Fully Automated Robotic Screening for the Identification of Positive Allosteric Modulators of N-Methyl-D-Aspartate Receptor Using Calcium Flux Assays

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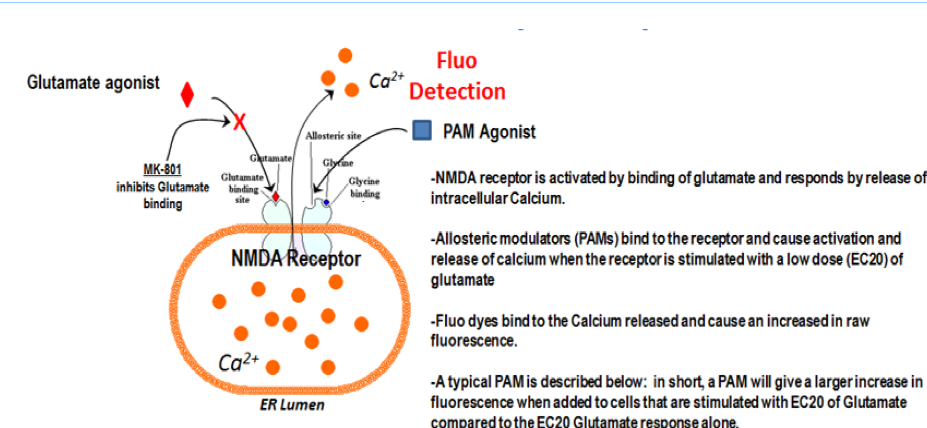
Abstract

