc-3', reverse (5'-gcggccgctcagctctccctatgacg-3')) for GRIN1 and ((forward (5'-tctagagccaccat ggcagagtgggctattg-3'), reverse (5'-gcggccgcttaaacatcagattcgatactag-3')) for GRIN2A).
- Full-length (wild-type and mutant) and truncated fragments were amplified from GRIN1 and GRIN2A clones using Hotstart PCR 2X Master mix.
- Use the standard PCR method to generate each fragment.
  1. Prepare PCR reagents as follows:
### Component Amount per reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot start polymerase</td>
<td>25 μL</td>
</tr>
<tr>
<td>Forward primer (10 μM)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Reverse primer (10 μM)</td>
<td>1 μL</td>
</tr>
<tr>
<td>DNA template (100 ng/μL)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>22 μL</td>
</tr>
<tr>
<td>Total volume</td>
<td>50 μL</td>
</tr>
</tbody>
</table>

2. Perform PCR reaction as shown below:

<table>
<thead>
<tr>
<th>Number of cycles</th>
<th>Temperature</th>
<th>Duration (Time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>10–20</td>
<td>95 °C</td>
<td>20 s</td>
</tr>
<tr>
<td></td>
<td>T&lt;sub&gt;m&lt;/sub&gt;-5 °C</td>
<td>20 s</td>
</tr>
<tr>
<td></td>
<td>72 °C</td>
<td>15–30 s</td>
</tr>
<tr>
<td>1</td>
<td>72 °C</td>
<td>3 min</td>
</tr>
<tr>
<td>1</td>
<td>4 °C</td>
<td>Infinite</td>
</tr>
</tbody>
</table>

3. Run PCR products on 0.9% agarose gels.

4. Purify PCR products from the gel using Gel Extraction kit and elute in 30 μL dH₂O.

5. Digest purified gene fragments, and pCDF1 and pCDH1 lentiviral expression vectors Plasmid with XbaI (5′) and NotI (3′) using the following reaction setup:

<table>
<thead>
<tr>
<th>Components</th>
<th>Reaction volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>Up to 50 μL</td>
</tr>
<tr>
<td>pCDF1 or pCDH1</td>
<td>3 μg</td>
</tr>
<tr>
<td>10× restriction buffer</td>
<td>5 μL</td>
</tr>
<tr>
<td>BSA (10 mg/mL)</td>
<td>1 μL</td>
</tr>
<tr>
<td>XbaI (20,000 U/mL)</td>
<td>2 μL</td>
</tr>
<tr>
<td>NotI (20,000 U/mL)</td>
<td>2 μL</td>
</tr>
</tbody>
</table>

6. Mix well and incubate at 37 °C for 2 h.

7. Add 2 μL calf intestine alkaline phosphatase (CIP-NEB) to vectors only, mix well, and incubate for an additional 30 min at 37 °C.

8. Run digested gene fragments and vectors on 0.8% agarose gel-EtBr and cut/purify using the Gel Extraction kit and elute in 30 μL of dH₂O.
9. Ligate purified vectors and gene products using the following reaction setup:

<table>
<thead>
<tr>
<th>Components</th>
<th>Reaction volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>Up to 20 μL</td>
</tr>
<tr>
<td>Digested vector</td>
<td>1 μL</td>
</tr>
<tr>
<td>Digested gene fragment</td>
<td>2–4 μL</td>
</tr>
<tr>
<td>5× ligase buffer</td>
<td>4 μL</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>2 μL</td>
</tr>
</tbody>
</table>

10. Transform *E. coli* chemically competent bacteria with 3–5 μL of the ligation mixture and incubate on ice for 30 min.

