Platform-dependence of activity of positive allosteric modulators of β1- and β2-containing $GABA_A$ receptor subtypes

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1 ABSTRACT

$GABA_A$ receptors are targets for drugs used for treatment of anxiety disorders, and for sedation. Many of the drugs in common use function as positive allosteric modulators (PAMs) of GABA, which potentiate the inhibitory activities of the ligand and channels within the central nervous system. Although some PAMs are relatively non-selective, others have been reported to preferentially potentiate channels containing β2 subunits over those containing β1 subunits. In this study, we tested 3 selective PAMs, loreclezole, tracazolate, and mefenamic acid, and 1 non-selective PAM, diazepam, for their effects on HEK293 and CHO cells expressing $GABA_A$ α1β1γ2L and α1β2γ2L subtypes. We utilized a membrane potential-sensitive dye on a FLIPR, and directly assessed electrophysiological responses on IonWorks Barracuda (IWB). In the FLIPR membrane potential assay, the β1- and β2-containing subtypes responded with similar maximal signal and EC_{50} values, thus demonstrating equivalent expression. The PAM results were largely consistent with previous reports: we observed that loreclezole, tracazolate, and mefenamic acid activated the β2-containing subtype with a higher E_{max} and lower EC_{50} than the β1-containing subtype, whilst diazepam potentiated both subtypes to a similar degree. However, when the same experiment was performed on IWB, similar degrees of potentiation at β1 and β2 were observed with all of the PAMs. Correct identities of the expressed subunits were confirmed by RT-PCR. Similar results in both FLIPR and IWB were obtained with the channels expressed in CHO cells, although in CHO cells the β2-containing channel exhibited a >4-fold higher signal amplitude than β1-containing subtype at maximal GABA concentrations. The apparent platform-specificity of the responses raises the possibility that the relative potencies of PAMs on different $GABA_A$ receptors are dependent on experimental conditions, and should be considered when attempting to profile compounds predicted to have PAM activity on $GABA_A$ receptors, particularly those containing the β1 subunit.

2 MATERIALS AND METHODS

$GABA_A$ subunit expression

Full length untagged cDNAs encoding $GABA_A$ subunits were cloned in pcDNA3 vectors downstream of a CMV promoter (GABRA1, GABRG2L) or pZeoSV2 downstream of an SV40 promoter (GABRB1, GABRB2). HEK293 cells were transiently transfected by MaxCyte electroporation with GABRA1, GABRB1 and GABRG2L to express $GABA_A$ α1β1γ2 and with GABRA1, GABRB2 and GABRG2L to express $GABA_A$ α1β2γ2L. Cells were allowed to recover 24-48 hours in DMEM/F12/10% FBS/PenStrep at 37°C, 5% CO_2 in 384-well multiwell plates or tissue culture dishes.

Molecular analysis

RNA was extracted from the transfected cells with an RNeasy RNA Isolation Kit (QIAGEN). RT-PCR was performed on the RNA with primers specific for the GABRB1 and GABRB2 cDNAs, and products were analysed by agarose gel electrophoresis and ethidium bromide staining

FLIPR assays

Cells plated at 30K/well were loaded with the Membrane Potential Red dye (Molecular Devices) in HEPES-Buffered Physiological Saline (HBPS) at 37°C for 30 min. Modulators or vehicle control were applied to the cells onboard a $FLIPR^{TETRA}$, and incubated for 5 min. GABA was then added at approximately EC_{10} for each channel. Serially diluted GABA was added in parallel to confirm the actual EC value.

