Development of Screening Tools to Identify Nicotinic Subtype-Selective Compounds

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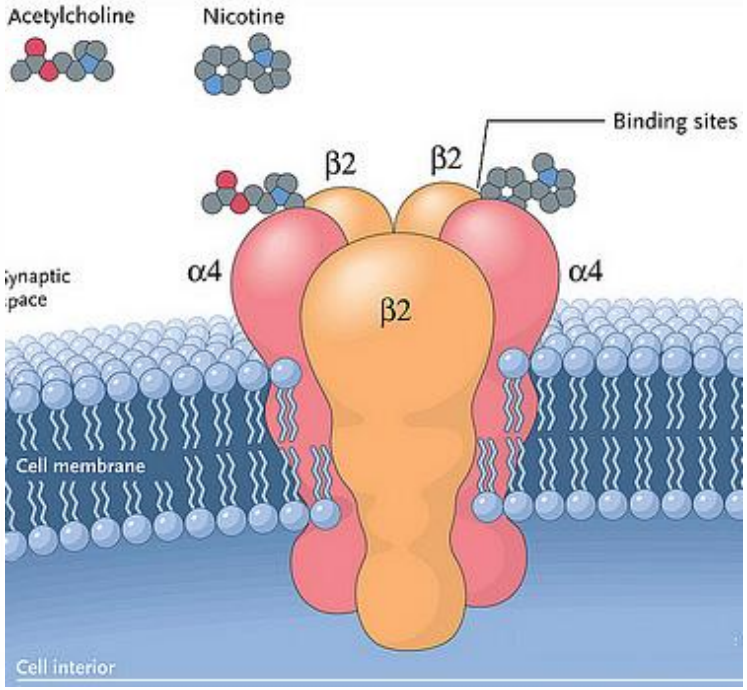
Tobacco Control Act – US FDA

- Signed into law 2009
- Authorized the FDA to regulate tobacco products to protect public health
- The Center for Tobacco Products (CTP) established to oversee implementation - including research
- CTP contracted with ChanTest to run the NicScreen project in August 2013, as part of an initiative to provide scientific support for developing tobacco product regulations

NicScreen Project Objectives

- **Primary NicSreen Objective:** Develop and validate bioassay screening tools for rapid identification of tobacco product constituents with nAChR subtype selective activity.
- **Phase I (9/2013 – 8/2014)** - Develop cell lines and high-throughput assays
- **Phase II (9/2015 – 8/2016)** – Pilot testing to determine the pharmacological profiles of different tobacco products
- **Phase III (9/2016 – 8/2017)** – Proof of concept study for high-throughput screening to evaluate tobacco product constituents for subtype selectivity. Develop methods for evaluating similarities and differences in the pharmacologic activity of marketed tobacco products and constituents

Nicotinic Acetylcholine Receptors Are Therapeutic Targets



- **Neuronal nicotinic receptors** - pentameric ligand-gated cation-selective channels
- **Subtypes defined by α and β** subunit composition, and functional properties (agonist-sensitivity, pharmacologic profile, Ca^{2+} permeability, and desensitization kinetics)

Cognition impairment in schizophrenia and Alzheimer's - α7 Agonists and PAMs in clinical trials (e.g., DMXB-A, galantamine, EVP-6124).

Parkinson's disease – transdermal nicotine and varenicline in clinical trials.

Depression – α4β2 antagonist to augment SSRIs in clinical trials (e.g., mecamylamine).

Tobacco addiction - α4β2 partial agonist (varenicline), marketed. Varenicline for treatment of alcohol, cocaine and methamphetamine addiction in clinical trials.

Inflammatory diseases (e.g. inflammatory bowel disease) - α7 receptor activation is anti-inflammatory in animal models.

Ion Channel HTS Screening & Profiling Platforms for Nicotinic Receptor Assays

- **Screening/Profiling Assays**
  - **Ligand Gated Channels**
    - Ion Works Barracuda (IWB): automated patch clamp
    - FLIPR: ion- or voltage-sensitive dyes
  - **Voltage Gated Channels**
    - IWB: automated patch clamp
    - FLIPR: ion-sensitive dyes (Ca^{2+}, Tl^{+})
- **Assay Modes**
  - agonist
  - antagonist
  - positive or negative allosteric modulator

IonWorks Barracuda

FLIPR^{TETRA}

Cell Line and Assay Validation Workflow

Cell line production:	Optimize recording conditions:	Functional Validation:	Reporting:
• cDNA cloning & transfection • Clone selection • Expression confirmation • Functional characterization • Stability and Scale-up	• Adjust stimulus parameters • Adjust intra- and extracellular solutions, holding potential • Conduct stimulus/response testing • Optimize cell density • Increase signal window and minimize variability (Z' factor optimization) • Evaluate DMSO tolerance	• Signal stability/uniformity • 2-Day plate uniformity tests (intra- and inter-day variability) • 2-Day reference compound testing: 3 – 10 cpds, 8 and 16 pt CRC	• Data analysis • Data QC • Report writing • Format • Report QC

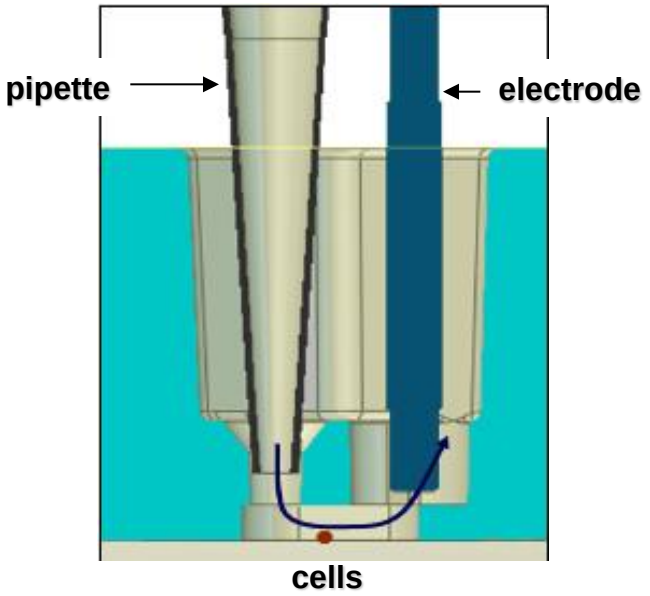
Assay Acceptance Criteria

Plate Level Parameters (FLIPR & IWB)	Value
Z' factor	≥ 0.5
CV for MAX Control	≤ 20%
CV for MIN Control	≤ 20%
Signal Window (SW)	≥ 3
EC_{50} for reference agonists	≤ 0.5 log*
IC_{50} for reference antagonists	≤ 0.5 log*
	*from historical mean

Well Level Parameter (IWB only)	Value
Rseal	> 100 MΩ
Current Amplitude	> 0.2 nA
Rseal Stability	< 50% decrease
Current Stability	< 50% decrease
Success rate (% valid wells)	≥90%

Validation data are obtained in independent experiments conducted on separate days

Ligand-Gated Assays in IonWorks Barracuda (IWB)



Continuous voltage-clamp current measurement with rapid solution addition for fast-desensitizing, ligand-gated channels

- Population Patch Clamp (PPC) and single hole recording modes (384-well patch plate)
- Controls single cell membrane potential and measures ionic currents in single-cell or cell population (≤ 64 cells/well).
- 384-channel pipettor, integrated 384-channel electronic head
- **Validated assays for multiple subtypes in ligand-gated 5-HT3R, $GABA_AR$, nAChR, NMDAR and TRP channels**

Throughput (manual plate handling)

