A GABA Ligand Gated Ion Channel Screen - Results and Best Practices

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Abstract

The GABA$_A$ receptors belong to a family of ligand-gated ion channels mediating fast synaptic transmission. They are drawing great attention in the pharmaceutical industry due to their potential role in the development of new therapeutics affecting anxiety, sleep disorders, and muscle relaxation. However, ligand-gated ion channel screening has been hampered by the lack of suitable high throughput electrophysiology platforms. The search for subtype-selective drugs for GABA$_A$ channels has been hampered by the lack of suitable high throughput electrophysiology platforms with the ability to interrogate ligand
gated ion channels.

Here we present the use of a novel electrophysiology screening platform integrating a microfluidics network for the study of GABA$_A$ receptor pharmacology. This platform features fast (100ms) solution exchange coupled with simultaneous data recording. A novel assay could monitor GABA response in real time, and obtain a 3 point EC50 dose curve within 1 minute.

The GABA$_A$ α1/β3/γ2 expressing HEK cells from Millipore were used for this study. The channel was targeted with agonists, including GABA and muscimol, inhibitors (picrotoxin, bicuculline, and gabazine), and positive modulators, including diazepam, zolpidem and chlordiazepoxide. The positive modulators produced concentration dependent augmentation of the GABA EC20 response. The pharmacology data determined using this method was consistent with the literature values obtained using other platforms. Statistical data for inter and intra-plate reproducibility, current stability, and Z'-values, is used to validate this approach.

Materials and Methods

Cells expressing GABA$_A$ (α1/β3/γ2-HEK, Millipore PrecisION Cat# CYL3053) were cultured in 225 cm^2 filter-top flasks containing DMEM/F12 glutamax, G-418, hygromycin B, and puromycin at 37°C and 5% CO_2. The cells were kept below 90% confluency. For cell isolation, flasks were first washed with 2 ml of Ca- and Mg-free PBS, followed by 5 ml of Detachin™ solution, after which cells were given two to five minutes of further Detachin™ treatment. Cell suspension in extracellular solution (5x10^6 cell/ml) was dispensed on an IonFlux plate.

Extracellular solution (mM): 137 NaCl, 4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, 9 glucose, pH 7.4 with NaOH.
Intracellular solution (mM): 130 K Aspartate, 5 $MgCl_2$, 5 EGTA, 4 Tris-ATP, 10 HEPES, pH 7.2 with KOH.

Compounds, GABA agonists (GABA, muscimol and isoguvacine hydrochloride), GABA inhibitors (bicuculline, and picrotoxin) and GABA positive modulators (diazepam, triazolam, and zolpidem) were all purchased from Sigma-Aldrich. GABA, isoguvacine were dissolved in deionized water to make a 10 mM stock solutions. The rest of the compounds were dissolved in DMSO to make a 1 mM or 10 mM stock solutions. Compounds were diluted serially into extracellular buffer. The highest concentration of DMSO in the assay was <0.1%, and DMSO vehicle controls were tested with no significant response.

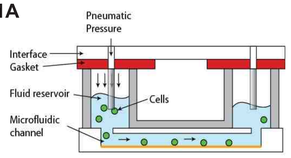
Whole-cell voltage clamp was established by applying suction (approximately 5 inHg) on the cells, while holding the membrane potential at -80 mV. Cells were clamped at -80 mV throughput the experiment and recorded for 10 second time intervals every 15 seconds. To evoke inward Cl- currents, GABA was applied for 3 seconds during the 10 second sweep. The resulting currents were sampled at 1 KHz. IonFlux platform features fast (<100ms) solution exchange coupled with simultaneous data recording, so that it was possible to monitor GABA response in real time.

References

1. L. Urban et. al (In press) Assay and Drug Development Technologies
2. E. Hollands et. al (2009) J. Biomol.Screen
3. B. Ebert, et. al(1997), Molecular Pharmacology
4. C. Bell-Horner, et. al(2000), Molecular Brain Research
5. E. Sannar, et. al(2002), J. of Pharmacology
6. S. Khom, et. al(2006), Molecular Pharmacology
7. R. Granja, et. al(1997), J. of Molecular Neuroscience

IonFlux System Description

1A



1B

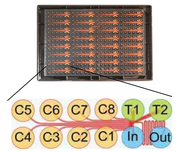
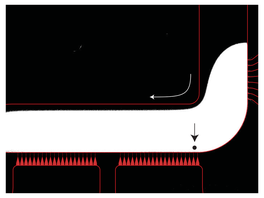


Figure 1. Pneumatic pressure controls rapid and precise microfluid delivery. A) A gasketed interface seals to an entire 384 well plate after it loads in the instrument. The interface includes pneumatic connections to each well, with positive and negative pressure regulation from computer-controlled electro-pneumatic regulators. Ag/AgCl electrodes are integrated in the interface. The IonFlux system includes up to 64 multiplexed amplifiers. B) Bottom-view of a 384-well plate filled with colored dye to visualize the microfluidic network patterns. Each 384-well plate includes 32 experimental zones, each containing 8 compound wells and 2 multi-cell trapping regions. Throughputs in excess of 1,000 data points per hour can be achieved.

