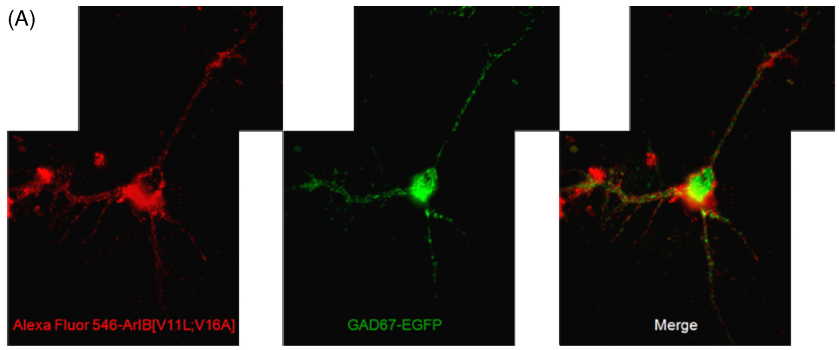

A, Cultured hippocampal neuron from the transgenic mouse strain FVB-Tg(GadGFP)45704Swn/J labeled with Alexa Fluor 546-ArIB[V11L;V16A] (red). The neuron was imaged live at 40x magnification using a cooled CCD camera. B, Cultured hippocampal neuron from Sprague Dawley rat showing neuronal processes labeled with Cy3-ArIB[V11L;V16A] (red) and immuno-labeled for synaptic vesicle protein-2 (green). Image was taken at 63x magnification using a cooled CCD camera.

Fig. 1.

1.10

Labeling α7 nAChRs on hippocampal neurons using fluorescent analogs of α-conotoxin ArIB[V11L;V16A]

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Hippocampal neurons are known to express several subtypes of nicotinic acetylcholine receptors (nAChRs). Among these, α4β2 and α7 are the most predominantly expressed subtypes. α7 nAChRs are expressed on several populations of neurons in the hippocampus but particularly on GABAergic interneurons where activation of α7 receptors induces the release of GABA. In the dentate gyrus of the hippocampus, presynaptic α7 receptors function at the mossy fiber-granule cell synapse to modulate glutamate release and thereby regulate granule cell activity. We evaluated the efficacy of two fluorescent derivatives of α-conotoxin ArIB[V11L;V16A] for detecting α7 nAChRs on cultured hippocampal neurons from mice and rats. ArIB[V11L;V16A] is a synthetic analog of a peptide isolated from the venom of the marine cone snail *Conus arenatus*. We conjugated ArIB[V11L;V16A] with two fluorescent dyes to produce Cy3-ArIB[V11L;V16A] and Alexa Fluor 546-ArIB[V11L;V16A]. Both fluorescent conjugates are ~1,500-fold more selective for α7 than for other nAChR subtypes as determined by functional studies of nAChRs heterologously expressed in *Xenopus laevis* oocytes. In addition, kinetic studies indicate that the binding of both conjugates is only slowly reversible. We used a combination of live-cell imaging and immunohistochemistry to evaluate the suitability of Cy3-ArIB[V11L;V16A] and Alexa Fluor 546-ArIB[V11L;V16A] for labeling α7 nAChRs. Hippocampal neurons from the transgenic mouse strain FVB-Tg(GadGFP)45704Swn/J that express EGFP as a reporter for glutamic acid decarboxylase-67 (GABAergic interneurons) were labeled with Alexa Fluor 546-ArIB[V11L;V16A] and imaged live. Neurons from Sprague Dawley rats were fixed, labeled with Cy3-ArIB[V11L;V16A], and stained with markers for either synaptic vesicle protein-2 (SV2) or postsynaptic density protein (PSD95). Labeling of neurons was observed using both fluorescent α-conotoxins and labeling was prevented by pre-incubation with α-bungarotoxin. The results demonstrate that Cy3-ArIB[V11L;V16A] and Alexa Fluor 546-ArIB[V11L;V16A] can be used to identify α7 nAChRs in cultured hippocampal neurons (Fig. 1).

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1.11

Radioligand binding characterization of [^{3}H]-A-998679: A novel positive allosteric modulator of α4β2 nAChRs

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Positive allosteric modulators (PAMs) have recently been identified that enhance responses of the α7 nAChR and other downstream events. Similarly, selective PAMs have also been identified for α4β2 nAChRs that do not possess intrinsic activity at the receptor on their own but potentiate the effects of agonists such as acetylcholine or nicotine. A-998679 is a close analog of NS-9283 (A-966933) which potentiates agonist responses at α4β2, but not at other heteromeric receptors. To further elucidate the interaction of this PAM with α4β2 nAChRs, A-998679 was radiolabeled. The present study characterized the ability of [^{3}H]-A-998679 to bind to native and recombinant nAChR α4β2 receptors. In membrane preparations

from HEK-293 cells expressing ferret α4β2 nAChRs, [^{3}H]-A-998679 bound to a high affinity site with a K_d of 2.8 nM and a B_{max} of 6405 fmol/mg. Unlabeled A-998679 (and related analogs) displaced binding with a K_i value of 7 nM. Association and dissociation curves were monophasic, with extremely fast on-rate and relatively slow off-rate. We also evaluated binding interactions using membranes from native tissues. In membranes prepared from human frontal cortex, [^{3}H]-A-998679 showed saturable binding with a K_d of 60 nM and a B_{max} of 2900 fmol/mg protein. However, specific binding was relatively poor in rat membranes, which bound [^{3}H]-cytisine with high affinity—the basis of this difference remains to be elucidated. In summary, our studies demonstrate, for the first time, that [^{3}H]-A-998679 is a relatively high affinity binding tool that may be useful in further examining interactions of PAM with α4β2 nAChRs.