- 10 plates/day/instrument
- 3200 compounds/day
- 16,000 compounds/week

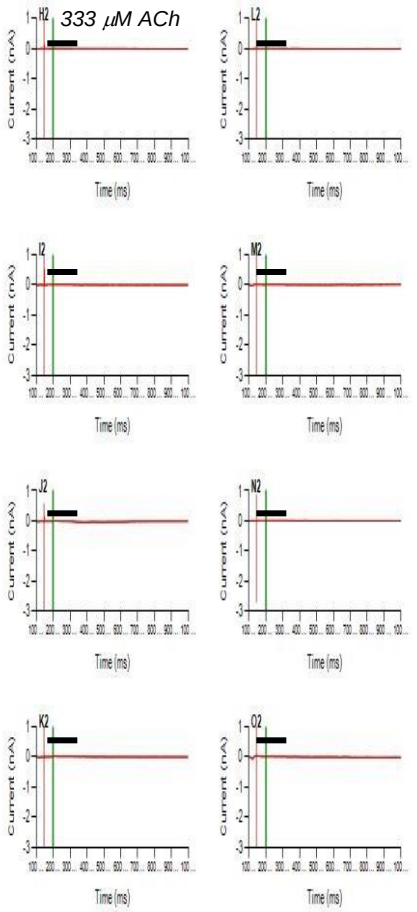
Timeline for screening 100,000 cpd library ~7 weeks

Nicotinic Cell Lines and Assays

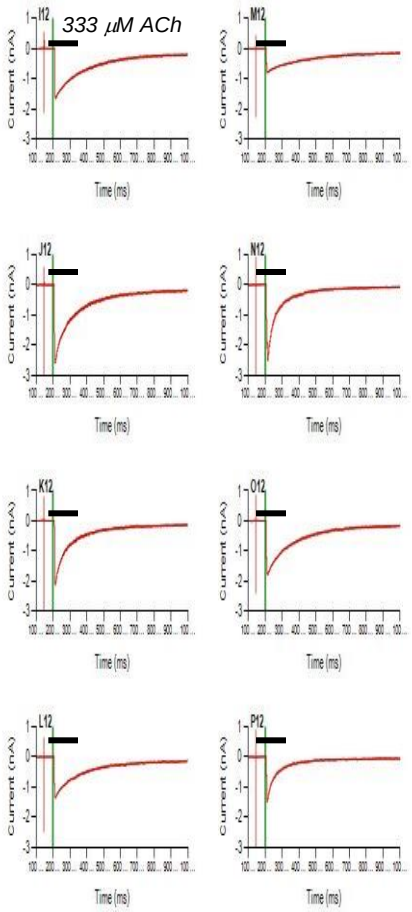
nAChR Subtype	Parental Cell	Assays
α3β4	CHO	IonWorks Barracuda, FLIPR
α4β2	CHO	IonWorks Barracuda, FLIPR
α3β4α5	CHO	IonWorks Barracuda, FLIPR
α7	CHO	IonWorks Barracuda, QPatch, FLIPR
α6/3β2β3^{V273S}	HEK293	IonWorks Barracuda, FLIPR

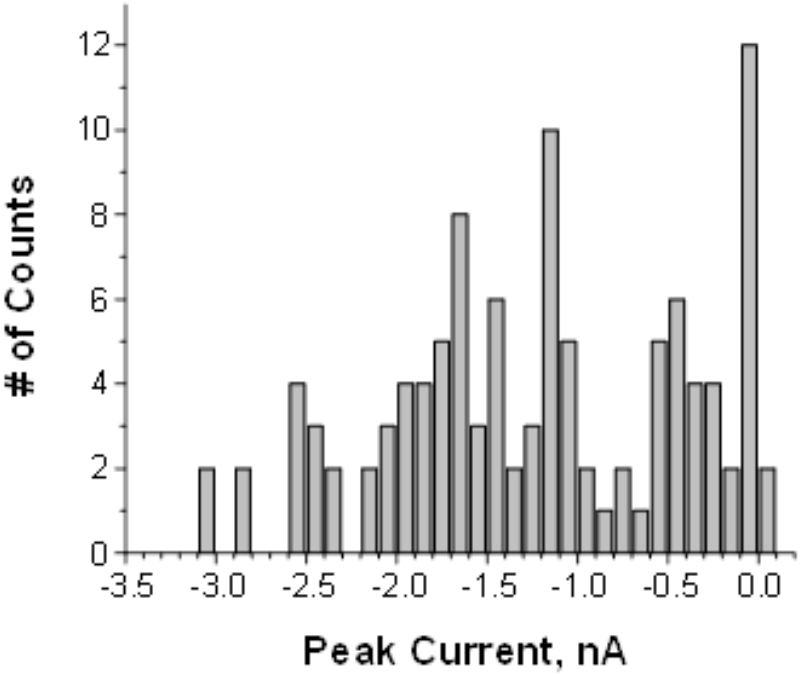
α4β2: Ionic Current Distribution in IonWorks Barracuda (Single Cell Mode)

Untransfected CHO Cells



α4β2-CHO Cells Clone #26





Stimulation – 0.3 mM ACh
Mean peak ±SD = 1.21 ± 0.82 nA

α4β2: Signal Uniformity

IonWorks Barracuda (PPC)

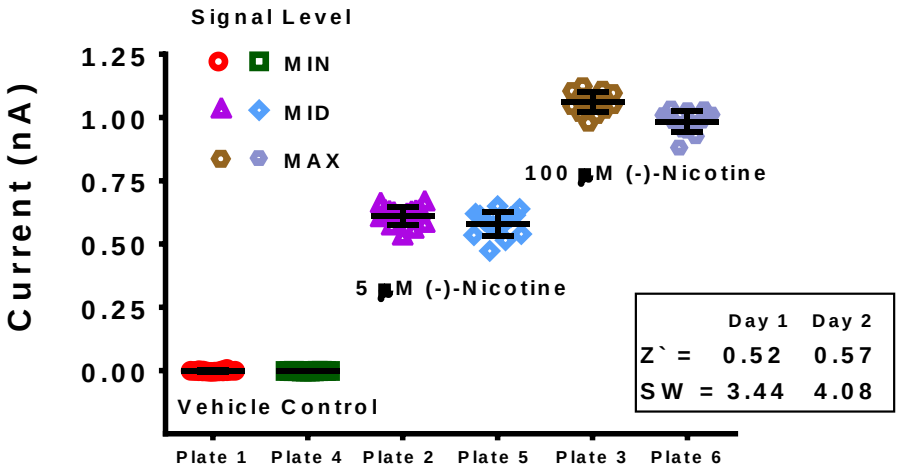


	Plate 1	Plate 4	Plate 2	Plate 5	Plate 3	Plate 6
Mean	-0.001421	-0.002157	0.6130	0.5803	1.061	0.9834
Std. Deviation	0.002791	0.0009560	0.03558	0.04661	0.03884	0.04179
Std. Error of Mean	0.0006977	0.0002390	0.008896	0.01165	0.009709	0.01045

