Automated electrophysiology screening of gain- and loss-of-function variants in the N-methyl-D-aspartate receptor GRIN gene family

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N-methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors composed of two glycine-binding GluN1 subunits (encoded by GRIN1 gene) in combination with two glutamate-binding GluN2 subunits (encoded by GRIN2A, GRIN2B, GRIN2C or GRIN2D genes).

Rare de novo variants of the GRIN genes have been associated with neurodevelopmental disorders (NDD) and epileptic encephalopathy. Functional and pharmacological analysis of these variants can be used to understand the variant properties and classify the GRIN variants as Gain of Function (GoF) or Loss of Function (LoF) based on the overall net actions of a protein. The classification of these variants from patients may provide diagnostic advantages and, together with precision medicine approaches, could enable the development of a personalised therapy.

Until now, the functional analysis of the GRIN variants has been restricted to the conventional manual patch-clamp and two electrode voltage clamp (TEVC) techniques, which suffer from low throughput. Here, using automated patch-clamp technology, we describe the functional and pharmacological characterization of two GRIN2B variants, E413G (LoF) and S810R (GoF), and compare the results generated by automated patch clamp using HEK cells (at Sygnature Discovery) to data generated using manual patch clamp and TEVC at the Emory lab (*Myers et al*., 2023; *Platzer et al*., 2017).

The aim of this work was to demonstrate the capability of the automated patch-clamp system to identify variants with the LoF and GoF classifications. In future, the establishment of a higher throughput assay will enable a faster evaluation of the GRIN variants, where multiple variants can be assessed simultaneously, while obtaining robust data.

1 Methods

The functional analysis of these variants includes six different assays:

1. Glutamate EC_{50} assay
2. Glycine EC_{50} assay
3. Mg^{2+} IC_{50} assay
4. Deactivation time-course following glutamate removal assay
5. Open probability assay
6. Cell surface protein expression assay

Using these assays, the measurements of the variants were compared to the corresponding GRIN wild type (WT) to determine the effects of the variant, which is expressed as a fold-change. The results were compared to those published in literature (obtained by manual patch clamp, TEVC or β-lactamase assays) by the Emory lab.

Whole cell voltage clamp recording (Assays 1-5):

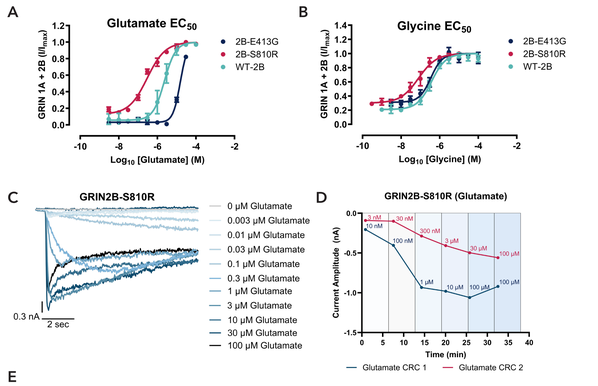
- HEK-GRIN1A expressing stable cell line was transiently transfected with the GRIN2B WT or variant of interest and used in Assay's 1-4.
- GRIN2B WT or variant of interest was co-expressed transiently with GRIN1A-A652C and used in Assay 5.
- Experiments were carried out at room temperature using multi-hole chips on the SyncroPatch 384i automated electrophysiology platform.
- To monitor currents, steady state voltage was held at -60 mV.
- Data analysis was performed using Data Control 384 v3.1 (Nanion) and Prism V10.2.3 (GraphPad).

Cell surface protein expression assay (Assay 6):

- HEK cells were transiently co-transfected with untagged GRIN1A WT and either GRIN2B WT or the variant of interest. GRIN2B constructs were extracellularly (N-terminus) tagged with a HiBiT tag.
- Cells were assessed in the presence and absence of lytic reagent to quantify both cell surface and total tagged protein levels.
- HiBiT expression was assessed using the Nano-Glo® HiBiT Extracellular and Lytic Detection System (Promega) and luminescence measured at room temperature.
- The luminescence was measured on the PHERAstar FSX microplate reader.
- Data analysis was performed using Prism V10.2.3 (GraphPad).
- β-lactamase assay is also under development.

2 Glutamate and Glycine Assay

An increase of glutamate or glycine EC_{50} reflects a decrease in agonist potency (promoting LoF), whereas a decrease of glutamate or glycine EC_{50} reflects an increase in potency (promoting GoF).