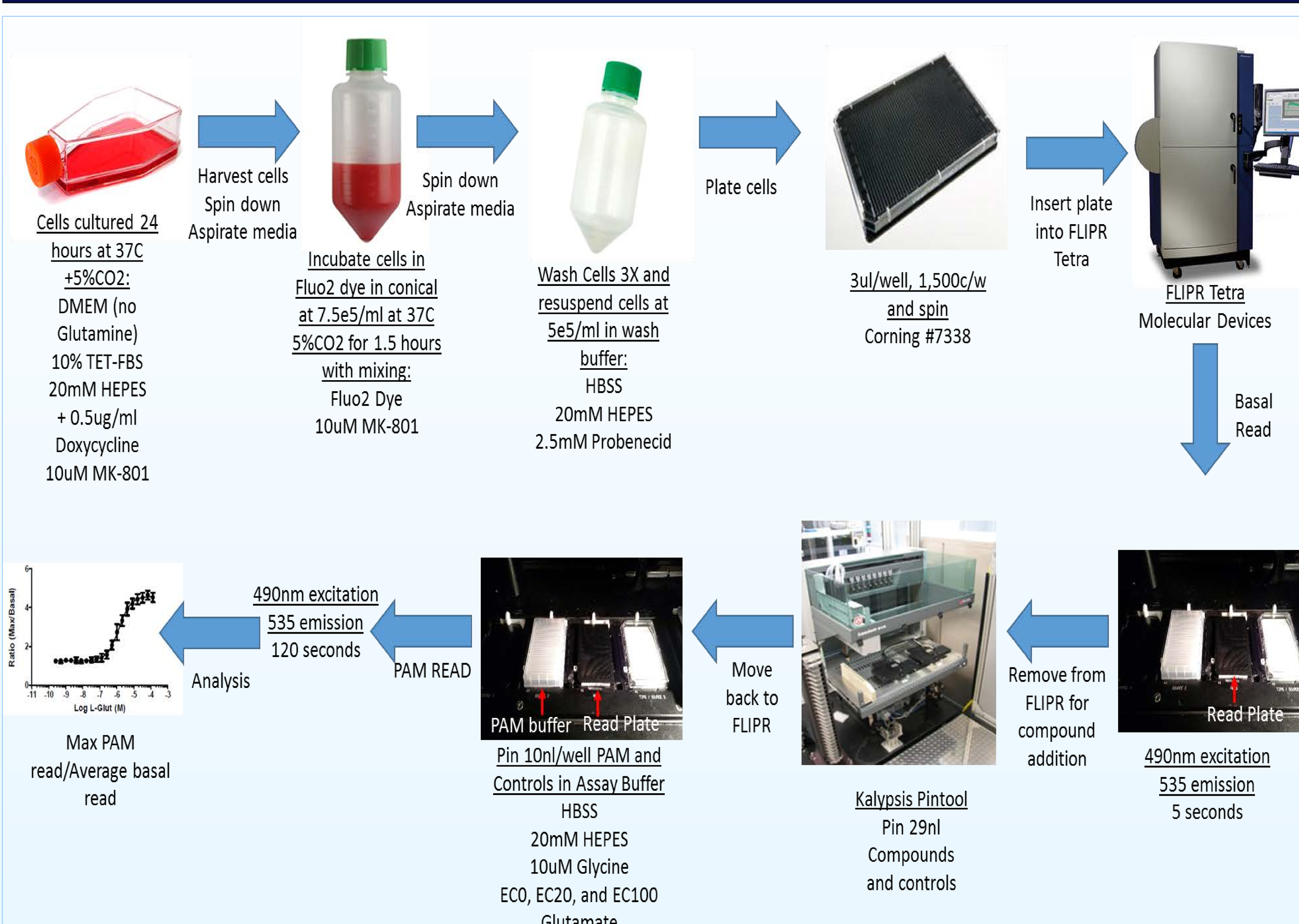
N-Methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors that play an important role in synaptic plasticity and learning and memory function which, when impaired, can contribute to the cognitive deficits seen in Alzheimer's disease and schizophrenia. Corrective measure using Positive allosteric modulators (PAMs) of NMDAR can therefore be useful therapeutic agents to restore function. Our fully automated novel high throughput screening effort utilized calcium flux readout to determine active PAMs of NR1/NR2A (NMDAR receptor subunits) expressed in HEK cells stimulated with glutamate. Greater than 810,000 compounds were screened in 1536 well format and we identified 864 NMDAR-PAMS with EC50 activity <10uM. Follow-up testing on several series of compounds in calcium flux assays demonstrated EC50 values between 0.49 and 10uM. Ultimately a series of 6 unique chemotypes of interest were identified that are now being pursued in MOA studies at Lilly. Assay miniaturization, uHTS, secondary and tertiary assay outcomes will be described demonstrating the successful collaboration between academia and industry.

1. NMDAR PAM Assay Principle And HTS 1536 well protocol



Order	Step	Condition	Comments
1	Dox treat cells	T175s	Cells are grown in growth media in 0.5ug/ml Dox +10uM MK-801
2	Incubation	Overnight	37C+5%CO2
3	Fluo2 addition	conical	Resuspend cells at 500,000/ml in Fluo2+10uM MK-801
4	Incubation	1.5 hours	37C + 5% CO2
5	Wash Cells	2 times	Wash Buffer (HBSS, 20mM Hepes, 2.5mM Probenecid)
6	Cell dispense	3uL/well	500,000 cells/ml
7	FLIPR TETRA Basal Read	FLIPR	5 second read (Raw 1 read)
8	Pintool	30nL/well	Compound addition outside the FLIPR
9	FLIPR TETRA PAM Pintool	15nL/well	Pam Stimulus and controls addition in Assay buffer (HBSS, 20mM Hepes, 10uM glycine)
10	FLIPR TETRA Read	120 seconds	PAM cycle of the single read (Raw 2 read)
11	Data collection	490ex/530em	Ratio of Raw2 and Raw1