11. Heat shock bacteria: ligation mix using 42 °C for 1 min, then put mixture back on ice for 2–3 min.

12. Add 400–500 μL Super Broth to each tube and incubate in a 37 °C shaker for 1–2 h.

13. Spread 150 μL of transformed bacteria on LB/Amp plates and incubate overnight in a 37 °C incubator.

14. Pick a few colonies and grow in 2× Super Broth media containing 100 μg/mL Amp overnight in a 37 °C bacterial shaker.

15. Purify plasmids from bacteria using Plasmid preparation kit and elute DNA in 40–50 μL of dH₂O. Sequence each construct to ensure proper sequence (see Note 1).

3.5 Cell Culture and Transient Expression

1. Grow HEK293T cells in complete RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) and split 1–10 the day prior to transfection.

2. Grow Melanoma cells in RPMI-1640 and supplemented with 10% fetal bovine serum.

3. Maintain A375Cell, 501Mel, SK-Mel-2Cell cells (purchased from National Cancer Institute, Division of Cancer Treatment, Developmental Therapeutics Program, Frederick, MD) in RPMI-1640 and supplemented with 10% FBS.

4. Co-transfect HEK293T cells at a 6:1 ratio of reagent to constructs (μL:μg) using 2–6 μg of GRIN1 and GRIN2A constructs, or at a 1 to 1 ratio for expression studies and functional analysis.

5. Incubate transfected cells overnight at 37 °C plus CO₂ in a humidified incubator.
GluN1 and GluN2A expression were then tested by western blot (WB) analysis (see detailed protocol below). Primary antibodies used to interrogate specific protein complex formation for both normal phenotype and dominant negative phenotypes (Fig. 1a–c) and signal transduction pathway analysis (Fig. 1d) were anti-GluN1, anti-GluN2A, anti-GluN2A, anti-P-p38 MAPK, anti-p38, and anti-GAPDH.

1. Seed HEK293T cells in a T75 flask at 1 × 10⁶ cells 24 h prior to transfection.
2. Transfect cells as described above to detect expression of GluN1 and GluN2A to help analyze NMDAR complex formation, phenotype and ionic influx of calcium.
3. Incubate cells overnight at 37 °C in 5% CO₂ and humidified incubator.
4. Gently wash transfected cells twice with 6 mL 1× PBS.
5. Lyse cells using 1.0 mL 1% NP-40 lysis buffer (1% NP-40, 50 mM Tris–HCl pH 7.5, 150 mM NaCl, Complete Protease Inhibitor tablet, EDTA-free, 1 μM sodium orthovanadate, 1 mM sodium fluoride, and 0.1% β-mercaptoethanol) per T-75 flask for 20 min on ice.
6. Scrap lysed cells using a cell scraper and then transfer into a 1.5 mL microcentrifuge tube.
7. Centrifuge cell extracts for 10 min at 14,000 × g at 4 °C.
8. Immunoprecipitate 800 μL of cell supernatant overnight using 20 μL of anti-GluN1 or GluN2A and 30 μL of 50% slurry of Protein A/G beads.
9. Wash the immunoprecipitates (IPs) three times using 800 μL per wash.
10. After the final wash, add 50 μL of 2× SDS Sample buffer with β-ME to each IP and heat at 95 °C for 5 min.
11. Centrifuge Heated IPs at 10,000 × g for 1 min prior to WB analysis.
12. Load 10 μL of IP samples onto 4–20% gradient SDS gels and run for approximately 35 min at 130 V.
13. Transfer material for western blot analysis using a semidry western blot system with 1× transfer buffer, two thick blot papers, and a nitrocellulose membrane.
14. Once transfer is done, wash membrane briefly before blocking with a 5% dry milk solution (w/v) dissolved in 1× Tris Buffered Saline with Tween-20 (TBST) (0.05% Tween-20 (1× TBS) for 30–60 min.
15. Wash membranes quickly 2–3× in 1× TBST prior to primary antibody incubation (see Note 2).
Fig. 1 Somatic mutations in GRIN2A have adverse effects on receptor function and formation. Mutant forms of GRIN2A binding GRIN1 with reduced affinity, thus causing decreased NMDAR complex formation. (a) HEK293T cells were transiently transfected with WT GRIN1 and GRIN2A (WT or mutants) or empty vectors as control and immunoprecipitated with anti-GRIN1. Immunoprecipitates were probed with anti-GRIN2A and anti-GRIN1 to confirm binding. Lysates were probed with anti-GRIN2A, and anti-GAPDH as a loading control. (b) HEK293T cells were transiently transfected with WT GRIN1 and GRIN2A (WT or mutants) or empty vectors as control and immunoprecipitated with anti-GRIN2A. Immunoprecipitates were probed with anti-GRIN1 and anti-GRIN2A to confirm binding. Lysates were probed with anti-GRIN1, and anti-GAPDH as a loading control. (c) HEK293T cells
3.7 Pooled Stable Expression