FLIPR

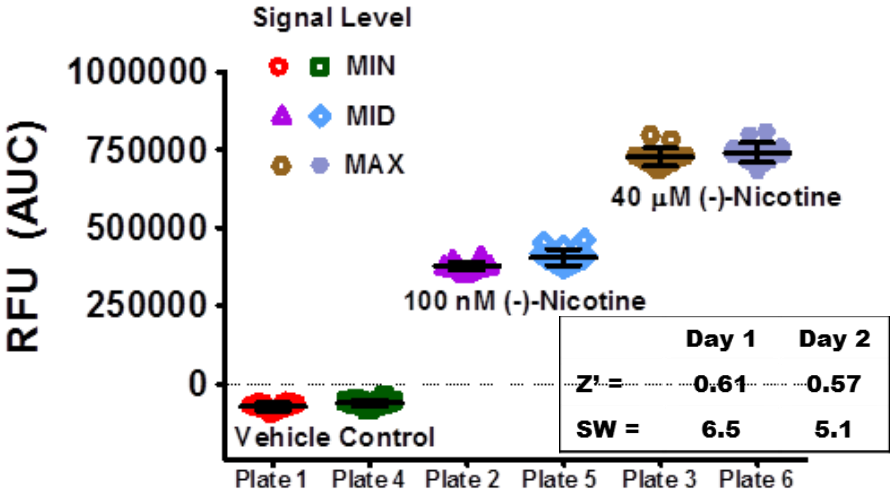
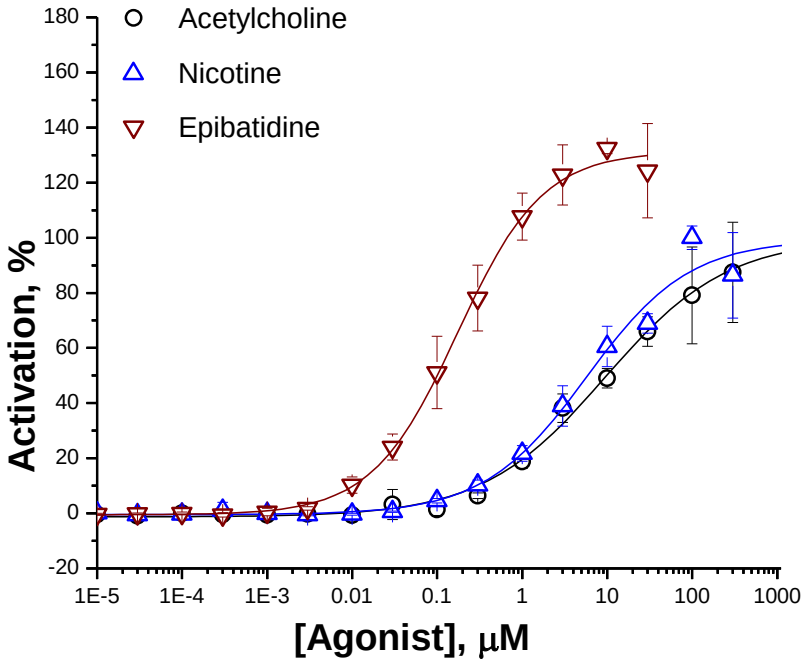


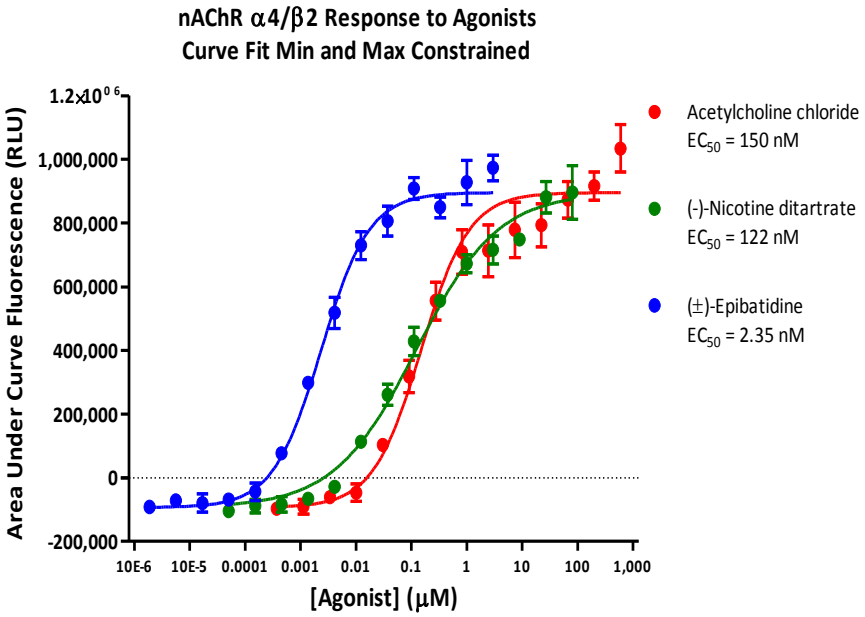
	Plate 1	Plate 4	Plate 2	Plate 5	Plate 3	Plate 6
Mean	-72975	-60804	377535	404558	725187	739139
Std. Deviation	11779	10828	14235	25919	29369	32169
Std. Error	2945	2707	3559	6480	7342	8042

α4β2: Agonist Assay 16 point CRC

IonWorks Barracuda



FLIPR MP-Assay

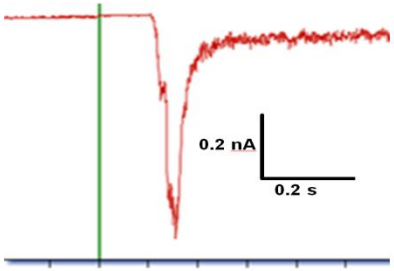


Reference Agonist	EC_{50}, µM		
	IWB	FLIPR	Xenopus*
Acetylcholine	9.4	0.15	4
Nicotine	5.7	0.12	1
Epibatidine	0.17	0.0024	ND

