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Positive allosteric modulators as an approach to nicotinic acetylcholine receptor- targeted therapeutics: advantages and limitations

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Abstract

Neuronal nicotinic acetylcholine receptors (nAChR), recognized targets for drug development in cognitive and neuro-degenerative disorders, are allosteric proteins with dynamic interconversions between multiple functional states. Activation of the nAChR ion channel is primarily controlled by the binding of ligands (agonists, partial agonists, competitive antagonists) at conventional agonist binding sites, but is also regulated in either negative or positive ways by the binding of ligands to other modulatory sites. In this review, we discuss models for the activation and desensitization of nAChR, and the discovery of multiple types of ligands that influence those processes in both heteromeric nAChR, such as the high affinity nicotine receptors of the brain, and homomeric α7-type receptors. In recent years, α7 nAChRs have been identified as a potential target for therapeutic indications leading to the development of α7-selective agonists and partial agonists. However, unique properties of α7 nAChR, including low probability of channel opening and rapid desensitization, may limit the therapeutic usefulness of ligands binding exclusively to conventional agonist binding sites. New enthusiasm for the therapeutic targeting of α7 has come from the identification of α7-selective positive allosteric modulators (PAMs) that work effectively on the intrinsic factors that limit α7 ion channel activation. While these new drugs appear promising for therapeutic development, we also consider potential caveats and possible limitations for their use, including PAM-insensitive forms of desensitization and cytotoxicity issues.

Keywords

Alzheimer’s disease; schizophrenia; drug development; electrophysiology; modeling

1. Introduction

Many drugs, like nicotine, were used socially or clinically long before their mechanisms of action were identified and understood. Now that we begin to understand the molecular substrates that mediate the effects of nicotine in the brain and on human behavior, we

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recognize that there is tremendous therapeutic potential for new agents targeting neuronal and somatic nicotinic-type receptors, all of which are ligand-gated ion channels activated by the neurotransmitter acetylcholine (ACh). In order to advance nicotinic receptor-based therapeutics, we now face the traditional challenges of drug development: optimizing drugs for receptor subtype specificity, potency, pharmacokinetic properties, and, most importantly, for every hypothetical indication we need to consider the optimal mechanism of action.

Numerous distinct nicotinic acetylcholine receptor (nAChR) subtypes can be identified based on subunit composition and stoichiometry [1], and for particular indications we identify broad classes of nAChR that can be classified as distinct pharmacological subtypes to be either targeted or avoided for the sake of reducing side effects. The first two classes of nAChR to be identified and pharmacologically characterized were the primary mediators of synaptic transmission outside the central nervous system (CNS), at neuromuscular junctions and autonomic ganglia. As drugs are being developed for CNS indications ranging from analgesia to schizophrenia, both of these peripheral nicotinic systems are potential sources for off-target effects. Most nAChR function in the CNS is dichotomized between two broad classes of nAChR: heteromeric (primarily α4-containing) and homomeric α7 receptors. Both of these receptor subtypes are most likely to be activated by ACh in the micromolar range. However, while α4* receptors initially open with high probability [2], they relax into high affinity desensitized states that resist further activation. In contrast, α7* nAChR open with very low probability, and, although they are profoundly desensitized by high concentrations of agonist, their desensitization is readily reversible [3].

One question at the core of therapeutic drug development is how a drug will affect the endogenous signaling mediated by the natural activator. The naïve assumption is that an agonist will replace or augment the stimulation provided by natural activator. However, for most nAChR subtypes a primary effect of the prolonged presence of an agonist is to produce desensitization, decreasing the effect of the normal fluctuations in ACh signaling [4]. In this regard, the effect of an agonist may be similar to that of an antagonist, as is the case with two drugs commonly used to paralyze the neuromuscular junction, succinylcholine (a potent neuromuscular receptor agonist), and tubocurarine (a competitive antagonist). Likewise, treatments for nicotine addiction include both partial agonists of α4* receptors, such as varenicline and the putative α4* receptor antagonist bupropion [5]. However, for this indication it is not the natural ACh-mediated signaling the drugs are intended to oppose, but the pulsatile stimulation of nAChRs delivered by cigarette smoking.