As per the methodology of cloning the GRIN1 or GRIN2A genes, we used lentiviral constructs (either HIV-based or FIV-based) allowing for the use of two different antibiotic selections for dual-infection.

3.7.1 Lentiviral Production in HEK293 Cells

1. Day 0. Seed T-75 flasks with 2–3 million HEK293T cells per flask and incubate until they are >70% confluent.
2. Day 1. Add 8.6 μg of FIV-34 N plus 1.4 μg of pVSV-G plus 2 μg of expression/stable plasmid (pCDF1 expression vector) followed by the addition of 500 μL of Opti-MEMI.
3. Mix DNA and Opti-MEMI mixture well and then add 24 μL of Turbofect to mix and gently mix.
4. Incubate for 15–20 min at room temperature and then add DNA/Turbofect mixture to cells.
5. Incubate cells and mixture for 60+ h before harvesting medium.
6. Day 4. Carefully remove medium from flask and add to 15 or 50 mL conical tubes and centrifuge at 1000 × g for 5 min.
7. Carefully remove tubes from centrifuge and filter the medium through a 0.45 μm filter attached to a sterile 20 mL or 50 mL syringe (see Note 3).

3.7.2 Lentiviral Production in 31T or SK-Mel-2 Cells

Lentivirus for GRIN1 and GRIN2A (wild-type or mutants) and empty vector control were used to sequentially infect 31T or SK-Mel-2 cells as described below:

1. Seed 31T or SK-Mel-2 cells at 1.5 × 10^6 cells per T75 flask 24 h prior to infection.
2. Day 0. Plate cells into T-75 flasks at a density of 2 × 10^6 cells so that the day of infection the cells are >80% confluent before transducing (infecting) with virus.
3. Day 1(2). Make infection cocktails of complete medium for your cells of interest plus harvested virus from Day 3 above plus polybrene (Hexadimethrine bromide). Tend to try two to three different viral ratios if a new cell line or a new batch of virus is made. Use a total of 8–10 mL of viral mixture at this ratio for transduction when using a T75 flask. All viral cocktails contain 8 μg/mL of

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Fig. 1 (continued) were transiently co-transfected with WT GRIN1 and GRIN2A (WT or mutants) or empty vectors as control and stimulated with 200 μM NMDA in the presence of Ringer’s solution for 60 min. Lysates were probed with anti-P-p38 MAPK, anti-p38 MAPK, and anti-GRIN2A to confirm expression. Lysates were probed with anti-Tubulin as a loading control. (d) HEK293T cells were transiently co-transfected with WT GRIN1 and WT GRIN2A and increasing amounts of empty vector or W372X and immunoprecipitated with anti-GRIN1. Immunoprecipitates were probed with anti-GRIN1 and anti-GRIN2A to confirm binding. Lysates were probed with anti-GRIN1, and anti-GAPDH as a loading control.
polybrene final concentration (stock solution is at 8 mg/mL made up in water and filter sterilized). This is stored at 4 °C.

4. Day 2 (3). Change medium on infected cells to complete medium.

5. Day 3 (4). GFP lentivirus: keep incubating for an additional 48–72 h and then visually inspect cells using a fluorescent microscope.

