

Recording of GABA_A receptor-mediated Cl⁻ currents using the IonFlux™ platform

Introduction

GABA (γ -amino butyric acid) is the primary inhibitory neurotransmitter in the central nervous system. GABA_A receptors belong to a family of ligand gated ion channels mediating fast synaptic transmission. They are of major importance as pharmacological targets for anxiolytics (e.g. benzodiazepines), schizophrenia, and sleep aids. At least nineteen different individual GABA_A receptor subunits assemble into pentameric structures in different combinations to form the native receptor (α 1-6, β 1-3, γ 1-3, δ , ρ 1-3, plus minor subunits)¹. When activated, these receptor/channels conduct a Cl⁻ current that desensitizes at higher GABA concentrations, with a characteristic rate for different subunit combinations. Receptors containing α 1-5 subunits, any β subunits, and the γ 2 subunit are the most prevalent in the brain. These receptors are sensitive to benzodiazepine modulation. 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 channels.

In this report, we present assay and pharmacology data for GABA modulators of a specific GABA_A receptor (α 1/ β 3/ γ 2) expressed in HEK 293 cells using the IonFlux electrophysiology platform. The IonFlux platform is a desktop instrument (Fig. 1) that utilizes microfluidic compound delivery on timescales below 100ms, facilitating the recording of fast activating ligand gated ion channels. A large number of cells (20 per ensemble) under voltage clamp can be exposed to a compound within a short time scale in parallel across the plate. Continuous recording, coupled with fast solution exchange, enables high throughput screening against ligand-gated ion channel targets.



Figure 1: The IonFlux system utilizes a “plate reader” format to simplify workflow and increase throughput. Systems are available with 16 and 64 amplifiers. Throughputs of 10,000 data points per day can be achieved.

Materials and Methods

Cells

Cells expressing hGABA_A (Millipore PreciSION™ hGABA_A α 1/ β 3/ γ 2-HEK Recombinant Cell Line, Cat# CYL3053) were cultured in 175 cm² filter-top flasks containing DMEM/ F12 glutamax, G-418, hygromycin B, and puromycin at 37°C and 5% CO₂. 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 treated with Detachin™ solution for two to five minutes. After release, the cell suspension was spun for 90 seconds (1000 rpm) prior to being resuspended in extracellular solution (5x10⁶ cell/ml).

The extracellular solution contained (mM): 137 NaCl, 4 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 HEPES, 9 glucose, pH 7.4 with NaOH. The intracellular solution for the whole cell voltage clamp contained (mM): 130 K Aspartate, 5 MgCl₂, 5 EGTA, 4 Tris-ATP, 10 HEPES, pH 7.2 with KOH. Cell suspension in extracellular solution was dispensed into an IonFlux plate.

Experimental Procedures

The IonFlux plate layout consists of units of twelve wells; two wells contain intracellular solution (cytosolic compartment), one contains ECS plus cells, eight contain ECS plus compounds of interest, and one well is for waste collection. Cells are captured from suspension by applying suction to microscopic channels in ensemble recording arrays. Once the array is fully occupied, the applied suction breaks the cell membranes of captured cells, establishing whole cell voltage clamp. For compound applications, pressure is applied to the appropriate compound wells, introducing the compound into the extracellular solution rapidly flowing over the cells.

For recording GABA_A currents, cell arrays were voltage clamped at a holding potential of -80 mV.

Compounds

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

Software

Data analysis and graphical presentation were performed using a combination of IonFlux software, Microsoft excel, and Origin Lab. All data are shown with arithmetic mean $\pm$ standard error of mean (SEM). For each ensemble of cells that had seal resistance per cell of $R_{\text{seal}} < 60\text{M}\Omega$, the data was excluded from subsequent analysis.

Results

A half plate experiment with rapidly activating inward currents evoked by 10 μM GABA are shown in Figure 2, as well as a screen capture of the IonFlux software. We recorded the response to increasing GABA concentrations applied for a 3s interval as shown in Figure 3. Typical GABA-evoked inward Cl⁻ currents increased to a maximum in $200 \pm 50\text{ms}$. The current on-time is influenced by both the ion channel kinetics and solution exchanges speed and is slower with respect to

buffer exchange times. The washout time was approximately 500ms.