An important alternative to the therapeutic development of agonists has been the development of positive allosteric modulators (PAMs), which can synergize and augment natural signals rather than oppose or attempt to replace them, while potentially preserving the topological distribution and rhythm of those natural neurotransmitter-mediated signals. Since PAMs bind to sites away from well-conserved agonist binding domains, increased receptor subtype selectivity may be an additional advantage of allosteric modulators. A proven area of success for the therapeutic development of PAMs is in regard to sedative-hypnotic drugs, such as barbiturates and benzodiazepines targeting $GABA_A$ receptors, which belong to the same superfamily of ligand-gated ion channels as nAChRs [6]. Despite $GABA_A$ PAMs having been known and used clinically for decades, nAChR PAMs have only relatively recently been identified.

In this article we review new advances in the development of nAChR PAMs, potential therapeutic applications, and possible limitations for their use. We will discuss the fundamental differences between heteromeric and homomeric nAChRs in the context of receptor models and energy “landscapes”, discussing how PAMs may alter receptor function to promote either short-term or sustained levels of increased ion channel opening.

2. Basic principles of allosteric proteins and modulation

The term “allosteric” was first introduced following observations that bacterial enzymes were inhibited by the end product of synthetic pathways, even though the end product had limited structural similarities with the enzyme active site substrate [7]. The inhibition appeared to be non-competitive with substrate, which led to hypotheses that the non-competitive inhibitor produced conformational alterations in the protein [8] and the formulation of the well-known Monod, Wyman, Changeux (MWC) model of protein allostery [9–11]. The basic concept proposed that proteins are dynamic structures existing in multiple discrete functional states or conformations, all of which are accessible to the protein under resting conditions, and that binding of a ligand alters the resting equilibrium by reversibly stabilizing the protein in the conformation to which the ligand has greatest affinity. The conformation of each subunit was proposed to be constrained by the other subunits, and protein symmetry always conserved. Therefore, the binding of ligand at one site was predicted to alter the affinity of the other binding sites within the oligomer for the ligands. The model was soon applied to hemoglobin to describe the cooperative nature of oxygen binding, and to proteins involved in signal transduction, including membrane receptors as diverse as G-protein coupled receptors and ligand-gated ion channels, including nAChRs [10, 11].

The MWC concept of allosteric proteins has enjoyed widespread acceptance. Unfortunately, the use of the term “allosteric” to describe ligands and sites of ligand interaction has become somewhat ambiguous. The term “allosteric ligand” is commonly used to refer to any ligand that binds to any site on the receptor and has the effect of promoting a specific conformation [12]. Under this definition, with the exception of true channel blockers all ligands including agonists, antagonists, and modulators are probably “allosteric”. However, the term “allosteric ligand” is also commonly used to describe ligands that modulate or regulate the protein through sites that are distinct from traditional agonist binding sites in protein receptors [10, 11]. The terms “orthosteric” and “allosteric” are sometimes used to differentiate between canonical agonist binding domains and sites located elsewhere from which ligands positively or negatively modulate the efficacy or potency of ligands binding to the primary agonist binding sites on the receptor. These terms are basically inaccurate, but can be useful to distinguish agonists from modulators so long as it is appreciated that both types of ligand may produce global changes in protein conformation that influence receptor function.

In the case of nAChR, the distinction can be made between the primary allosteric sites where the binding of agonists or partial agonists is both necessary and sufficient to produce ion conducting conformations of the receptors. In contrast, ligands binding to modulatory allosteric sites are unable to produce receptor activation on their own, but facilitate agonist-evoked responses by modifying energy barriers associated with transitions between functional conformations (see below). However, observations that some positive allosteric modulators may produce activation of $GABA_A$ [13] and α7 receptors [14] independent of traditional agonist, blur the functional distinction between agonist and modulator. Nonetheless, such observations are entirely consistent with the MWC model since an allosteric modulator would alter the resting distribution of functional states of the receptor, perhaps by enhancing the small probability of spontaneous openings in the absence of orthosteric agonist.