6. Puromycin lentivirus: change medium to complete medium with the appropriate amount of puromycin as determined by kill curve (1–40 μg/mL) (see Note 4).

7. Days 5–8. Check that cells are dying off and that your control flask (no virus added to same cells infected) is dead or almost completely dead. After the control flask is completely dead then the cells that were infected can be harvested by either picking of individual clones or pooling all the cells together or both. These are your pooled clones to be used for functional assays.

8. Determine stable expression of GluN1 proteins (wild-type) and GluN2A (wild-type or mutants) by immunoprecipitation and SDS-PAGE analysis.

9. Then perform immunoblotting with anti-GluN1, anti-GluN2A, and anti-GAPDH to show equivalent expression among pools. (Described above) (see Fig. 2a–b).

10. Determine stable expression of GRIN2A (wild-type or mutants) by RT-PCR analysis of mRNA from 31T or SK-Mel-2 stable pooled clones using GRIN2A-specific primers and GAPDH primers as a loading control (data not shown).

3.8 Proliferation Assays

1. To examine growth potential of cells stably expressing GRIN1: GRIN2A (wild-type and mutants), seed pooled 31T or SK-Mel-2 pooled clones into 96-well plates at 300 cells per well in either 1%, 2.5% or 10% serum-containing medium.

2. Incubate cells for 13–17 days in 96-well plates covered loosely with saran wrap and a damp paper towel (reduces evaporation in wells).

3. Analyze samples every 48 h by lysing cells in 50 μL 0.2% SDS/well and incubating for 2 h at 37 °C.

4. Add 150 μL/well of SYBR Green I solution (1:750 SYBR Green I diluted in dH₂O).

5. Analyze plates using a fluorescence reader.

6. Plot data for proliferation assays using MicroSoft Excel to show growth or lack of potential for each stable pooled clone (Fig. 3a–b).
Fig. 2 Melanoma cells expressing mutant forms of GRIN2A have increased ability for anchorage-independent growth. Stable melanoma cell lines 31T and SK-Mel-2 expressing WT GRIN1 and GRIN2A (WT or mutants) or empty vector control and immunoprecipitated with anti-GRIN1 and anti-GRIN2A. Immunoprecipitates were probed with anti-GRIN2A and anti-GRIN1 to examine NMDAR complexes in 31T (a) or SK-Mel-2 (b).

Fig. 3 Expression of GRIN2A mutants in melanoma cell lines results in similar proliferation. Proliferation assay of stable pooled clones 2359 (31T) or SK-Mel-2 cells expressing WT or mutant forms of GRIN2A and empty vector control. Cells seeded in a 96-well plate were measured for proliferation using dilute SYBR Green I over 7–8 days.
3.9 Migration Assays

1. To pre-condition migration wells (8.0 μm), add 500–600 μL of warmed (37 °C) RPMI-1640 for 30–60 min at 37 °C.

2. Once pre-conditioned, carefully remove media from the top chamber and add 750 μL of RPMI-1640 supplemented with 10% FBS to the bottom chamber.

3. Seed 31T or SK-Mel-2 pooled clones into pre-conditioned migration wells at 30,000–100,000 cells per well in the serum-free medium in the top chamber and incubate for 24–48 h prior to harvesting (see Note 5).

4. For antagonist studies use a final concentration of 10 μM TCN-213 dissolved in DMSO in the top and bottom chambers of the Boyden chamber.

5. Use DMSO as a negative control for antagonist assays at <0.02% (vol/vol).

6. Once incubation times are up remove inserts and washed-differentially fix using a Hema 3 Stat Pack as per manufacturer’s protocol.

7. Analyze inserts and count for cells migrated per field of view using ImageJ (NIH software) (see Fig. 4a–b).

3.10 Soft Agar Assay

1. Prior to starting a soft agar assay, preheat a clean water bath to 45 °C.

2. Prepare 2.5% Bacto-Agar (H2O) (1.5 g/60 mL dH2O) and autoclave to dissolve and sterilize. Cool to 45 °C before use (keep in 45 °C water bath throughout the procedure).