Figure 2: Screen shot from IonFlux showing inward Cl⁻ current during exposure of HEK cells expressing GABA_A ($\alpha 1/\beta 3/\gamma 2$) 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).

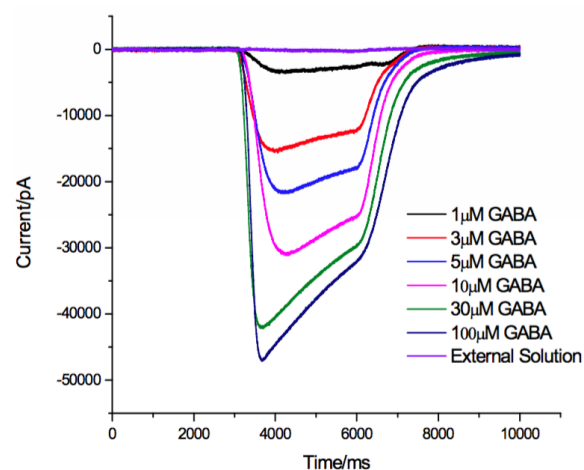


Figure 3: Inward Cl⁻ current from one ensemble of cells exposed to GABA with increasing concentrations (1 μM to 100 μM).

To evaluate the suitability of screening against GABA_A activity using IonFlux, we measured the magnitude of the stimulus response (10 μM GABA application) for 128 cell ensembles across 8 plates, with the comparison to a negative control (the application of 1% of DMSO in ECS buffer). Statistical data for inter and intra-plate reproducibility, current stability, and Z'-value were calculated to validate the protocol. We obtained an average Z' value of 0.58 (Figure 4). The consistency and precision of GABA assays on IonFlux proves that it is possible to conduct robust pharmacology studies of GABA_A receptors. Data on the pharmacology of a

number of test compounds was obtained as described in the following sections.

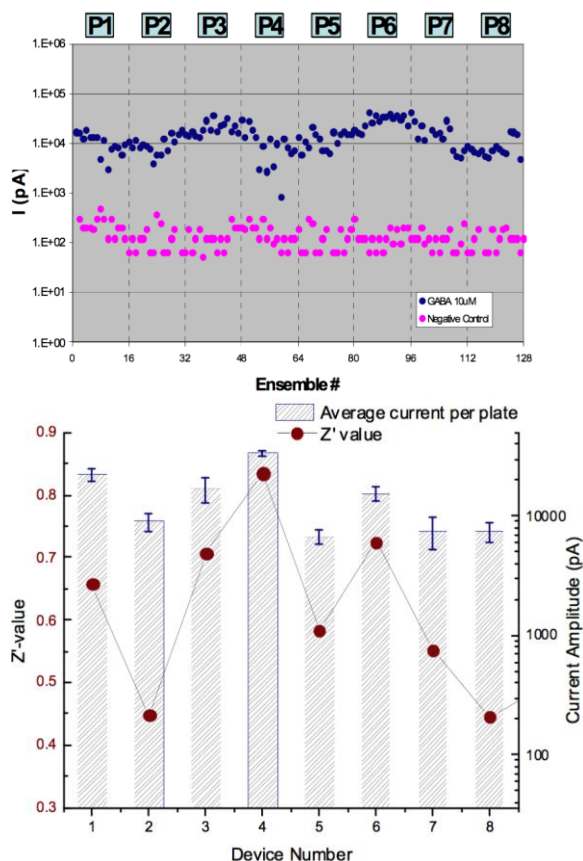


Figure 4: Plate-to-plate consistency in current intensity: IonFlux data showing the magnitude of the stimulus response (10 μ M GABA application) for 128 cell ensembles across 8 plates, and comparison to a negative control (1% DMSO application). Z' values (DMSO vehicle vs. 10 μ M GABA) of 8 plates were 0.66, 0.45, 0.71, 0.83, 0.58, 0.72, 0.55, and 0.44. Average Z' = 0.58 ± 0.15 .

GABA_A Agonist Pharmacology

GABA, muscimol and isoguvacine evoke a concentration dependent inward Cl^- current. For each experiment, a cell ensemble was exposed to increasing agonist concentrations. Sweeps showing the response to increasing concentrations of muscimol and isoguvacine are shown in Fig. 5. Note the difference in current on times based on the gating kinetics for the different agonist compounds.