2.1. Models of heteromeric nAChR activation and desensitization

The functions of allosteric proteins, including nAChRs and other ligand-gated channels, are typically modeled under the theoretical framework of MWC allostery. Discrete functional states (i.e. protein conformations) are defined, with basic models of nAChR required to

account for resting closed states (C), open states (O), and desensitized states (D). There are sufficient data, from both heteromeric muscle-type and neuronal nAChR [15–17], to indicate the existence of two distinct open states which have similar ionic conductance, but different mean channel open times. In Figure 1 we represent these two open states as O*, short-lived openings that occur in isolation, and O′, longer openings that sometimes occur in bursts. Because the behavior of individual receptor molecules are stochastic in nature, typical Markov assumptions are made to describe the interconnection between these several states and may, with sufficient data, allow assignment of rate constants for the interstate conversions. A complementary perspective is provided by Eyring rate theory, which describes the relationships among the receptor states in regard to their respective free energy levels and intervening energy barriers. The heights of the energy barriers are inversely proportionate to the logs of the transition rate constants in standard presentations of Markov models.

In Figure 1 we show hypothetical energy landscapes that provide a qualitative description of key features associated with heteromeric nAChR, such as α4-containing neuronal nAChR. Heteromeric nAChR have two agonist binding sites and, therefore, can have three different levels of agonist occupancy, with 0, 1, or 2 agonist molecules bound. The relative stabilities and interconversion rates between the states depend on the level of agonist occupancy [10, 18]. Therefore, to clarify the presentation of these landscapes, we have separated the distinct profiles that would be associated with the different levels of agonist occupancy by a given agonist. In reality, the overall free energy of the unliganded condition is probably higher (more positive) than the average free energy corresponding to the singly liganded condition, which in turn is higher than the doubly liganded condition. For simplicity, the C state corresponding to each level of agonist occupancy is used to align the energy landscapes, and barriers between states are meant to represent the net transition, including any intermediate states such as the recently proposed flip state between resting closed and open states [19]. Recent work with tethered agonists has provided the additional perspective and complexity that within the orthosteric site a single ligand can bind in multiple orientations, each of which may be associated with a unique energy landscapes [20].

With no agonist molecules bound (left-most profile in Figure 1A), receptors are most stable in the resting closed state, with some equilibration between the resting closed and the desensitized states, and vanishingly low probability of channel opening (P_{open}). With a single agonist binding site occupied (middle scheme, Figure 1A), there is an increased probability of channel opening, particularly to the brief O* state. Early studies that first identified the two distinct open states associated the short-lived events with singly liganded receptors [17], and it was unclear from those studies whether or not singly liganded receptors could also open to the longer-lived state. When experiments were conducted at increasing concentrations of agonist, the frequency of long-lived openings increased, essentially as the probability of having doubly bound receptors increased. Using conditional elimination of single agonist binding sites in muscle-type receptors, we have recently shown [21] that while singly bound receptors are most likely to open to the O* state, they can also open to the O′ state. The probability of opening to the longer-lived O′ state from activation at one binding site increases under conditions of saturated agonist binding to that site. In addition, those experiments demonstrated that even singly bound receptors were ultimately most stable in the D state, as shown schematically.

Receptors with two ligands bound have a high probability of converting to the long-lived O′ open state, as represented in the right-most scheme in the Figure 1A. It has been reported [2] that following the rapid application of a high concentration of agonist, sufficient to saturate the binding sites and fully populate the resting closed state, α4β2 nAChRs have approximately an 80% probability of synchronously passing into open states prior to

reaching an equilibration which will ultimately favor the D state. Receptors with both agonist sites bound may also open to the O* state, but isolated brief openings are only about 10% as common as longer openings or bursts [17, 22]. Conversions occurring between the O* and O′ would be undetectable as unique events in single channel recordings and would add to the apparent open duration of O′ events.