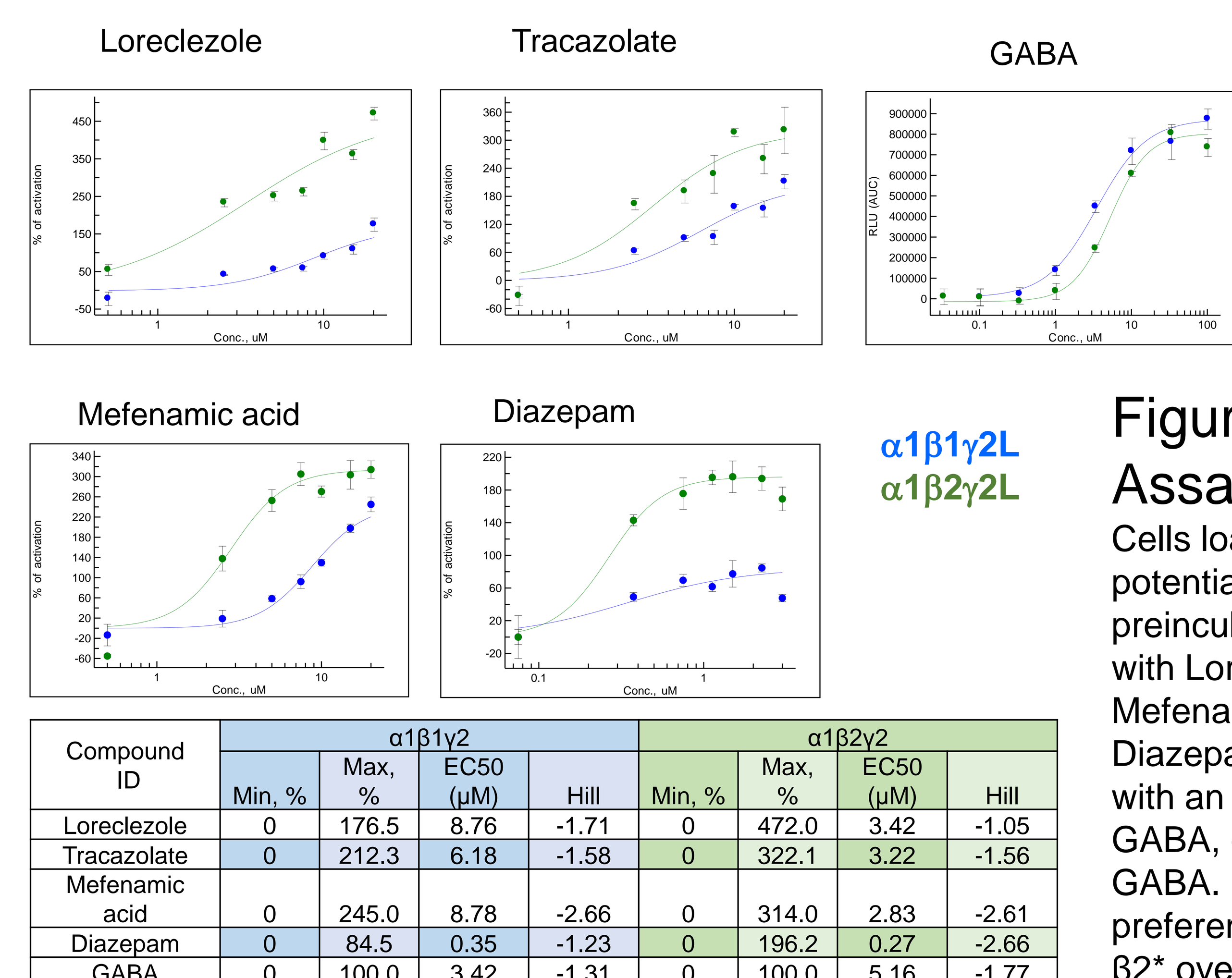
IonWorks Barracuda analysis

Cells were detached with Accutase and resuspended in Extracellular Buffer, HBPS (composition in mM): NaCl, 110; Na-Gluconate, 50; KCl, 4.0; $CaCl_2$, 5; $MgCl_2$, 1; HEPES, 10; Glucose, 10; pH adjusted to 7.4 with NaOH. Intracellular solution (mM): KCl, 140; $MgCl_2$, 5; EGTA, 1; HEPES, 10; pH adjusted to 7.2 with KOH. Cells were analysed in Population Patch Clamp mode with whole-cell recording configuration established via patch perforation. Test compound, agonist, and control formulations were supplemented with 200 μM $LaCl_3$ to reduce current artifacts. Each test compound application will consist of the addition of 20 µL of 2x concentrated solution to the total 40 µL of final volume of the PPC well. Two recordings (scans) are performed; one scan during application of test compound to detect agonist effects and a second scan five minutes after test article application during positive agonist control application to detect antagonist effects. Inward Cl^- current are recorded at holding potential of -70 mV.