The comparison of the magnitude and time course of the response to three agonists at a concentration of 3 μ M is shown in Fig. 6. The observed potency rank was muscimol > GABA > isoguvacine, which agreed with the literature^{2,3}. Concentration response curves were analyzed using a Hill fit in Origin, yielding EC₅₀ values

of 1.7 μ M (muscimol), 3.3 μ M (GABA), and 11.2 μ M (isoguvacine) (Figure 6).

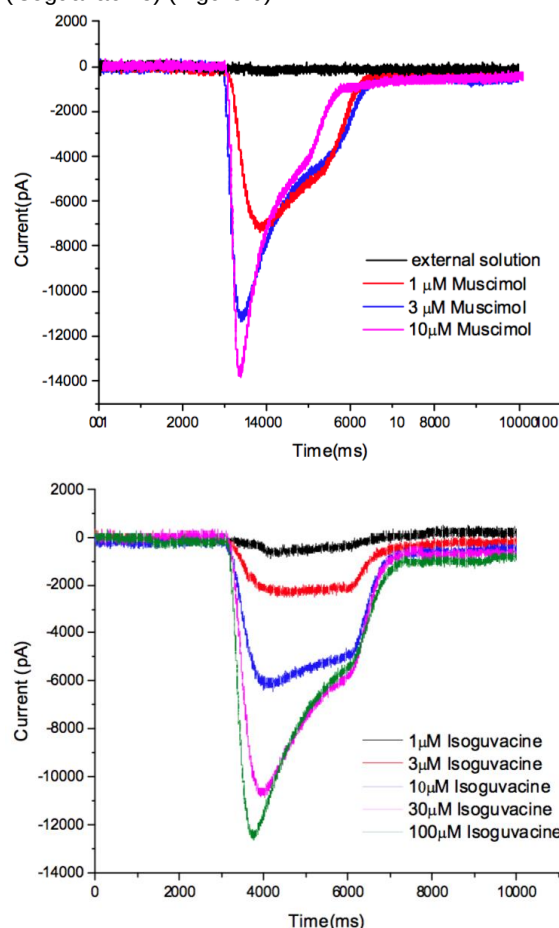


Figure 5: Response of a cell ensemble exposed to a control application of external buffer, followed by increasing doses of two agonists: muscimol 1-10 μ M (left) and isoguvacine 1-100 μ M (right).

GABA_A Antagonist Pharmacology

GABA_A antagonists, picrotoxin and bicuculline were studied by co-applying the compounds with 10 μ M GABA (EC₈₀). Picrotoxin was co-applied with GABA without pre-incubation. Bicuculline was pre-incubated for 3 minutes before being co-applied with GABA. An example of a screen capture during the experiment and the dose-response curve from the picrotoxin experiments are shown in Figure 7. Note that in the current trace chart (left panel of the screen capture) the difference between a set of cursors for each sweep that is recorded (cursors at 1s and 4s on the sweep graph). Therefore, the points on the current trace chart are each 15s apart, because a 10s sweep was recorded every 15s. The values below the graph (-10 to -20nA) are peak values from GABA responses, while the

points close to 0 nA are recorded during the washout period. Example sweeps for bicuculine and an EC50 fit are shown in Figure 8.