2. NMDAR PAM HTS Assay Protocol

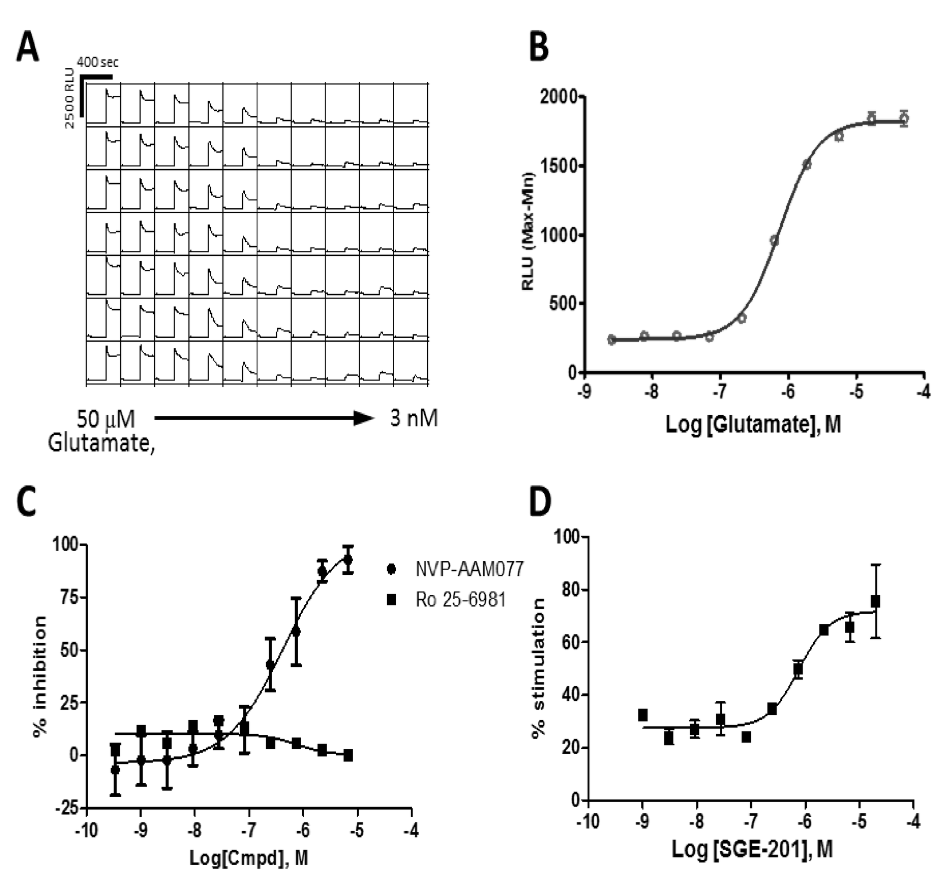


3. NMDAR PAM HTS Campaign

Step	Screen type	Target	Number of compounds tested (9.6µM)	Selection criteria	Number of selected compounds	Assay statistics Z'	Assay statistics S/B
1	Primary screen PAM	NMDA	810,512	35%[a]	5,517 (0.68%)	0.60± 0.06	2.84 ±0.43
2a	Confirmation 3X PAM	NMDA	5,434	35%[b]	1,475 (27%)	0.63± 0.06	2.96 ±0.18
2b	Counterscreen 3X PAM	HEK	5,434	38%[c]	844 (16%)	0.65 ±0.04	2.88 ±0.06
3a	Titration Assay PAM	NMDA	864[d]	EC_{50}< 10µM	362 (42%)	0.61± 0.05	2.72 ±0.11
3b	Counterscreen Titration PAM	HEK	864[d]	EC_{50}< 10µM	33 (3.8%)	0.70± 0.04	2.94 ±0.06