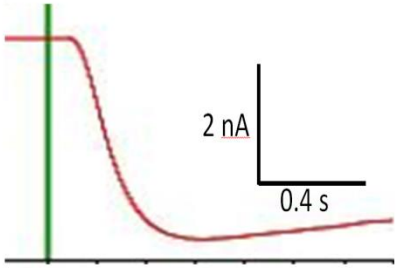
*Moroni et al., 2006

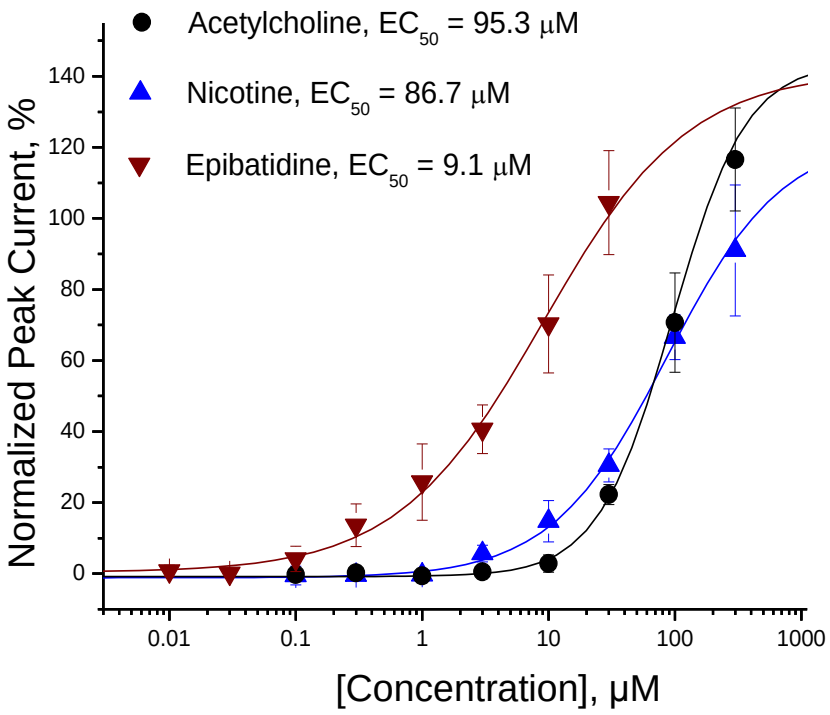
IWB: α7-CHO Agonist Assay

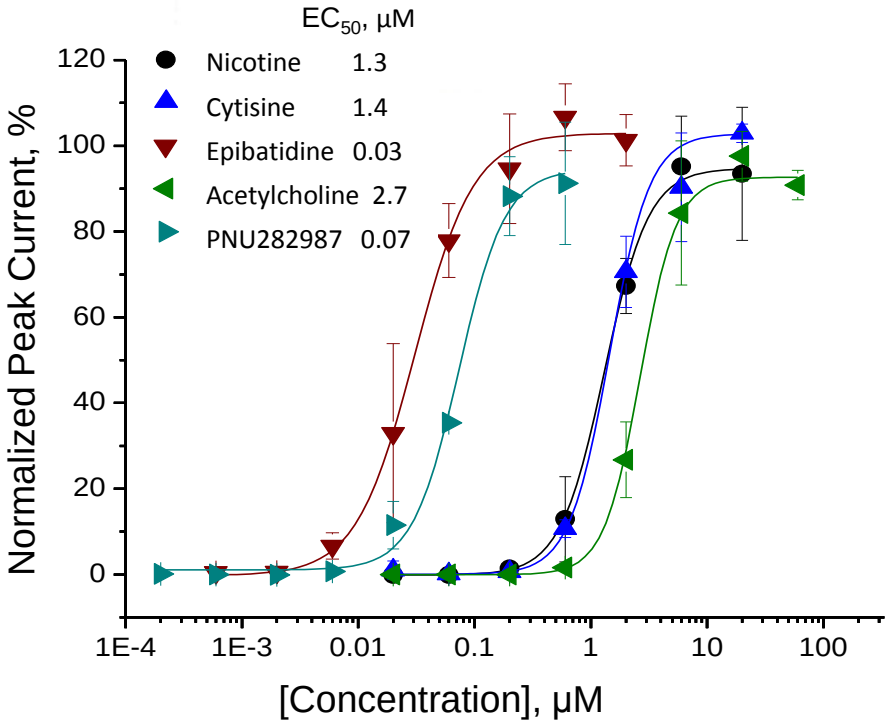
Max Response, no PAM



Max Response, PNU120596 (6 µM)

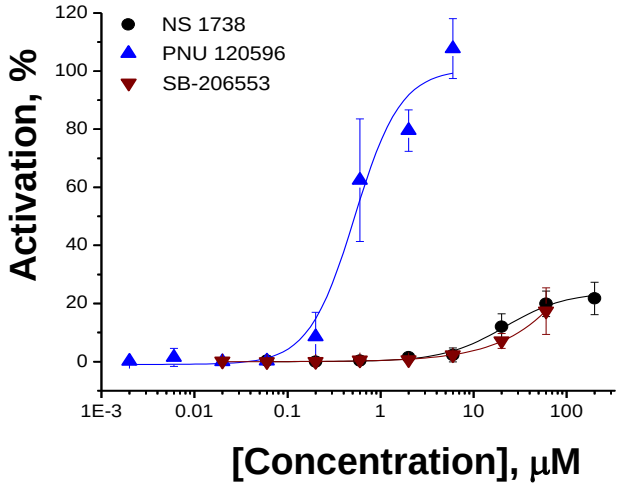




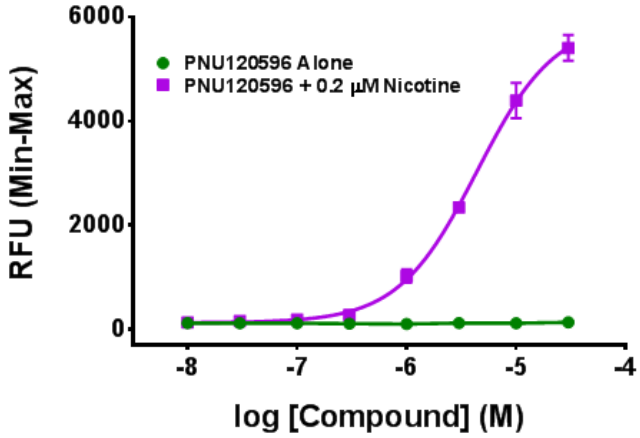


α7 Positive Allosteric Modulators

IonWorks Barracuda



FLIPR

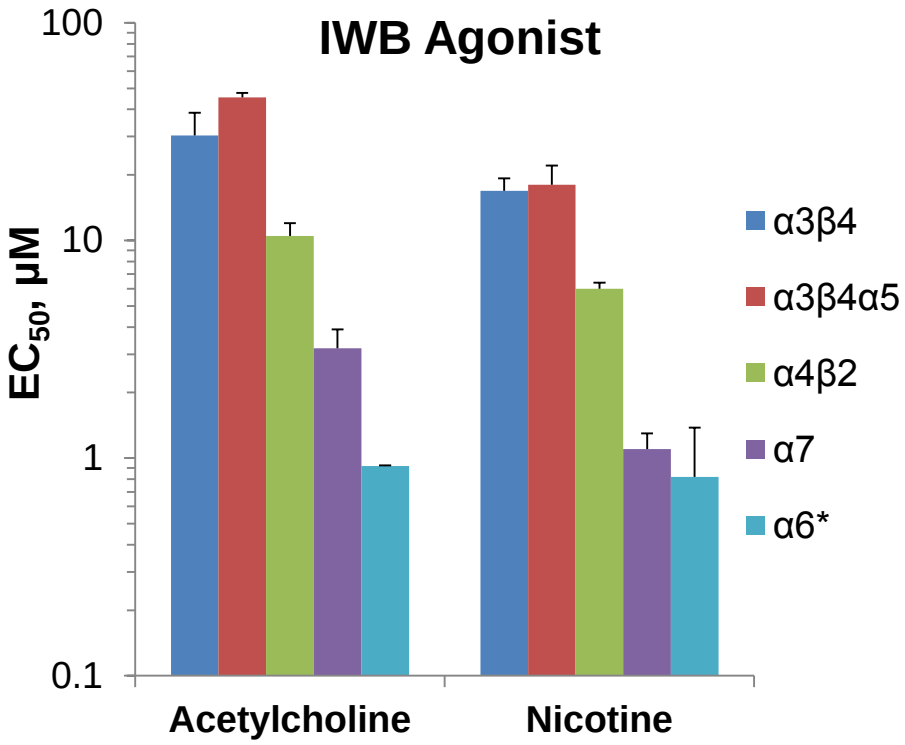


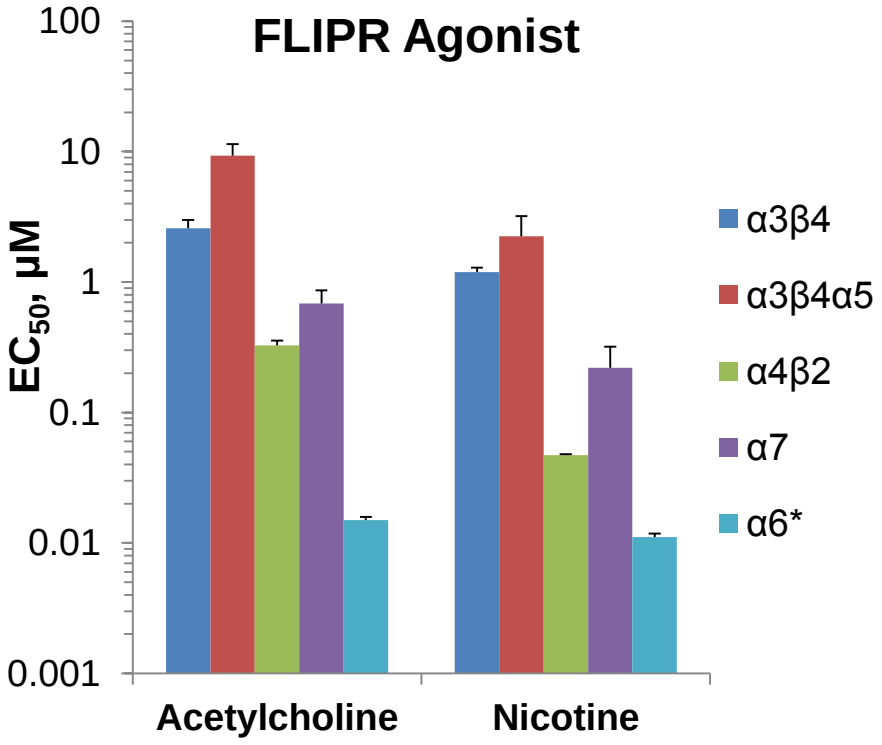
Reference Compound	EC_{50}, μM		
	IWB	FLIPR	Published
NS 1738	21.7	ND	12.5*
PNU 120596	0.53	3.6	0.216†
SB-206553	>5 μM	ND	45‡