E

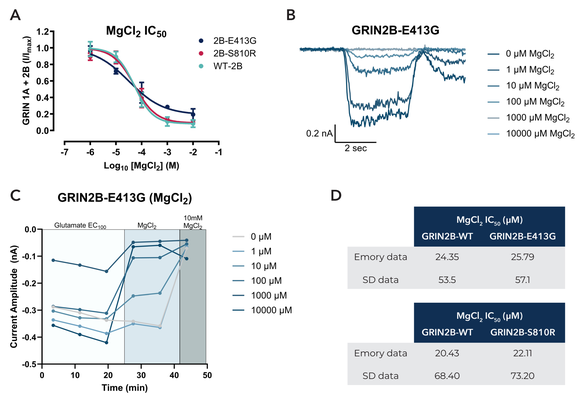
		GRIN2B-WT	GRIN2B-E413G
Glutamate EC_{50} (µM)	Emory data	1.29	73.93
	SD data	1.87	45.3
Glycine EC_{50} (µM)	Emory data	0.35	0.32
	SD data	0.36	0.36

		GRIN2B-WT	GRIN2B-S810R
Glutamate EC_{50} (µM)	Emory data	1.39	0.023
	SD data	1.32	0.265
Glycine EC_{50} (µM)	Emory data	0.32	0.0064
	SD data	0.35	0.12

Figure 1: Glutamate and Glycine assay.
Concentration-response curves for (A) glutamate in the presence of EC_{100} glycine and (B) glycine in the presence of EC_{100} glutamate. The mean ± S.E.M. are shown for n≥2 wells. Example glutamate (C) current traces and (D) time-courses where up to 5 concentrations of glutamate were applied per well and glycine was present at EC_{100}. (E) The tables show the calculated agonist EC_{50} values, with data obtained in-house (Sygnature Discovery) and from Emory.

3 Mg^{2+} Assay

The concentration dependent effect of $MgCl_2$ is assessed and although a change in Mg^{2+} potency is not expected in all variants, an increase in potency can reflect a LoF, whereas a decrease in potency can reflect a GoF.



D

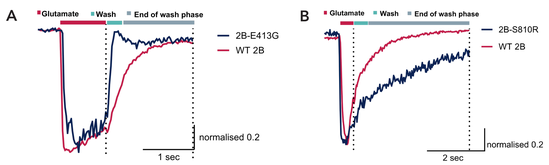
	$MgCl_2$ IC_{50} (µM)	
	GRIN2B-WT	GRIN2B-E413G
Emory data	24.35	25.79
SD data	53.5	57.1

	$MgCl_2$ IC_{50} (µM)	
	GRIN2B-WT	GRIN2B-S810R
Emory data	20.43	22.11
SD data	68.40	73.20

Figure 2: Mg^{2+} assay.
(A) Concentration-response curves for $MgCl_2$ in the presence of $EC_{50\text{-}100}$ glutamate and EC_{100} glycine. The mean ± S.E.M. are shown for n≥2 wells. The $MgCl_2$ curve for the variants were obtained from two different experiments, thus the average WT-2B $MgCl_2$ is shown. Representative (B) current amplitudes from a single well per concentration and (C) response time-courses from the average data. (D) The table shows the $MgCl_2$ IC_{50}, with data obtained in-house (Sygnature Discovery) and from Emory.

4 Deactivation Assays

Two different application protocols are under development that adjust the solution application speed and wash volumes to capture either fast or slow deactivation kinetics. Generally, either a faster or slower deactivation time course in comparison to the WT can be characteristic of LoF and GoF, respectively.



C

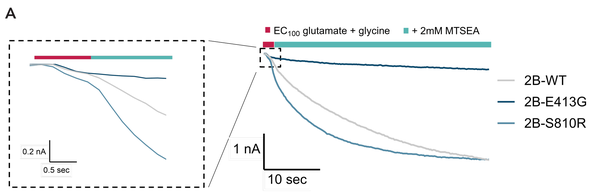
	tau_w (s)	
	GRIN2B-WT	GRIN2B-E413G
	fast component	fast component
Emory data	0.57	0.020
SD data	0.38	0.05

	tau_w (s)	
	GRIN2B-WT	GRIN2B-S810R
	fast component	fast component
Emory data	0.71	8.37
SD data	0.42	1.30

Figure 3: Deactivation assay.
The deactivation time-course following rapid glutamate removal for GRIN2B WT and the (A) GRIN2B-E413G and (B) GRIN2B-S810R variants. Normalised responses show activation by EC_{100} glutamate and EC_{100} glycine using wash-out with physiological solution in presence of 50 µM DL-APV. (C) The fitted tau values are summarised. Deactivation tau_w were derived by fitting the deactivation time course during the wash period with a single or bi-exponential function. Dashed vertical lines show the area analysed.

5 Open Probability

Increases in the calculated open probability of the variants in comparison to WT can be associated with a GoF, whereas decreases can reflect a LoF. Optimisation of the application methods allowed for successful measurement of the open probability of the 2B-WT and 2B-S810R variant, but not the 2B-E413G variant. Further optimization is ongoing to investigate the discrepancy in measurements between labs.



B

	P_{open}	
	GRIN2B-WT	GRIN2B-E413G
Emory data	0.03	0.03
SD data	0.024	0.11

	P_{open}	
	GRIN2B-WT	GRIN2B-S810R
Emory data	0.043	0.082
SD data	0.024	0.032

Figure 4: Open probability assay.
(A) Representative current traces in the presence of EC_{100} glutamate and glycine followed by the covalent modifying reagent, MTSEA. (B) Summary of the open probability calculated in-house (Sygnature Discovery) and from Emory (mean for n≥2 wells). The channel open probability was estimated using the equation $[(\gamma_{MTSEA}/\gamma_{Control}) \times (1/Potentiation)]$, where γ_{MTSEA} and $\gamma_{Control}$ were the single channel chord conductance values estimated from GluN1/GluN2A receptors and *Potentiation* is the ratio of current in the presence of 2 mM MTSEA to current in the absence of MTSEA; γ_{MTSEA} /$\gamma_{Control}$ was 0.67 (*Yuan et al.*, 2005).