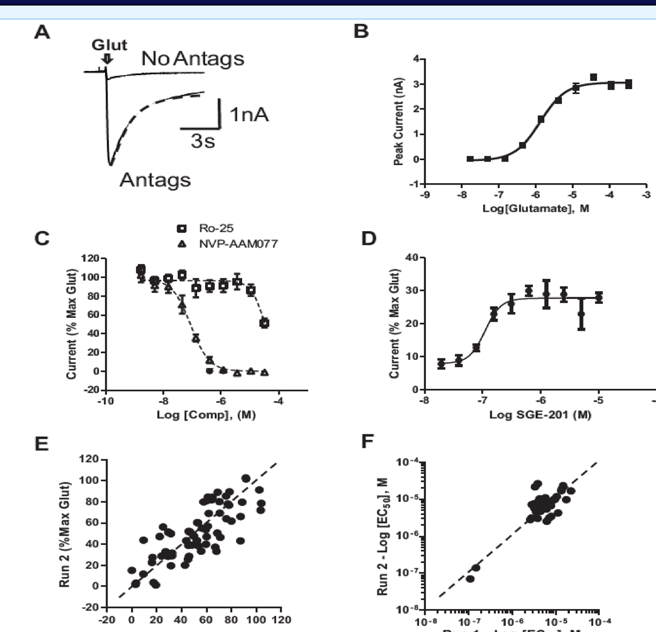
A list of the number of compounds tested at each stage of the HTS campaign. Also listed are the assay statistics for each stage along with the hit cutoffs and number of selected compounds. The screening concentrations for each step are also provided. a, interval-based cutoff; b, primary hit cutoff; c, DMSO, sample field cutoff; d, 96 µM start concentration for titration assays.

4. NMDAR Pharmacological Validation of the FLIPR 384 Assay



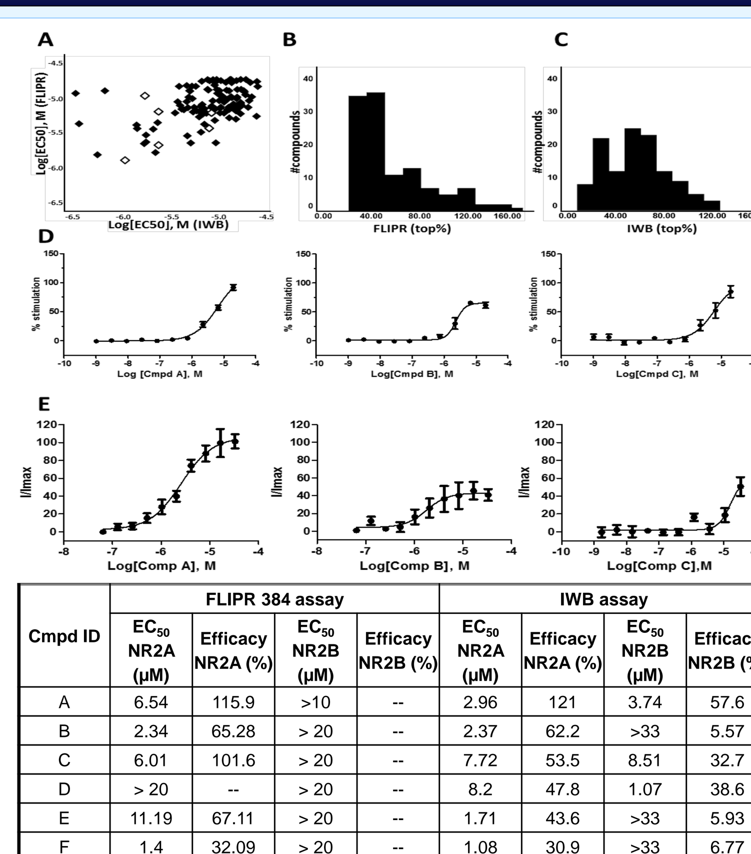
A) FLIPR traces corresponding to a glutamate concentration response curve in 384 format (maximum starting concentration was 50 µM). (B) Analysis of 16 glutamate CRCs generated in the same experiment; the average and SEM for each concentration point are represented, EC50 (glutamate) = 0.736 µM (n = 16). (C) Inhibition caused by the control antagonists NVP-AAM077 and Ro 25-6981 on the NR1/NR2A cell line is shown. (D) Example of a concentration–response curve obtained for SGE-201. Results (n = 2) were normalized and represented using GraphPad Prism software. One hundred percent stimulation was 20 µM glutamate (EC100), and 0% stimulation was EC20 (0.2 µM).