PNU 120596 was the most effective PAM

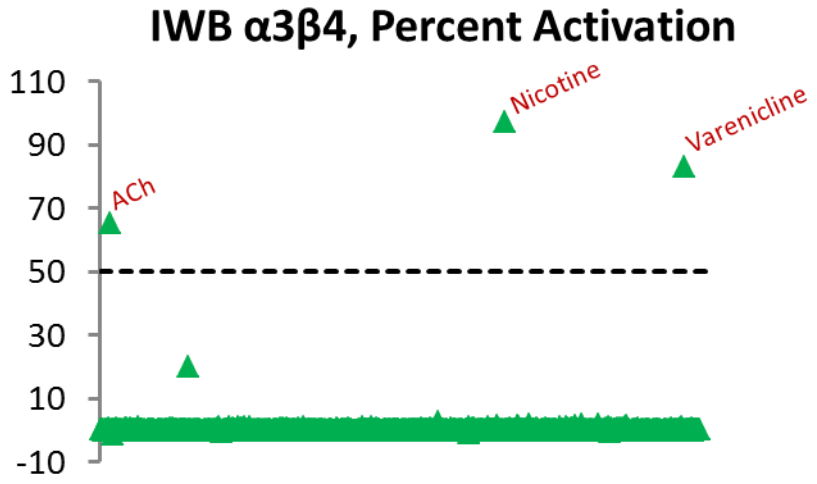
*Friis et al. 2008. QPatch ionic current
†Hurst et al. 2005. FLIPR Ca^{2+} flux
‡Roncarati et al., 2008. FLIPR Ca^{2+} flux

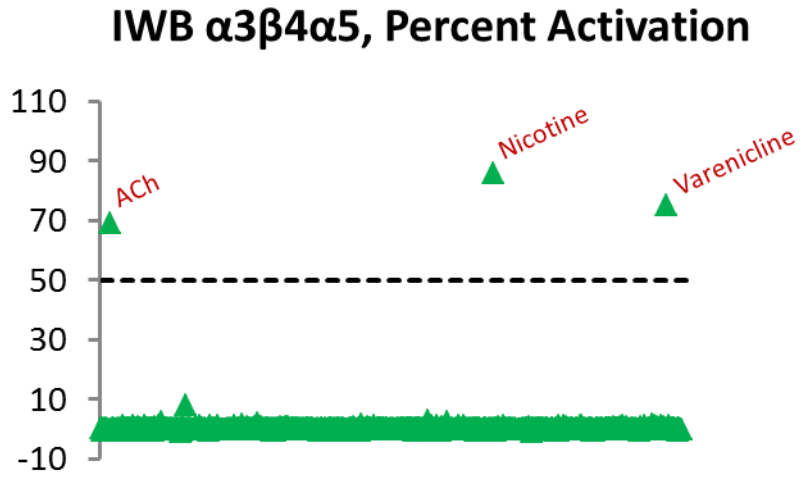
Agonist Profiles

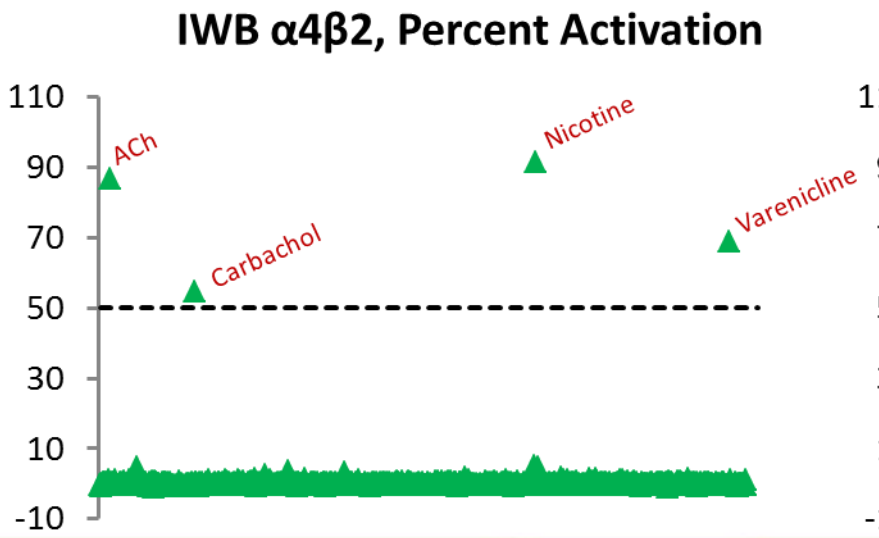


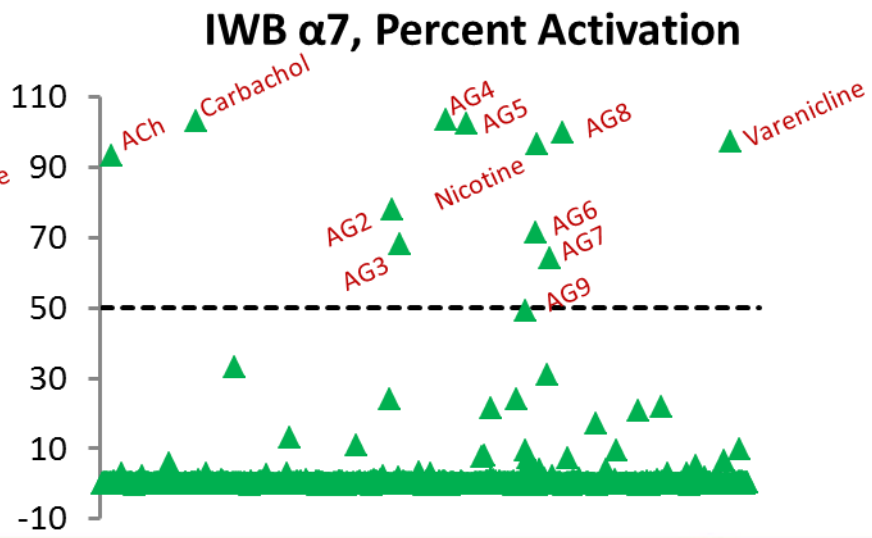


IWB: Subtype Selectivity Pilot Study: ENZO Library Agonist Mode SP Screen, 786 Drugs @ 60 µM