The model as presented in Figure 1 assumes that the agonist binding steps proceed from a rapid change in agonist concentration and all receptors are initially in the resting closed state. Therefore, the affinity associated with the association and dissociation steps shown below the state-transition landscapes would correspond with the micromolar ACh potency for channel activation. However, it is known that agonists bind with much higher apparent affinity once the receptors have relaxed into the low energy D state sometime after the initial presentation of agonist. This could be modeled by assigning different association and dissociation rates to each of the states in the model. However, if dissociation rates from the O and D states are so slow that they approach zero, then the apparent high affinity of the receptors at equilibrium might be related to the slow conversion between the D and C states, with dissociation still occurring primarily from receptors in the C state.

2.2. Modeling potential mechanisms of positive allosteric modulators

Allosteric modulators may affect receptor function in any of several ways that could have the net effect of increasing channel activation under particular sets of conditions. One requirement for “true” positive allosteric modulators is that they are distinct from agonists, having no intrinsic channel activation properties. Therefore, we can consider PAM mechanisms in terms of modifying the normal receptor mechanisms discussed above.

One potential effect of a PAM would be to enhance agonist binding to the resting state of the receptor, increasing potency. This was proposed to be the mechanism for benzodiazepine enhancement of $GABA_A$ receptor function [23]. This particular form of modulation would be most effective at increasing responses to weak stimuli, with little or no effect on the response to strong stimuli, since the energy landscape under the conditions of receptor saturation would be unchanged. It is generally believed that the relatively large therapeutic index associated with benzodiazepines, compared to other $GABA_A$ modulators such as barbiturates, may be due to the fact that they do not produce more $GABA_A$ receptor activation than would be created by high concentration of GABA alone.

Positive allosteric modulators are commonly observed to increase agonist efficacy, at least in regard to the peak current evoked by an agonist application. Two ways they might potentially do so are illustrated in Figure 1B as alternative energy landscapes for the doubly liganded condition. In the scheme on the left, the energy barrier from C to O′ is reduced, allowing for faster opening after a rapid increase in agonist concentration. The energy barrier from O′ to D is increased, which would have the effect of increasing mean channel open time and the probability of multiple openings prior to desensitization. These alterations in the energy profile would produce a transient increase in P_{open}, conditional on all receptors initially being in the C state. Note, however, that in this scheme the absolute disposition of the four states is unchanged from the initial condition in Figure 1A. Therefore, even with these changes to the energy profile, the receptors would ultimately relax to the same equilibrium conditions, strongly favoring occupancy of the D state.

The scheme on the right in Figure 1B allows for increased P_{open} by destabilizing the D state relative to the other states in the model. As drawn, this would primarily be associated with increasing the exit rates out of the D state. A PAM working through this mechanism might have a relatively small effect on the peak transient current evoked by a rapid agonist application, but would have a large effect under equilibrium conditions, producing a

significant amount of steady-state current. However, since, as mentioned above, α4-containing nAChRs naturally open with high probability upon stimulation with ACh, the potential range for enhanced activation of these receptors may be limited.

The two hypothetical energy landscapes in Figure 1B represent mechanisms that could produce either transient or steady-state potentiation, and both assume that the PAM effects are on the same population of receptors that are functional in the absence of the PAM. However, it has been reported in some systems that there are two pools of surface nAChRs and that only a small percentage (10–20%) are capable of being activated at all [2, 24]. Hypothetically, PAMs might also work by switching receptors from the non-activatable pool into the activatable population. It may be especially important to consider this possible mechanism for agents that are known to have potential effects on cellular signal transduction, such as genistein, a tyrosine kinase inhibitor and an α7 potentiator [25].

2.3. Models for the unique properties of homomeric α7-nAChR and potential mechanisms for α7 PAM activity

The hypothetical energy profiles illustrated for heteromeric nAChR in Figure 1A are unlikely to be applicable to homomeric α7 nAChR. Since α7 nAChR do not require specialized subunits to provide the complementary surface of the ligand-binding domain, there are five putatively equivalent binding sites per receptor rather than the two likely nonequivalent sites in heteromeric nAChR [26]. Additionally, α7 nAChR manifest a unique "fast" desensitized state, entry into which appears to be concentration-dependent, suggesting that the state is stabilized by high fractional occupancy of the agonist binding sites [27]. Figure 1C illustrates a series of hypothetical energy landscapes for α7 nAChR as related to the level of binding site occupancy by a full agonist.