2A



2B

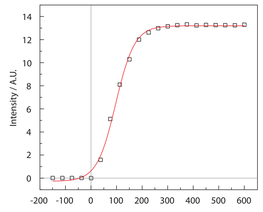
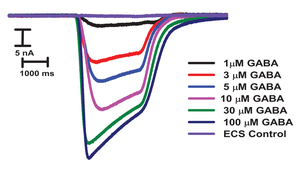


Figure 2. Rapid Compound Application[1]. A) An outline of the microfluidic walls is shown in black and overlaid with a fluorescence image of compound being applied in the main channel (white arrow for flow direction). Compound inlets C1-C8 are located upstream (right side of figure) and cell trapping arrays (T1 and T2) are on the bottom of the figure. When a compound solution is applied, it contacts the wall that traps the cell ensemble and extends across 80% of the cross-section of the main channel, while EC solution from the inlet well flows alongside and occupies the remaining 20% of the channel. B) Fluorescent changes as a function of time were measured from a 5 µm diameter spot (dark arrow in Fig. 2A) at the trapping site. The increase in fluorescence (ON time) was fit by a Boltzmann sigmoid and yielded a time constant of 36.9 +/- 0.9 ms (standard deviation across 3 applications)..

GABA Channel Recording and GABA EC50

3A



3B

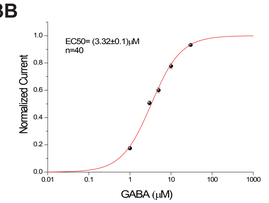
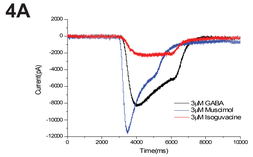


Figure 3. Representative sweeps (3A) showing the response of the same cell ensemble exposed to increasing GABA concentrations. Figure 3B shows average response to GABA (n = 30 EC50 experiments), standard error, and a fit function (EC50 = 3.4 µM, R2 =0.995) as compared to a literature value of 3 to 8 µM[2,3].

GABA Agonist Pharmacology

4A



4B

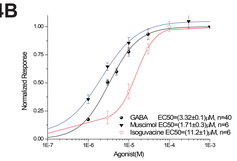
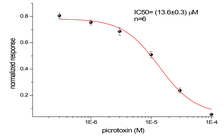


Figure 4. Response of a cell ensemble exposed to 3 µM muscimol, 3 µM GABA, and 3 µM isoguvacine, showing the different potency of the three compounds (4A), and dose response (4B). The observed potency was muscimol>GABA>isoguvacine. EC50 values were consistent with literatures[2,3]

GABA Inhibitor Pharmacology

5A



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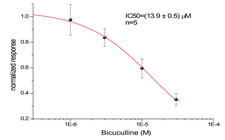


Figure 5. Response of a cell ensemble exposed to 10 µM GABA co-applied with increasing concentrations of picrotoxin(8A top) and bicuculline (8A bottom). (Bicuculline was preincubated for 3 minutes before co-applied with GABA). A hill fit (8B) provides an IC50 value of picrotoxin at 14 µM, as compared to a literature value of 2.5 to 7 µM[2,4], and bicuculline at 2 µM[2,4].

Consistency in GABA Current Intensity

6A

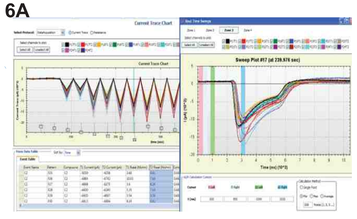


Figure 6A. Screen shot from IonFlux showing inward Cl$^-$ currents during exposure of ensembles of 20 HEK cells expressing GABA$_A$(α1/β3/γ2) to 10 µM GABA. Cursors are set in the sweep plot window (right) determined a value for the GABA-evoked current plotted as point values against time (left panel).