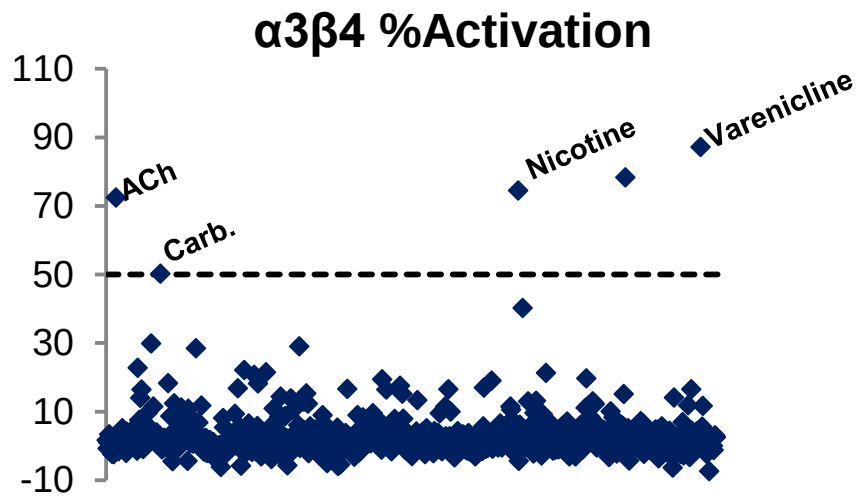


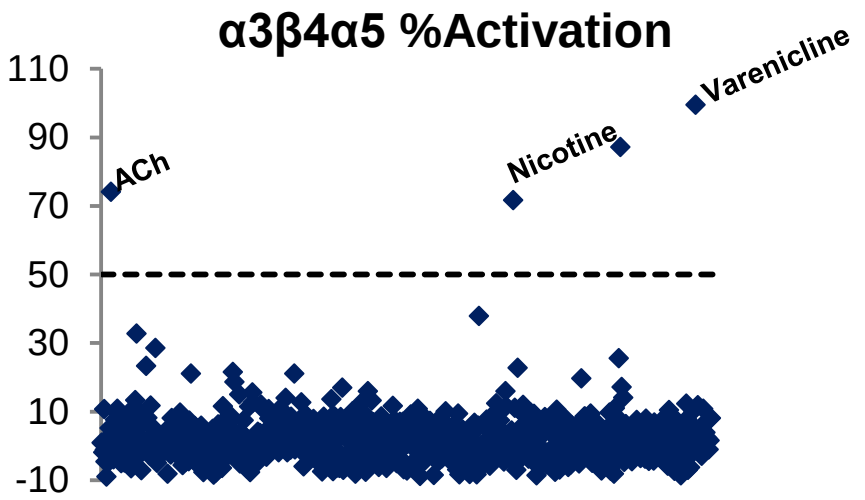


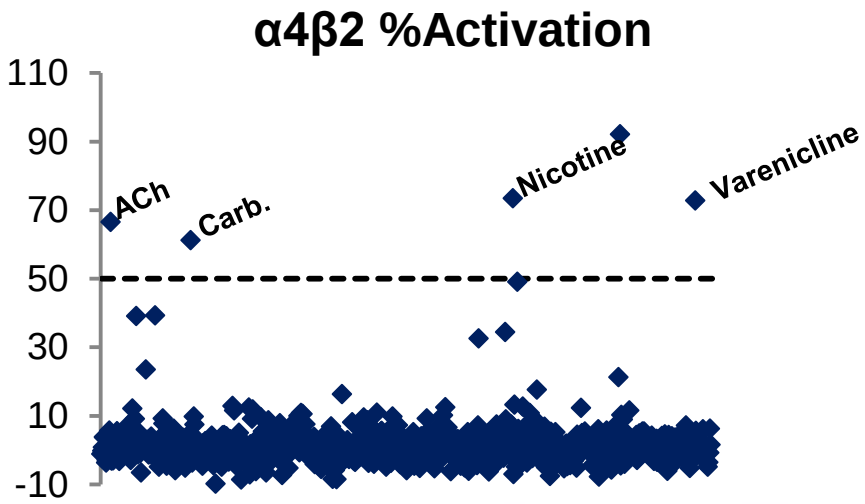


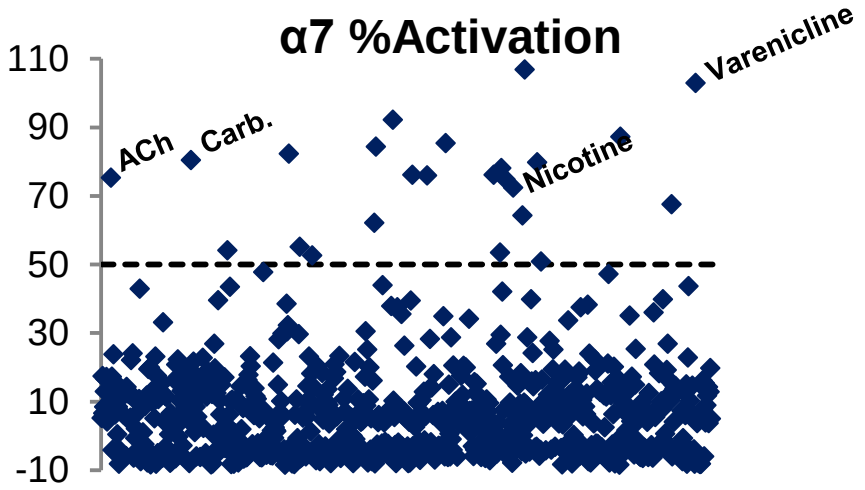


FLIPR: Subtype Selectivity Pilot Study: ENZO Library Agonist Mode SP Screen, 786 Drugs @ 12 µM