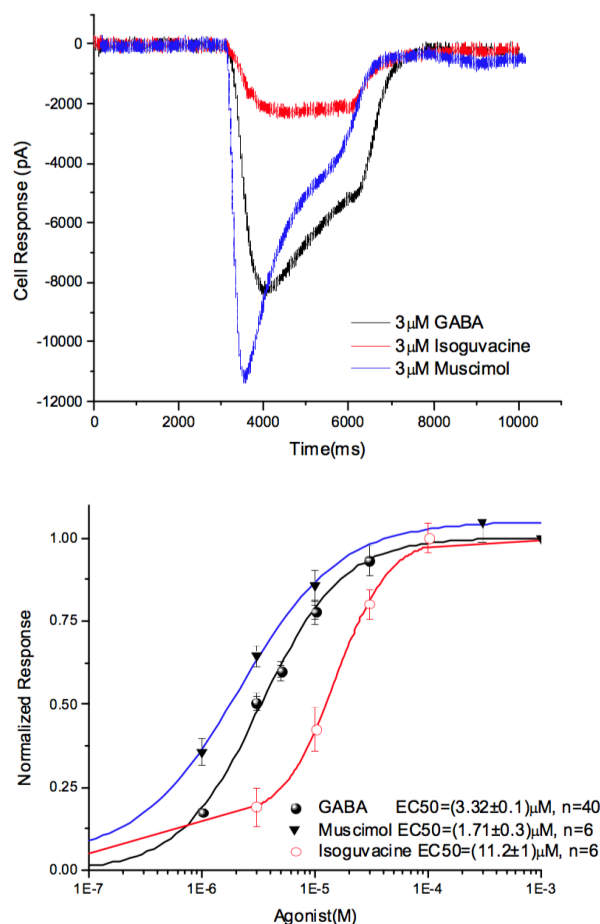


Figure 6: Response of a cell ensemble exposed to 3µM muscimol, 3µM GABA, and 3µM isoguvacine, showing the different potency of the three compounds (left). Dose response curves (right) were fitted using a 4-parameter logistics equation using Origin. Average efficacies were measure as follows: muscimol EC50=1.71 µM (n=6, Hill slope= 1.21, R2=0.996), GABA EC50= 3.32 µM (n=40, Hill slope=1.204, R2=0.995), and isoguvacine EC50 = 11.2 µM (n = 6, Hill slope= 1.16, R2=0.994). Reported EC50 values are as follows: muscimol: 1~ 4 µM, GABA: 3~8 µM, and isoguvacine: 30~ 150 µM.

GABA_A Positive Modulators

The interaction with benzodiazepines has been a major subject in studies on GABA receptors, due to the therapeutic effects of benzodiazepines as anxiolytics, sedative hypnotics, and muscle relaxants. We studied three benzodiazepines: diazepam and triazolam, which contain the 1,4 benzodiazepine structure, and zolpidem, which contains an imidazopyridine structure¹.

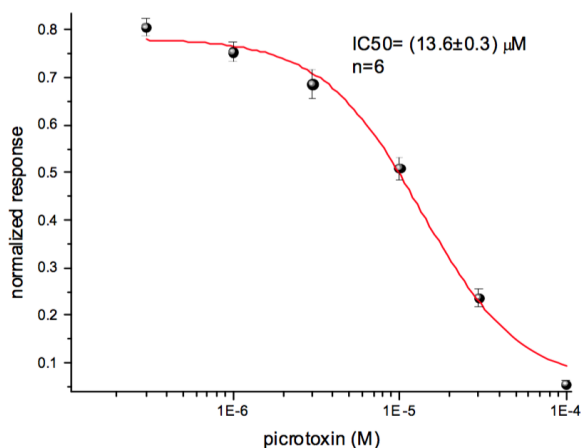
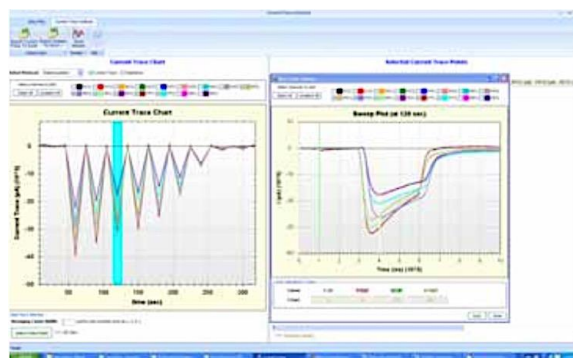


Figure 7: Screen shot from IonFlux showing inhibition of GABA response with picrotoxin. Response of a cell ensemble exposed to 10 µM GABA co-applied with increasing concentrations of picrotoxin (left). Dose response analysis yielded an IC50 value of 13.6 µM (n=6), as compared to a literature value of 2.5 µM ~7 µM^{2,4}.

All three modulator compounds were pre-incubated for 1 minute before being co-applied with 1 µM GABA (EC20), and the results are shown in Figure 9. Diazepam enhanced the maximum Cl⁻ current of α1/β3/γ2 receptors by more than 100% (left), with an EC50= 425 nM, as compared to reported literature values of 120 to 210 nM^{2,7}. Zolpidem enhanced the maximum Cl⁻ current by ~100% (right), with a measured EC50 = 84.6 nM, as compared to literature values of 70-150 nM^{2,5}. Triazolam enhanced the maximum Cl⁻ current by ~100%, with an EC50= 12.1nM, as compared with reported values of 22 to 45 nM^{5,6}.