3. Make Nutrient Mix: (see Note 6): 60 mL of 2× RPMI media (1.2 g powdered media into 58.8 mL sterile dH2O, plus 1.2 mL of 100× Glutamine), 30 mL of FBS, 150 mL of 1× RPMI.

Fig. 4 Melanoma cells expressing mutant forms of GRIN2A have adverse effects on receptor function in cellular migration. Melanoma cell line 31T (a) stably expressing GRIN1 and GRIN2A mutants or empty vector were seeded into Boyden chambers in 0.5% serum and grown for 48 h before staining and counting. Melanoma cell line 31T (b) stably expressing GRIN1 and GRIN2A mutants or empty vector were seeded into Boyden chambers plus and minus 10 μM TCN-213 for 48 h before staining and counting. Error bars are representative of n = 3 (s.d.) (*p < 0.01 students t-test)
4. Mix Bacto-Agar mix into nutrient mix for a 0.5% agar concentration, and swirl gently (avoid making bubbles). **Keep this mix at 45 °C** or it will cool and harden. This volume (300 mL) will be enough for up to 200 wells in 24-well plates.

5. Pipet 1 mL nutrient-agar mix into each well (24-well plate) without bubbles—try to pop any that may get into the wells with sterile microtips.

6. Allow agar to cool/harden at room temperature (should only take ~10 min), and then store at 37 °C until cell mixes are ready.

7. Trypsinize and count cells. You will need to make up a mix for plating cell samples in duplicate.

8. Dilute the 0.5 mL cell suspension into 1 mL nutrient-agar mix (has to be a 1/3 dilution for a final agar concentration of 0.33%) and swirl gently to mix.

9. Immediately layer 0.5 mL of cell-agar mix into each well already containing the 0.5% nutrient-agar base.

10. Allow several minutes at room temperature to cool/harden, and then wrap plates in saran wrap and place at 37 °C to grow—check for colonies every few days starting 1 week after plating (**see Note 7**).

11. To stain the colonies, add 500 μL crystal violet staining solution (0.005% C.V. with 0.25% formalin) on the top of soft agar.

12. Incubate dishes for 1 h in a 37 °C incubator.

13. To test anchorage independence of 31T or SK-Mel-2 pooled clones, plate cells in triplicate at 1000 cells/well and in top plugs consisting of sterile 0.33% Bacto-Agar (BD, Sparks, MD) and 10% FBS in a 24-well plate. The lower plug contains sterile 0.5% Bacto-Agar and 10% fetal bovine serum.

14. After 2 weeks, photograph the colonies and count colonies by quantifying using ImageJ (NIH software) (**see Fig. 5a–b**).

### 3.11 Lentiviral shRNA

Constructs for stable depletion of *GRIN2A* were confirmed to efficiently knock down *GRIN2A* at the RNA level. Lentiviral stocks were prepared as described below:

1. Day 1. Seed fresh HEK293T cells in T75 flask/per shRNA lentivirus at 2 × 10⁶ cells and incubate overnight.

2. Day 2. Transfect HEK293T (~70–80% confluence) using either TurboFect or Arrest-In.

3. In Tube A add 3 μg of shRNA vector, 3 μg of pHRCMV-8.2ΔR, and 0.3 μg of pCMV-VSV-G and then add 500 μL of OptiMEM-I serum-free medium.

4. In Tube B mix 500 μL OptiMEM-I serum-free medium and 40 μL of TurboFect (or Arrest-In). Allow this to incubate for 5 min at room temperature.
5. Add contents of Tube B to Tube A and incubate at room temperature for 20–40 min.

6. Add the mixture (~1 mL) to the HEK293T cells and incubate for 48 h.

7. Before adding the transfection mixture to the HEK293T cells you can change the media to lower serum containing media (from 10% to 2–3% serum) if you desire. This will allow the cells to still produce virus at a good rate but will stop them from getting to over-crowded.