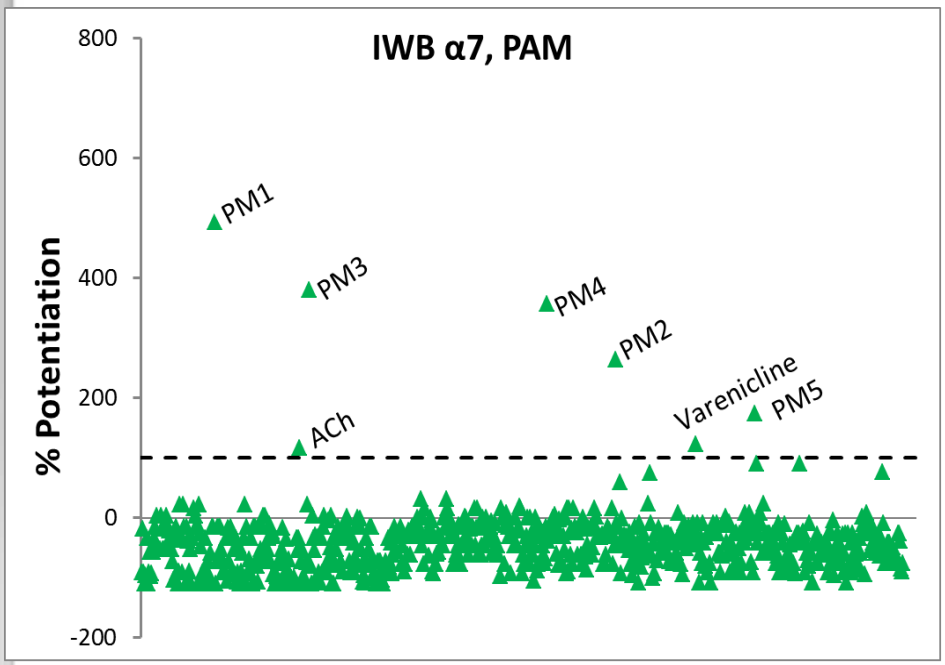


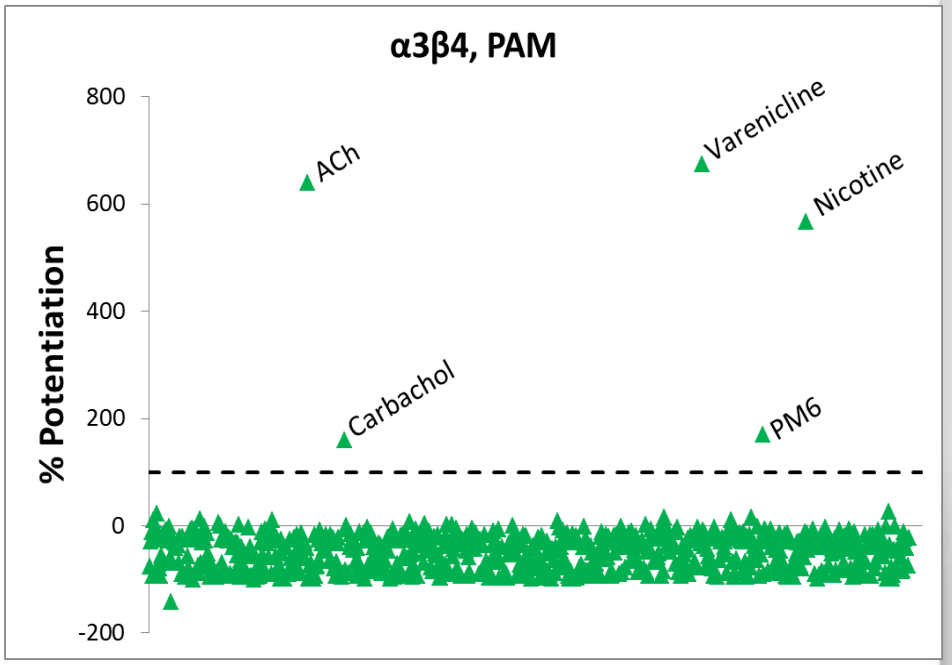






IWB: Enzo Lib. Screen (60 µM) – PAM Mode

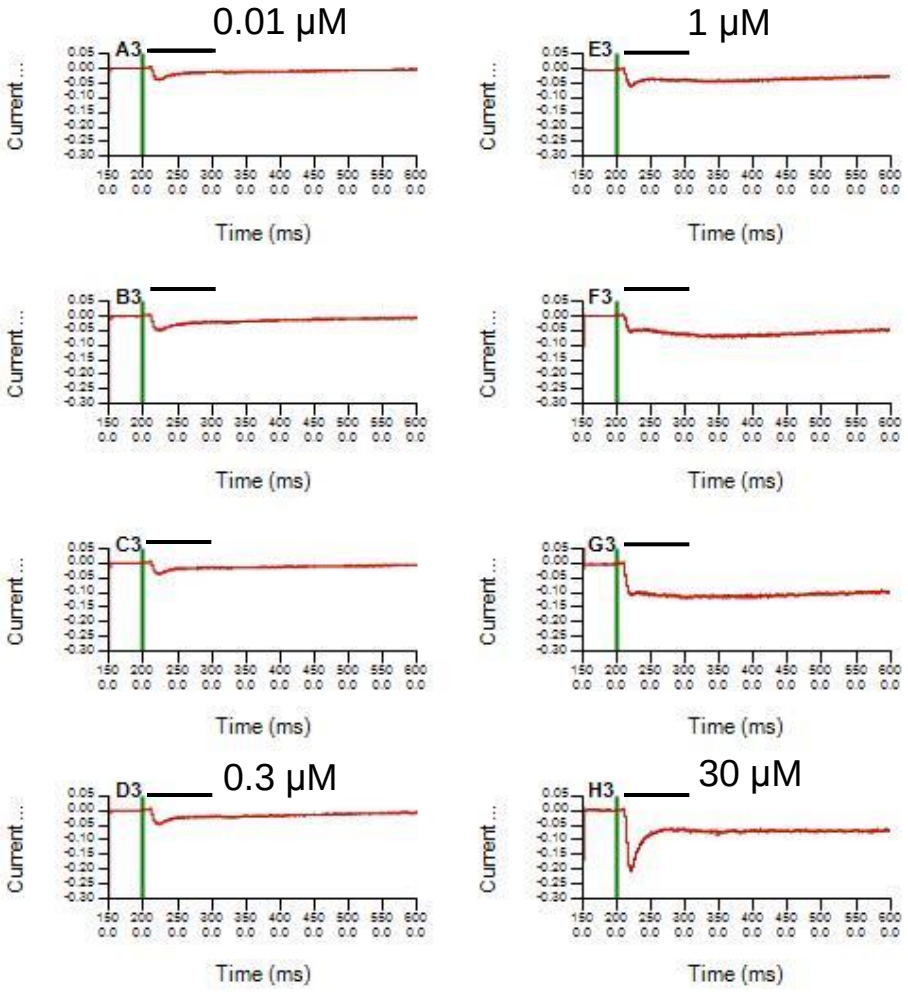


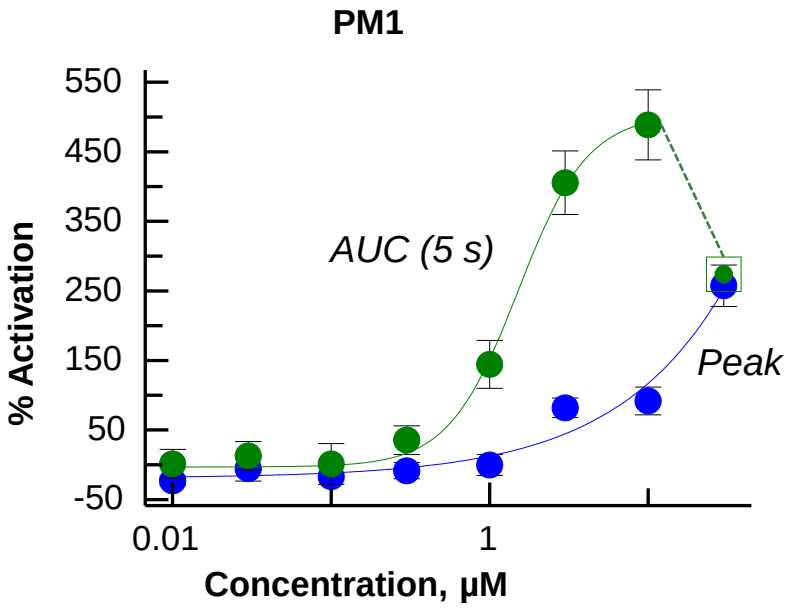


α7-Selective PAMs	
PM1	antineoplastic, EGFR inhibitor
PM2	antineoplastic, kinase inhibitor
PM3	phosphodiesterase inhibitor
PM4	microtubule disruptor
PM5	corticosteroid

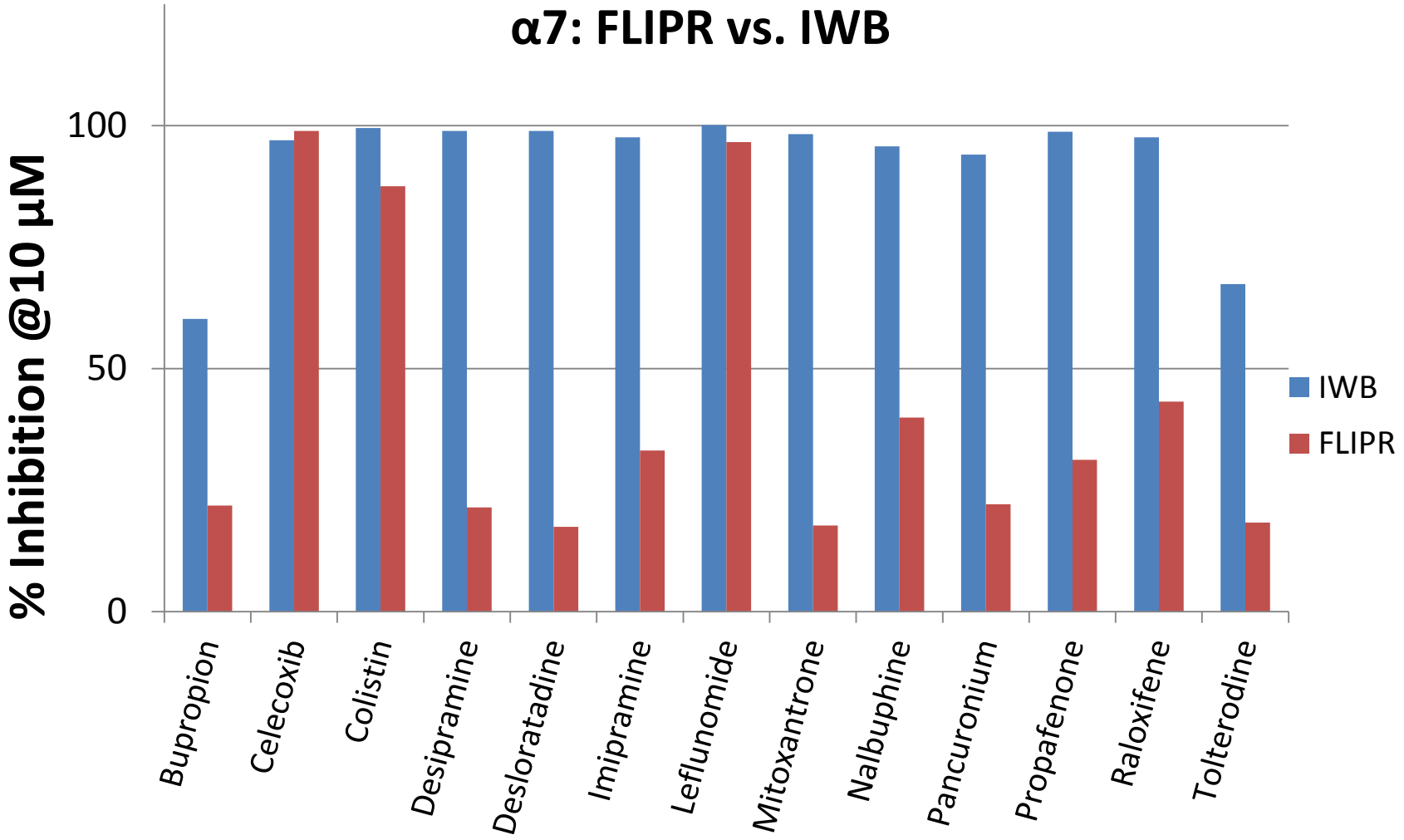
α3β4-Selective PAM	
PM6	antibacterial, KCNQ1 channel activator