The IonFlux microfluidic platform has eliminated latency times between compound application/removal operations. In contrast, platforms that rely on fluid handlers to apply compounds require a minimal wait time on the time scale of seconds to load a new set of pipettes, aspirate and dispense subsequent compounds, as well as scheduling conflicts when a larger number of cells are being assayed. Using

microfluidic delivery, we performed a fast cumulative dose response experiment by applying increases dose of GABA (1 μ M, 3 μ M and 10 μ M GABA) within 15 seconds (Figure 10).

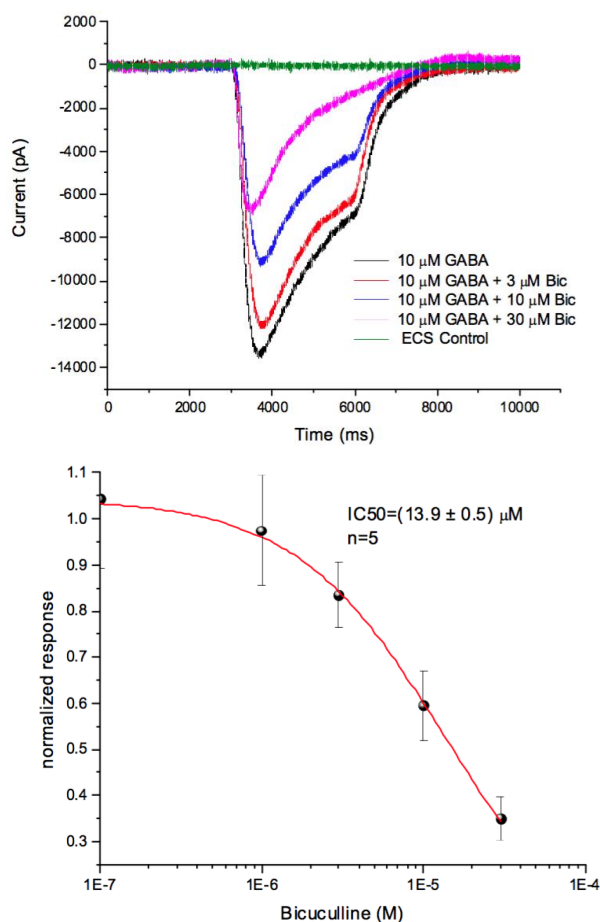


Figure 8: Response of a cell ensemble exposed to 10 μ M GABA co-applied with increasing concentrations of bicuculline (left). Each concentration of bicuculline was pre-incubated for 3 min before the GABA co-application. A Hill fit provided an IC50 value of 13.9 μ M (n=5), as compared to a literature value of 2 μ M².

Discussion

We have used the IonFlux automated electrophysiology platform to perform assay consistency and pharmacology studies on a specific GABAA receptor (α 1/ β 3/ γ 2). GABA signaling is the major mediator of CNS inhibition, important for understanding and treating conditions such as anxiety, schizophrenia and possibly neuropathic pain. The GABAA assay on IonFlux showed a high level of consistency, with an average Z'= 0.58. We also present pharmacological data for two antagonist compounds

and three positive allosteric modulator compounds, showing good agreement with literature values.