4 CONCLUSIONS

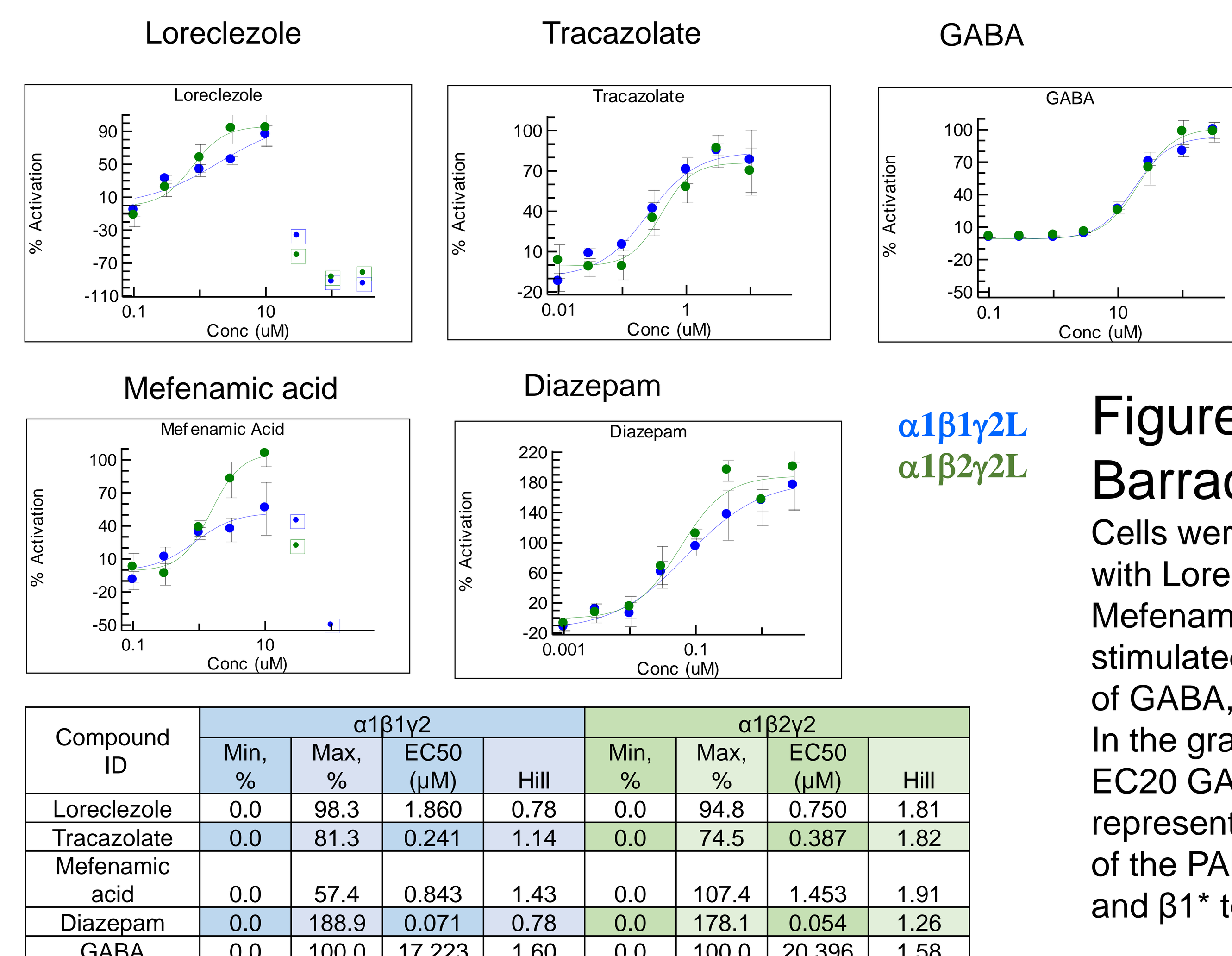
- PAMs potentiated $GABA_A$ β2* to a greater degree than $GABA_A$ β1* in FLIPR assays
- PAMs potentiated $GABA_A$ β1* and β2* to similar degrees in IonWorks Barracuda assay.
- Appropriate expression of each β subunit was demonstrated by RT-PCR.
- Choice of assay platform may exaggerate or minimize selectivity of PAMs for β1 and β2-containing $GABA_A$ receptors.