6B

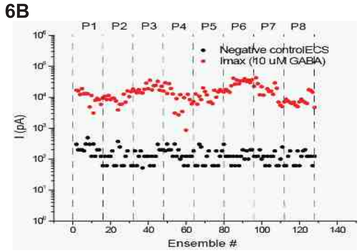


Figure 6B. Plate-to plate consistency in current amplitude: IonFlux data showing the magnitude of the stimulus response (10 µM GABA application) for 128 cell ensembles across 8 plates, and comparison to a negative control (ECS buffer application).

6C

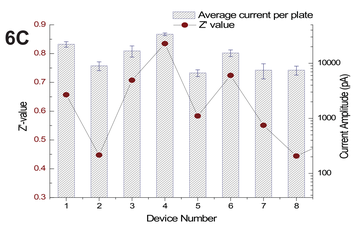


Figure 6C. Average current and Z'-values per plate: Z'-values were obtained by comparing the distribution of 10 µM GABA applications with control application of ECS buffer (left axis, scatter plot). The overall Z'-value for the run was 0.58 ± 0.15. Also plotted are average current per ensemble and standard deviation of the same (bar chart, right axis).

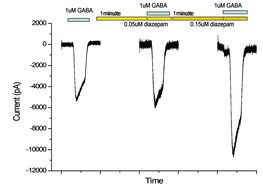
Conclusions

- We presented pharmacological data for agonists, inhibitors and three positive allosteric modulators of the GABA(α1/β3/γ2) receptor ion channel.
- The GABA assay showed a high level of consistency, with an average Z'=0.6 for GABA applications across all recordings, and pharmacology data that is in good agreement with the literature.
- Parallel microfluidic perfusion in the IonFlux system allows for fast exchange of solutions simultaneously across the whole plate, enabling unique ligand-gated channel assay protocols: fast EC50 shift assays and open channel modulation. Synchronous perfusion also improves assay throughput and data quality.

GABA Positive Modulator Pharmacology

Protocol 1: Pre-incubation

7A



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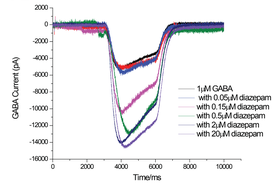

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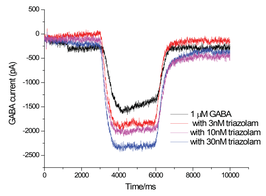

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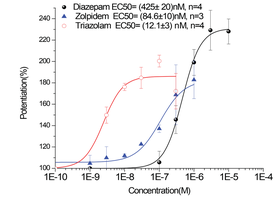
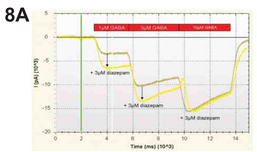


Figure 7. Pharmacology of the benzodiazepine GABA modulators. A) Protocol setup: pre-incubation with the modulator for 1 minute, followed by a co-application with 1 µM GABA(EC20). B) and C) Response of a cell ensemble exposed to 1 µM GABA with increasing diazepam (7A) and triazolam (7B) concentrations. D) EC50 of diazepam, zolpidem, and triazolam. Experimental values were 425 nM, 84.6 nM, 12.1 nM, in agreement with literature values of 160 nM, 70-150 nM, and 22-45 nM respectively [5,6,7].

Modulator Pharmacology: Unique Protocol Modalities

Protocol 2: A Fast EC50 Shift Assay

8A



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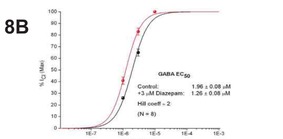
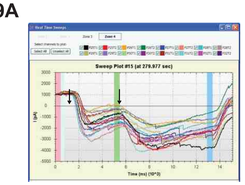


Figure 8. Cumulative dose response curves (1 µM, 3 µM and 10 µM GABA) were obtained in the presence of the benzodiazepine, diazepam (8A), reproducing the known shift in GABA potency attributable to positive allosteric modulators (8B)[2].

Protocol 3: An Open Channel Modulation Assay

9A



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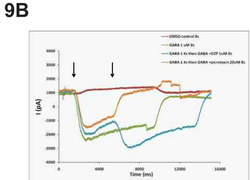

Figure 9: Open channel assay for studying the potency of GABA channel modulators. A) An example screenshot showing 1 µM diazepam co-applied with 1 µM GABA instantly potentiated GABA response. Cells were perfused in 1 µM GABA for 4 sec then switched to GABA-diazepam mix solution for 8 sec. Different traces in the graph are recordings from 16 different cell ensembles. B) Overlay of the responses of a cell ensemble to 1 µM diazepam (blue trace) and 20 µM picrotoxin (orange trace), as comparing to DMSO and GABA only controls.