α7 PAM Confirmation CRC: EGFR Inhibitor

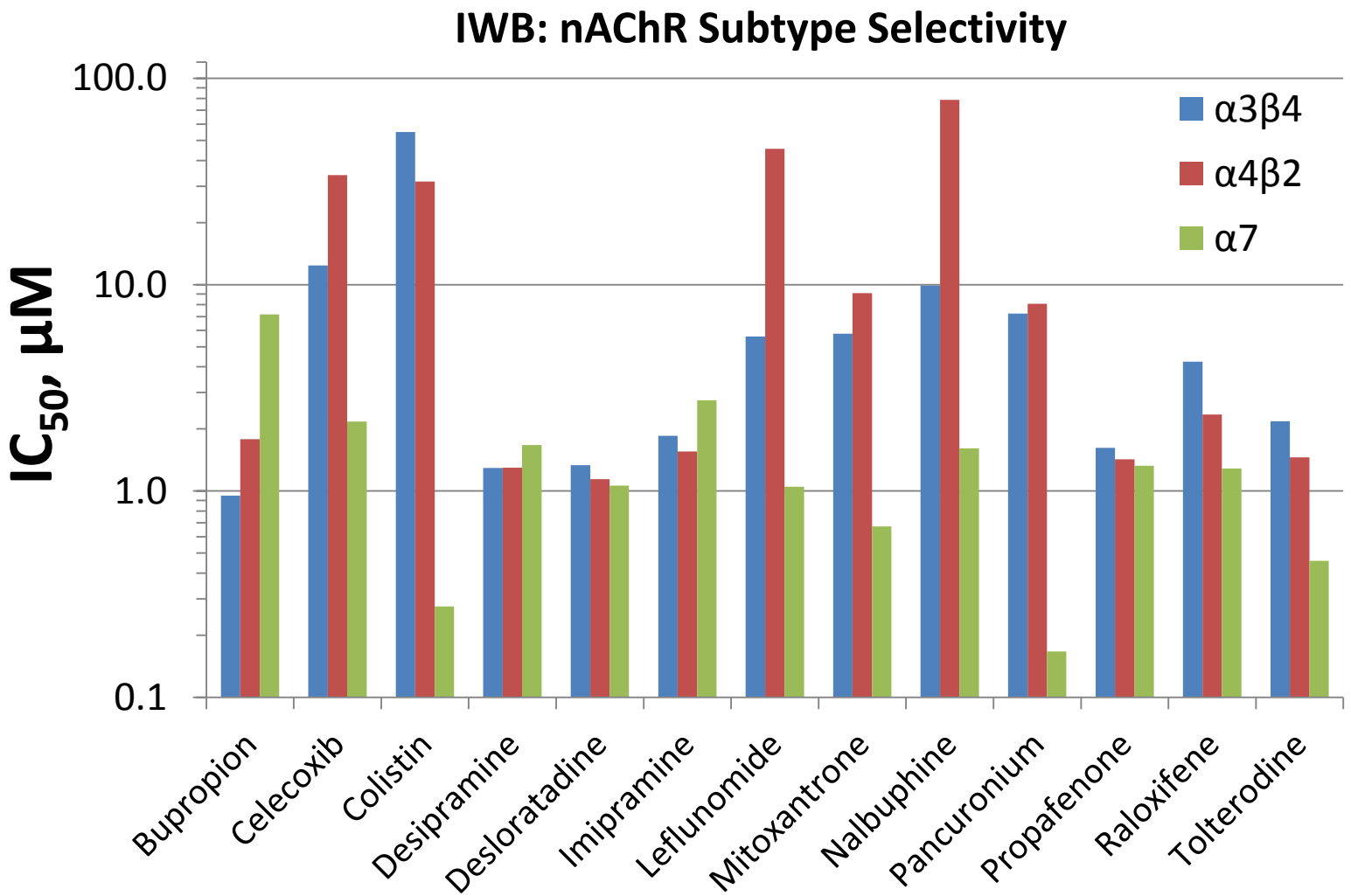




FLIPR vs. IWB Antagonist Screen

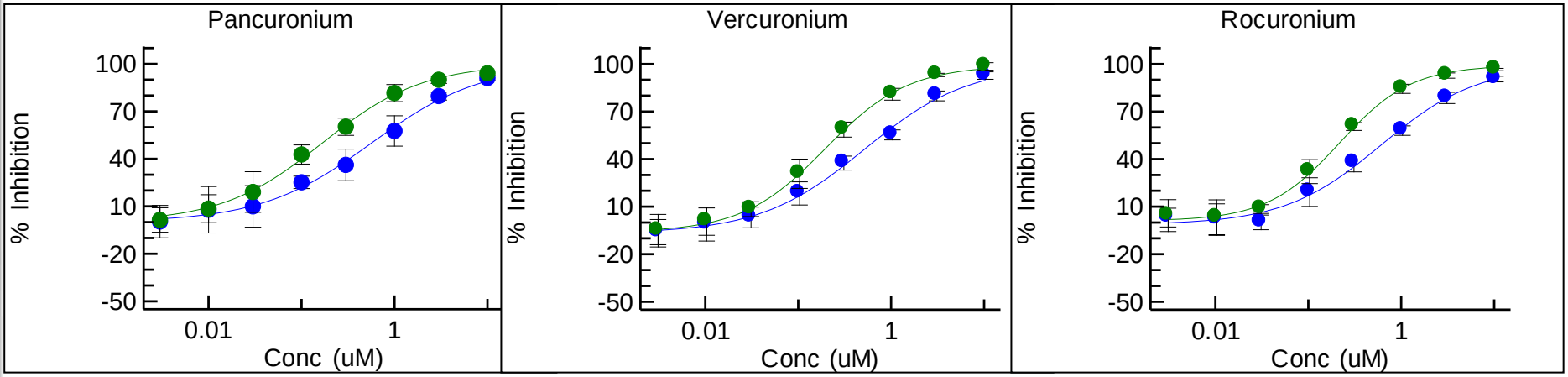


IWB: Antagonist Evaluation



IWB: Evidence for Pore Blocking by Nondepolarizing Neuromuscular Blocking Agents in α7 Receptors

Concentration-Response Curves: Peak current (blue) vs. AUC (green)



Drug	IC_{50}, µM (AUC)	IC_{50}, µM (Peak Current)
Pancuronium	0.17	0.57
Rocuronium	0.21	0.59
Vecuronium	0.19	0.50

nAChR Summary

- IWB and FLIPR platforms are suitable for high-throughput nAChR assays, but have different agonist/antagonist sensitivities
- IWB Agonist & antagonist sensitivity/selectivity of the α3β4, α3β4α5 and α4β2 IWB assays were similar to that of conventional e-phys.
- IWB Sensitivity of α4β2 and α7 subtypes positive allosteric modulators was similar to conventional e-phys.
- α6/3 β2 $\beta3^{V273S}$ Cell line showed the expected high sensitivity to agonists. EC_{50} values were similar to conventional e-phys.
- α7 Subtype-selective, novel agonists and PAMs were identified in the Enzo Library.

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