3 RESULTS



Compound ID	α1β1γ2				α1β2γ2			
	Min, %	Max, %	EC50 (μM)	Hill	Min, %	Max, %	EC50 (μM)	Hill
Loreclezole	0	176.5	8.76	-1.71	0	472.0	3.42	-1.05
Tracazolate	0	212.3	6.18	-1.58	0	322.1	3.22	-1.56
Mefenamic acid	0	245.0	8.78	-2.66	0	314.0	2.83	-2.61
Diazepam	0	84.5	0.35	-1.23	0	196.2	0.27	-2.66
GABA	0	100.0	3.42	-1.31	0	100.0	5.16	-1.77

Figure 1. FLIPR Assay
Cells loaded with membrane potential dye were preincubated for 5 minutes with Loreclozole, Tracazolate, Mefenamic Acid and Diazepam, then stimulated with an EC_{10} concentration of GABA, or a dilution series of GABA. All of the PAMs preferentially activated $GABA_A$ β2* over β1*.



Compound ID	α1β1γ2				α1β2γ2			
	Min, %	Max, %	EC50 (μM)	Hill	Min, %	Max, %	EC50 (μM)	Hill
Loreclezole	0.0	98.3	1.860	0.78	0.0	94.8	0.750	1.81
Tracazolate	0.0	81.3	0.241	1.14	0.0	74.5	0.387	1.82
Mefenamic acid	0.0	57.4	0.843	1.43	0.0	107.4	1.453	1.91
Diazepam	0.0	188.9	0.071	0.78	0.0	178.1	0.054	1.26
GABA	0.0	100.0	17.223	1.60	0.0	100.0	20.396	1.58

Figure 2. IonWorks Barracuda Assay
Cells were preincubated for 5 minutes with Loreclozole, Tracazolate, Mefenamic Acid and Diazepam, then stimulated with an EC_{10} concentration of GABA, or a dilution series of GABA. In the graphs, 0% reflects the signal at EC20 GABA alone, and 100% represents maximal GABA signal. All of the PAMs potentiated $GABA_A$ β2* and β1* to similar degrees.

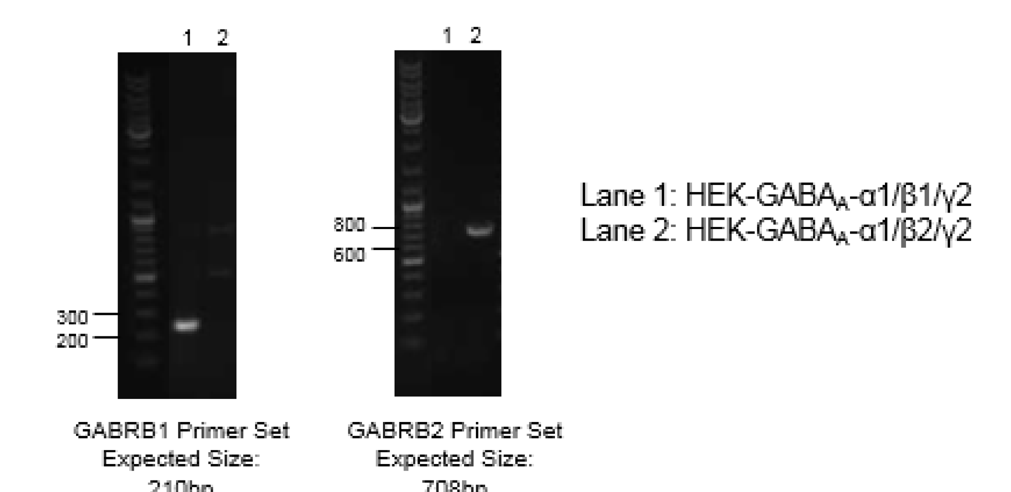


Figure 3. Molecular Confirmation of $GABA_A$ β subunit expression
Cells were subjected to RNA isolation, and RT-PCR was performed with primers specific for GABRB1 and GABRB2. In addition, full length products were amplified and subjected to DNA sequencing. Both methods demonstrated expression of the appropriate β subunits.

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