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1.12

α3* and α7* nAChR mediated Ca^{2+} transient generation in neuroblastoma IMR-32 cells

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α3* and α7* nAChRs are members of cys-loop ligand gated ion channel family implicated in the control of intracellular Ca^{2+} signaling regulation. Both subunits are also expressed in human neuroblastoma IMR-32 cells. In this study, we investigated and compared the intracellular global Ca^{2+} transient generation evoked by selective activation of α3* and α7* nAChR pathways in IMR-32 cells using Ca^{2+} imaging (FLIPR), and examining the effects of various inhibitors (all tested at 10 μM except as noted) of ER Ca^{2+} ATPase pump (CPA and 1 μM thapsigargin), Ca^{2+} induced Ca^{2+} release (ryanodine and dantrolene), Ca^{2+} channels (nitrendipine, diltiazem, and 100 μM Cd^{2+}), nAChRs (100 nM MLA and mecamylamine), and removal of extracellular Ca^{2+}. The activation of α3* pathway was obtained by agonists with the following rank order of potencies (pEC_{50}): epibatidine (7.6) > varenicline (5.9) > nicotine (5.0) > cytisine (4.7) in a concentration-dependent manner. As reported previously [1], the addition of selective α7 agonists alone had no effect on basal Ca^{2+}. In the presence of an α7 PAM (A-867744 or PNU-120596), α7 agonists concentration dependently evoked Ca^{2+} transients with the following rank order (pEC_{50}): A-795723 (8.7) > NS6784 [2] (7.3) ≈ PNU282987 (7.2). The effects of various inhibitors on the α3* and α7* mediated Ca^{2+} transient generation were examined on the responses evoked by varenicline (10 μM) and NS6784 (1 μM + α7 PAM), respectively. Removal of extracellular Ca^{2+} and pre-addition of MLA, but not CPA, thapsigargin, ryanodine, dantrolene, nitrendipine, diltiazem, Cd^{2+} or mecamylamine, attenuated or diminished the α7* agonist evoked Ca^{2+} transients. In contrast, removal of extracellular Ca^{2+}, diltiazem, nitrendipine, and mecamylamine inhibited the α3* mediated Ca^{2+} transients. Other compounds tested: Cd^{2+}, CPA, thapsigargin, ryanodine, dantrolene, and MLA had no effect. The effects of the Ca^{2+} channel blockers were also examined in HEK-293 cells, lacking endogenous Ca^{2+} channels, expressing human α3β4 nAChRs by Ca^{2+} imaging and in IMR-32 cells by patch clamp. Nitrendipine and diltiazem, but not Cd^{2+}, directly inhibited α3* agonist evoked responses. In summary, this study shows that α3* and α7* nAChR agonist evoked global Ca^{2+} transient generation in IMR-32 cells does not involve Ca^{2+} channels, intracellular Ca^{2+} stores, or Ca^{2+} induced Ca^{2+} release. However, these mechanisms may still be involved in other forms of nAChR mediated Ca^{2+} signaling.

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1.13

Positive allosteric modulation of α7 neuronal nicotinic acetylcholine receptors: Lack of mechanism-based evidence for cytotoxicity in PC12 cells and rat primary cortical neurons

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α7 nicotinic acetylcholine receptors (α7 nAChRs) play an important role in cognitive function. Positive allosteric modulators (PAM) amplify effects of α7 nAChR agonists and demonstrate potential as an approach for treatment of cognitive deficits in neuropsychiatric diseases. PAMs can either predominately affect the apparent peak current response (type I) or increase both the apparent peak current response and duration of channel opening due to prolonged desensitization (type II). The delay of receptor desensitization by type II PAMs raises the concern about the possibility of Ca^{2+}-induced toxicity through prolonged activation of α7 nAChRs. The present study addresses whether type I PAM [N-(4-chlorophenyl)]-alpha-[(4-chloro-phenyl)-amino methylene]-3-methyl-5-isoxazoleacet-amide (CCMI) and type II PAM 1-[5-Chloro-2,4-dimethoxy-phenyl]-3-[5-methyl-isoxazol-3-yl]-urea (PNU-120596), or 4-[5-(4-Chloro-phenyl)-2-methyl-3-propionyl-pyrrol-1-yl]-benzenesulfonamide (A-867744) could reveal differential cytotoxicity profiles. Studies were conducted using in vitro cell culture models—PC12 and rat cortical neuronal cells expressing endogenous α7 nAChR. Our results showed that neither type I nor type II PAMs had any detrimental effect on cell viability or cytotoxicity. In particular, type II PAMs did not affect neuron number and neurite outgrowth under conditions when nAChR activity was measured by α7 ligand sensitive Ca^{2+} influx and ERK1/2 phosphorylation. This study demonstrated that both type I and type II α7 nAChR selective PAMs, although exhibiting differential electrophysiological profiles, do not exert cytotoxic effects in cells endogenously expressing α7 nAChRs.

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1.14

A novel nicotinic antagonist protects the function of hippocampal slices against neurotoxic organophosphates

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Our group described that cembranoids, cyclic diterpenoids, of marine or terrestrial origin are noncompetitive nicotinic antagonists. The tobacco cembranoid (1S,2E,4R,6R,7E,11E)-cembra-2,7,11-