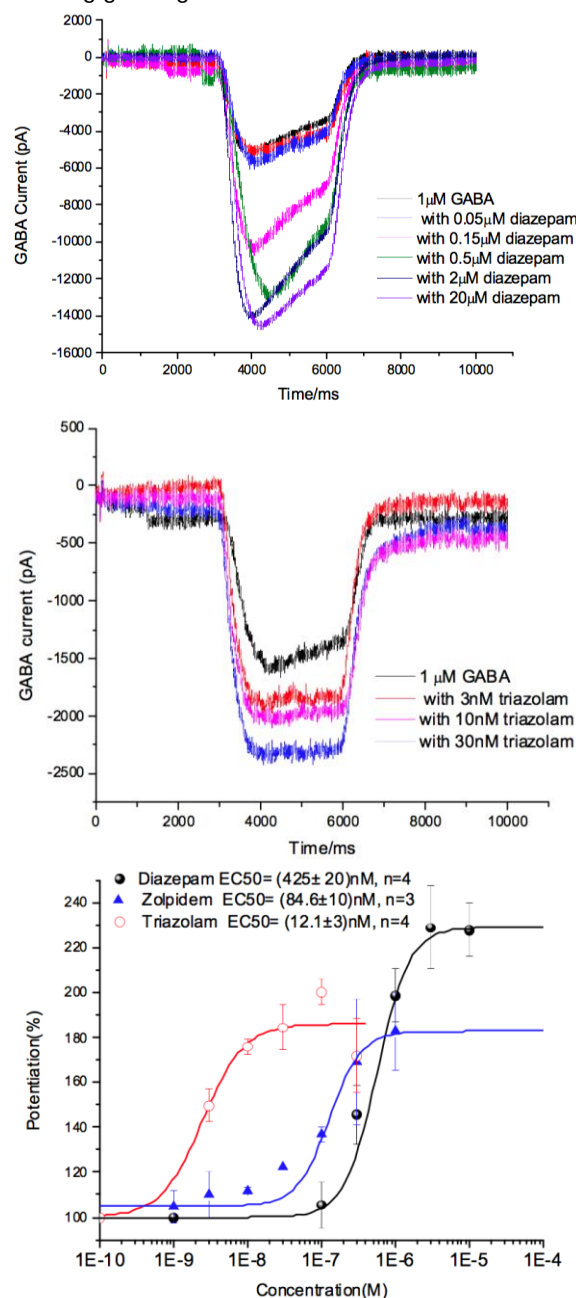


Figure 9: Response of a cell ensemble exposed to 1 μ M GABA (EC20) with increasing diazepam (top left) and triazolam (top right) concentrations. Dose response analysis (bottom) yielded EC50 of diazepam 425 nM (n=4, Hill slope= 1.28, R2= 0.977). The reported literature EC50 value is ~160 nM². EC50 of zolpidem was 84.6 nM (n=3, Hill slope= 1.25, R2=0.967). The reported literature EC50 values range from 70 nM to 150 nM^{2,4}. EC50 of triazolam was 12.1 nM (n=4, Hill slope =1.41, R2=0.970). The reported literature EC50 values range from 22 nM to 45 nM^{5,6}.

The IonFlux platform integrates microfluidic compound delivery with ensemble patch clamp measurements,

enabling continuous recording and fast compound applications without delays between compound additions. It is a flexible platform, with a 16 recording channel and a 64 recording channel version of the instrument. It can operate in an automated fashion by interfacing with fluid handlers, operating similarly to a plate reader device. As such, it addresses the need for high throughput screening for ligand-gated ion channels

References

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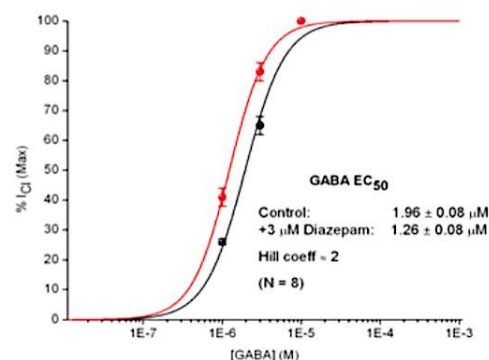
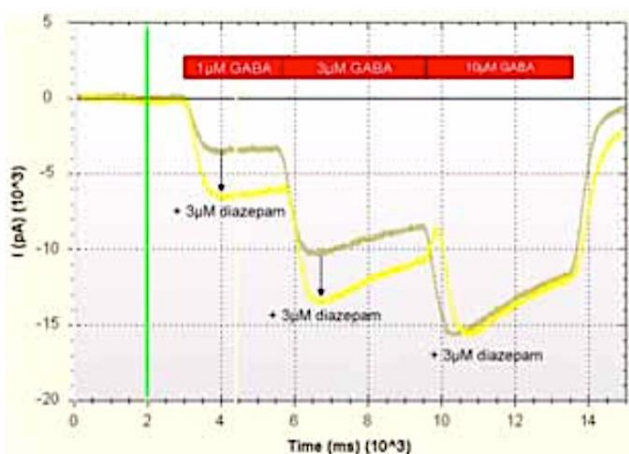


Figure 10: Cumulative dose response curves (1 μ M, 3 μ M and 10 μ M GABA) were obtained in the presence of the positive modulator diazepam. The shift in potency (GABA EC₅₀) can be readily measured (left), and results in an EC₅₀ shift that is consistent with literature values.