5. NMDAR Currents Recorded in PPC Mode On Ion Works Barracuda



(A) Representative NR1/NR2A currents activated by 300 µM glutamate. A robust current was recorded when 1 mM ketamine and 0.1 mM AP5 were used during the isolation procedure (no Antags), and only small current was observed without antagonists during the cell preparation (Antags). Current activated rapidly and decayed with t=2.19s (fit trace—dash line) in the presence of glutamate. (B) Concentration–response curve for activation of NR1/NR2A by glutamate. (C) Block of NR1/NR2A current by subtype-selective Antagonists (mean±SEM, n=8). (D) Potentiation of NR1/NR2A peak currents by SGE-201. Peak currents evoked by 0.2 µM glutamate (EC10) were potentiated by 2 min pre-incubation with SGE-201 dissolved in 0.1% BSA containing external solution. (E) Reproducibility of maximal efficacy values for a set of 64 compounds. Maximum efficacy was calculated as a percent of peak current obtained with saturating concentration of glutamate (300 µM). The correlation coefficient ($R2$) was 0.79; the dash line is at 45°. (F) Reproducibility of EC50 values for the same set of 64 compounds. Only the datapoints corresponding to compounds showing EC50 values of <33 µM are presented (n=38). $R2$ was 0.83; the dash line is at 45°. In all experiments, the external solution contained 30 µM glycine and the holding potential was -70mv.

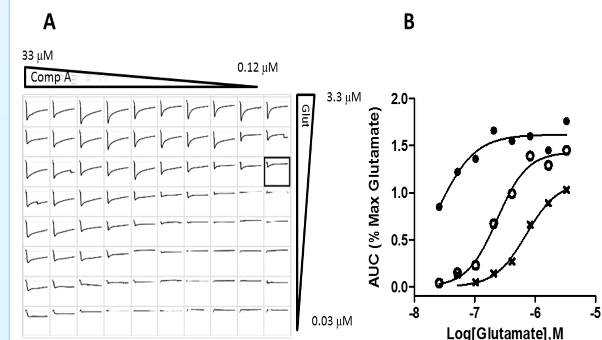
6. NMDAR PAM Confirmed Actives in FLIPR 384 and IWB assays



Cmpd ID	FLIPR 384 assay EC_{50} NR2A (µM)	FLIPR 384 assay Efficacy NR2A (%)	FLIPR 384 assay EC_{50} NR2B (µM)	FLIPR 384 assay Efficacy NR2B (%)	IWB assay EC_{50} NR2A (µM)	IWB assay Efficacy NR2A (%)	IWB assay EC_{50} NR2B (µM)	IWB assay Efficacy NR2B (%)
A	6.54	115.9	>10	--	2.96	121	3.74	57.6
B	2.34	65.28	> 20	--	2.37	62.2	>33	5.57
C	6.01	101.6	> 20	--	7.72	53.5	8.51	32.7
D	> 20	--	> 20	--	8.2	47.8	1.07	38.6
E	11.19	67.11	> 20	--	1.71	43.6	>33	5.93
F	1.4	32.09	> 20	--	1.08	30.9	>33	6.77