6 Cell Surface Expression

The surface expression was assessed by the HiBiT assays, with low and high surface expression associated with LoF and GoF, respectively.

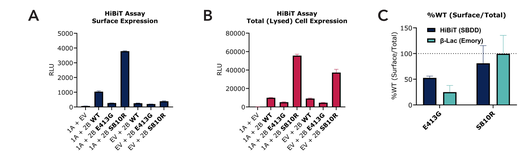


Figure 5: Cell surface assay.
(A) Surface HiBiT expression (Luminescence; RLU) (n=3 wells ± SD) in 1 independent experiment. Empty Vector (EV) controls were assessed in parallel. (B) Total HiBiT expression (n=3 wells ± SD) in 1 independent experiment in lysed cells (C) Surface expression is reported relative to total expression of GRIN2B variants (in lysed cells) and shown as mean percentage of GRIN2B WT (surface/total) for each variant (N=3 ± SD). β-lac data collected at Emory is also presented (N=7-9).

7 Classification Thresholds

A

	Support for LoF (high confidence)	Support for LoF (moderate confidence)	Support for GoF (high confidence)	Support for GoF (moderate confidence)
Glu/Gly potency ratio	↓ to 0.40 or more	↓ to 0.67-0.40	↑ to 2.5 or more	↑ to 1.5-2.5 fold
$MgCl_2$ IC_{50} ratio	↓ to 0.40 or more	↓ to 0.67-0.40	↑ to 2.5 or more	↑ to 1.5-2.5 fold
T_w deactivation ratio	↓ to 0.50 or more	↓ to 0.67-0.50	↑ to 2 or more	↑ to 1.5-2 fold
Open probability ratio	↓ to 0.50 or more	↓ to 0.67-0.50	↑ to 2 or more	↑ to 1.5-2 fold
Surface expression ratio	↓ to 0.50 or more	↓ to 0.67-0.50	↑ to 2 or more	↑ to 1.5-2 fold

B

	GRIN2B-E413G		GRIN2B-S810R	
	Emory Data	SD data	Emory data	SD data
Glutamate EC_{50} ratio	H (0.02)	H (0.04)	H (60.43)	H (4.98)
Glycine EC_{50} ratio	1.09	0.95	H (50.00)	H (2.94)
Mg^{2+} IC_{50} ratio	1.06	1.07	1.08	1.07
P_{open} (MTSEA) ratio	1.00	H (4.58)	M (1.91)	1.33
tau_w ratio	H (0.04)	H (0.13)	H (11.79)	H (3.1)
Surface/Total (B-lac or HiBiT) ratio	H (0.25)	M (0.53)	0.99	0.81
Count (High, Mod)	3,0	conflict	3,1	3,0
Outcome	LoF	uncertain	GoF	GoF
Charge transfer	0.008	0.06	18.9	3.4
Outcome	LoF	LoF	GoF	GoF
Final Call	Likely LoF	Possible LoF^{Yen}	Likely GoF	Likely GoF

Figure 6: Thresholds determination of GoF and LoF.
(A) The threshold for fold changes in the ratio between WT and variant to support characterisation of GoF or LoF with high or moderate confidence as defined in literature (Myers et al., 2023). (B) Assessment of variant-mediated fold changes supporting GoF and LoF are summarised for Sygnature Discovery data. Glutamate and glycine potency ratios are WT/variant. Mg^{2+} IC_{50}, open probability, weighted tau (τw), and surface expression fold effects are variant/WT. A high (H) or moderate (M) degree of confidence for the change is indicated; blue is LoF and green is GoF. Charge transfer is calculated as described by *Myers et al.*, 2023.

8 Summary

- We have demonstrated the ability of our SyncroPatch platform and cell surface assays to complete the 6 experimental assays that can be used to assign GoF and LoF to NMDAR function in heterologous assays (*Myers et al.*, 2023).
- Consistently with previous data, we characterised GRIN2B-E413G as LoF and GRIN2B-S810R as GoF.
- Optimisation is on-going to increase the confidence level in automated patch clamp data and identify limits of this platform in the characterisation of these measurements.
- Overall, the automated patch clamp platform provides the advantage to assess multiple variants as well as the WT simultaneously on the chip, which has the potential to expedite the assay throughput.
- In addition, complementary assays (such as TEVC) may be necessary for some variants with large changes in some parameters.

References

1. Myers et al., Human Molecular Genetics. 2023, PMID: 37369021
2. Platzer et al., Journal of Medical Genetics, 2017, PMID: 28377535
3. Yuan et al., Journal of Biological Chemistry PMID. 2005, 15970596