8. Day 4. To harvest the virus, carefully remove the medium from flask to 50 mL conical tubes.

9. Spin at 3000 × g for 10–15 min.

10. Carefully remove the supernatant and add to 20 mL syringe attached to sterile filter (0.45 µm).

11. Aliquot into sterile 1.50 mL tubes for small infections or sterile 15 mL conical tubes for larger infections.

12. Store at −80 °C.

13. Infect melanoma cell lines (31T, 39T, 501Mel, or 125T) with shRNA lentiviruses for each condition (vector and two-three different GRIN2A specific shRNAs).

14. Select stable pooled clones in the presence of 3 µg/mL puromycin containing normal medium for 3–5 days prior to determining knockdown efficiency.

15. Test stably infected pooled clones for proper knockdown of mRNA via qRT-PCR analysis (Fig. 6a–b).

16. Test stably infected pooled clones in functional assays, proliferation assay (see Fig. 6c–f).

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**Fig. 5** Melanoma cells expressing mutant forms of GRIN2A have adverse effects on receptor function in anchor dependency. Melanoma cell line 31T (a) and SK-Mel-28 (b) stably expressing GRIN1 and GRIN2A mutants or empty vector tested for colony formation of 31T or SK-Mel-2 cells in soft agar, respectively. Error bars are representative of n = 3 (s.d.) (*p < 0.01 Student’s t-test in quantitative graphs)
3.12 Reverse Transcription PCR

1. Extract total RNA from pooled clones of melanoma cells stably expressing wild-type or mutant \textit{GRIN2A} forms or knocked down for endogenous \textit{GRIN2A}.

2. Elute total RNA in 30 μL diethylpyrocarbonate (DEPC)-treated distilled H\textsubscript{2}O.

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**Fig. 6** \textit{GRIN2A} functions as a tumor suppressor in melanoma cells. Stable depletion of endogenous \textit{GRIN2A} in melanoma cells. Melanoma cells stably depleted of endogenous \textit{GRIN2A} were confirmed via qRT-PCR of all clones. (a–b) Analysis of knockdown used specific \textit{GRIN2A} primers and efficiency was compared to GAPDH levels in each sample, thus generating a fold change. Proliferation assay of wild-type 31T (c) or 39T (d) and mutant 501Mel (e) or 125T (f) expressing \textit{GRIN2A} cell lines depleted of \textit{GRIN2A}. Knockdown resulted in little to no change in proliferation for 125T or 501Mel melanoma cell lines expressing mutant \textit{GRIN2A} but increased proliferation for both 31T and 39T cell lines expressing WT \textit{GRIN2A}. Each cell line used pLKO.1 as an empty vector control.
3. To make cDNA use in qRT-PCR assay, aliquot 1 μg RNA into a labeled RNase-free tube on ice. Bring volume up to 8 μL with DEPC-H2O.

4. Add 1 μL 10× buffer and 1 μL DNase to the RNA and tap gently to mix. Incubate at room temperature for 15 min. Add 1 μL of 25 mM EDTA to the RNA mix and incubate at 65 °C to heat inactivate the DNase I for 10 min.

5. Place on ice for 1 min and then proceed to cDNA synthesis protocol.

6. For cDNA Synthesis, combine the following in clean DNase/RNase-free tube: 7 μL of DEPC-H2O, 1 μL of 10 mM dNTP, 1 μL of Primer (50 μM Oligo(dT) or 50 ng/μL random hexamers).

7. Tap mix well to combine and add 9 μL to the 11 μL RNA tube (see the DNase step above).

8. Incubate at 65 °C for 5 min, then place on ice for 1 min.

9. Prepare the cDNA synthesis mix. Add each component in the order shown as follows: 4 μL of 10× RT buffer, 8 μL of 25 mM MgCl2, 4 μL of 0.1 M DTT, 1 mL of RNase OUT, 1 mL of RT enzyme, 2 μL of DEPC-H2O.