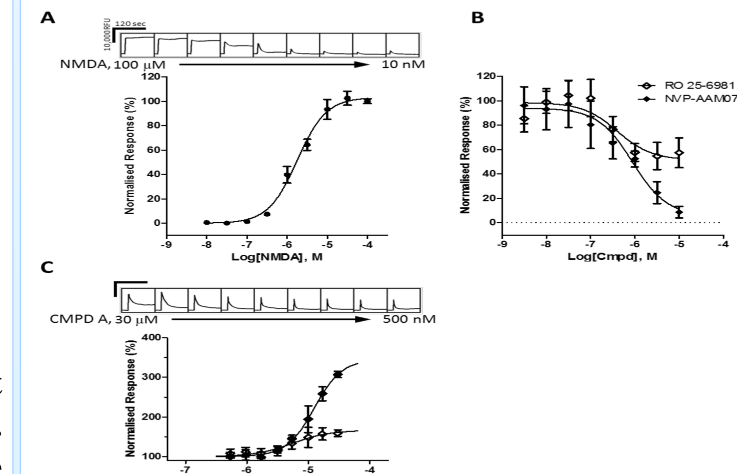
(A) Summary data of potency (EC50 values) of all tested hits (from HTS titration and selectivity assays) in the FLIPR 384 and IWB assays on the NR1/NR2A cell line. 119 compounds of EC50 values of <33 µM (IWB assay) and <20 µM (FLIPR assay). The open symbols represent data for the six selected seeds shown in F. (B,C) Frequency distribution histograms for the efficacy data (maximum top stimulation percent) for all compounds presented in A. Concentration–response curves for three of the selected seeds are presented in (D) for the FLIPR assay and in (E) for the IWB assay (data obtained on the NR1/NR2A receptor cell lines). Results were normalized to the top glutamate concentration measured in each of the two assays. (F) Pharmacological properties of a selected set of NMDAR-PAMs. Summary data on activity of six selected PAMs in the calcium flux and patch-clamp assays. Data obtained on the NR1/NR2A and NR1/NR2B cell lines are presented as average values (n = 2–6).

7. NMDAR PAM Effect of Compound A on Glutamate Curve



(A) Representative NR1/NR2A current trace recorded in matrix format. Glutamate was serially diluted in the vertical direction (8-point CRC; 1:2 dilutions from 3.3 µM top concentration). Compound A was diluted horizontally (9-point CRC; 1:2 dilutions from 33 µM top concentration). (B) Concentration response curve for activation of NR1/NR2A by glutamate (n = 4) in the control buffer (x) and in the presence of 2.1 µM (O) and 33 µM (•) compound A. The glutamate EC50 values were 0.7, 0.23, and 0.024 µM, respectively.

8. NMDAR PAM Potentiation of Native NMDAR by Compound A



(A) Concentration-dependent response to NMDA in rat cortical neurons; EC50 = 1.78 µM (± 0.11, n = 6). (B) NMDA responses induced by a fixed concentration (10 µM) are blocked by selective NMDA antagonists in a concentration dependent manner. (C) Compound A induced a robust potentiation of the response induced by a subthreshold NMDA concentration (1 µM). Data are normalized as percent increase over the response caused by test concentration (1 µM). The EC50 values were 5.52 (±3.29, n = 3) for peak amplitude analysis (open symbols) and 12.51 (±4.79, n = 3) for AUC analysis (filled symbols). The corresponding values for maximum potentiation are 166.4% (±13.7%) for peak amplitude and 345.1% (±45.84%) for AUC, respectively.

Conclusion

This effort culminated in the successful collaboration between Scripps and Eli Lilly. Ultimately, we demonstrate miniaturization and implementation of the NMDAR ion channel assay into a fully automated, 1536 well 810K compound HTS. We met the aims of this effort by isolating compounds that potentiate the glutamate response in the ion channel receptor NMDAR. Some of those compounds will be further analyzed for their effectiveness in treating diseases like schizophrenia and Alzheimer's.

Acknowledgement and Reference

This project was a joint collaboration with Eli Lilly and the Scripps Research Institute.
The data included is taken from the following publication:

Jambrina and Smith et al. An Integrated Approach for Screening and Identification of Positive Allosteric Modulators of N-Methyl-D-Aspartate Receptors. J Biomol Screen. 2016 Jun;21(5):468-79