10. Add 20 μL mix to each RNA mix and tap gently to combine.

11. For Oligo(dT)—incubate for 50 min at 50 °C.

12. For Random Hexamers—incubate for 10 min at room temperature, then 50 min at 50 °C.

13. Terminate reactions at 85 °C for 5 min—chill on ice.

14. Collect the reaction by a brief centrifugation, then add 1 μL RNase H to each tube and incubate at 37 °C for 20 min.

15. cDNA synthesis is complete: dilute product with 40 μL of sterile dH2O prior to use.

16. Amplify cDNA using the oligo dT20 primer supplied in the kit.

17. To test for loss of GRIN2A message, use 1 μL of cDNA in the PCR reaction with either GRIN2A primers or GAPDH primers.

### 3.13 Proliferation Assays of Stable Knockdown Cells

1. Stable pooled knockdown clones for GRIN2A were seeded into 96-well plates at 500 cells per well in either 1%, 2.5% or 10% serum-containing medium.

2. Wrap plates loosely in saran wrap with a damp paper towel and incubate at 37 °C for 6–8 days.

3. Analyze samples every 2–3 days by lysing cells in 50 μL 0.2% SDS/well and incubating for 2 h at 37 °C.

4. Add 150 μL/well of SYBR Green I solution (1:750 SYBR Green I diluted in dH2O).

5. Analyze plates using a fluorescence reader (Fig. 6c–f).
3.14 Calcium Assays

1. Seed HEK293T cells in 35 mm glass-bottomed culture dishes at a density of $1 \times 10^5$ cells per plate.

2. Transfect HEK293T cells transiently with GluN1 and either GluN2A WT or the following mutants (Q891X, R920K, W1271X).

3. Thirty six hours post transfection wash cells twice with Tyrode solution (5 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 1.9 mM CaCl$_2$, 5.6 mM glucose, buffered to pH 7.4 with Tris base) prior to loading with Fluo-3Am.

4. Load cells with 10 μM Fluo-3 AM diluted in Tyrode solution supplemented with 0.1% BSA for 45 min in the dark.

5. Wash cells subsequently with Tyrode-BSA twice followed by two washes of Tyrode solution alone.

6. Leave cells for 15 min in the dark to ensure complete hydrolysis of the acetoxymethyl ester (AM) groups.

7. Take cell images every 5 s over a period of 15 min and collect images on a Zeiss LSM-700 laser-scanning microscope with a 63× oil immersion lens in a single-track mode using excitation 488 nm and emission BP 505-530 filter sets.

8. Analyze images to determine how mutant GluN2A molecules function compared to wild-type GluN2A in calcium influx (Fig. 7).

4 Notes

1. Send several constructs of the same type for sequencing and then analyze sequence to ensure that the fragments contain either full length wild-type or one of the specific somatic mutations found during whole exome sequencing.

2. Once the secondary antibody incubation and final washes are done rinse three to four times in TBS or dH$_2$O prior to the addition of ECL reagent. Use premixed ECL reagent on membranes for 1–5 min depending on the strength of the antibody. This is to be tested prior to utilizing it for experiments.

3. Aliquot this into 1.5 mL eppendorff tubes and store at $-80$ °C until ready to do infection.

4. A kill curve needs to be done for every cell that has not been tested before in this lab. I usually do a range from 1, 2, 4, 5, 7, 10, 15, 20, 30, 40, 50, 100 μg/mL. Using puromycin, the cells will be dead after 2 days or almost if they are sensitive.

5. When using tumor lines for the first time test which cell number works best to give you a fair number of cells that migrated
within 24 h. Good numbers to start with range from 10,000 to 100,000 cells/well.

6. Make sure to filter components together and keep heated at 45 °C until you are ready to mix with preheated Bacto-Agar. Once you have made the Bacto-Agar nutrient mix keep heated at 45 °C so that it does not harden.

7. First time trials with new cells will require that you plate several different concentrations to optimize density for colony formation (500–5000 cells per well).

References