Key features of α7 activation and desensitization are consistent with the model in Figure 1C. The P_{open} of α7 receptors is very low under all conditions, and open times are very brief, usually less than 100 μs [3, 28]. These features are associated with the steep barrier for entry into the open state, which is located in a very shallow trough. In this model, we present the rapid form of desensitization unique to α7 as the D_s state and propose that it is analogous to the O′ state of heteromeric nAChR. The D_s state is more stable than the short-lived O* state, and it is most readily entered into with high levels of agonist occupancy. The true intrinsic desensitized state (D_i in Figure 1C) of α7 receptors is less stable than that of heteromeric nAChR, and so the absolute free energy difference between the D_i and C states at high levels of agonist occupancy is less for α7 than for the heteromeric nAChR modeled in Figure 1.

All of the general mechanisms for the potentiation of nAChR discussed above and illustrated in Figure 1 might be applied to α7-selective PAMs. Additionally, as will be discussed below, the intrinsically low P_{open} of α7 greatly broadens the range for possible potentiation of this important therapeutic target and suggests that these PAMs may draw on unique mechanisms. However, since α7 is a receptor with high calcium permeability, it must also be considered whether extreme increases in α7 channel activation may lead to unexpected, and potentially undesirable, effects. For this reason, drug development in this area benefits from the generation of compounds with widely different activity profiles and depends on the testing of such compounds in a variety of appropriate model systems.

3. Known allosteric modulators of heteromeric nAChR

Several ions and molecules have been identified to potentiate agonist-evoked responses of heteromeric nAChRs. However, the development of PAMs with activity on heteromeric nAChRs has developed slowly relative to the identification and development of α7 PAMs. In general, the known heteromeric receptor PAMs produce only modest potentiation and are

poorly selective for specific heteromeric receptor subtypes. In addition, several PAMs of α7 nAChR actually have negative effects on α4β2 and α3β4 mediated responses (see below).

Calcium ions were shown to allosterically potentiate nAChR-mediated responses from heteromeric α2-, α3-, and α4- containing receptors expressed heterologously and in native tissues including the medial habenula, chromaffin cells, and ganglion neurons [29–31]. Responses were potentiated by low millimolar concentrations (< 10 mM) of calcium, which are considered to be physiologically relevant, up to about 4-fold in a voltage-insensitive manner that depended on agonist concentration and extracellular, not intracellular, calcium [30]. Although at millimolar concentrations of external calcium single-channel conductance was decreased, increased frequency of single-channel openings, with little to no effect on open durations, was found to account for the potentiation [29, 31]. In habenular neurons, similar potentiation was observed with barium and strontium ions, but not magnesium ions [29], while in α3β4 receptors expressed in *Xenopus* oocytes, neither barium nor magnesium ions potentiated responses [30].

Zinc ions, which are found in high concentrations in many brain regions and are key functional components of many proteins, were shown to potentiate α2-, α3-, α4- containing nAChR in a biphasic nature, with potentiation occurring between 1 to 100 μM and inhibition occurring at higher concentrations. Responses of α4β4 receptors were potentiated about 5-fold with more modest potentiation observed on α2β4, α2β4, α3β4, and α4β2 receptors. Zinc did not potentiate but rather inhibited α3β2 receptors with an IC_{50} of ~100 μM. Both potentiation and inhibition of responses by zinc were voltage independent [32]. Similar to its effects on α3β2 receptors, ionic zinc inhibited α7 mediated responses with an IC_{50} of 27 μM in a voltage-insensitive manner [33]. With site-directed cysteine mutations and reactive sulfhydryl reagents, residues E59 and H162 on the α4 subunit were found to be critical for potentiation by zinc, potentially forming part of the zinc binding site at subunit interfaces not containing the orthosteric site [34]. Potentiating effects of lead ions were observed on α3β2 receptors at concentrations between 1 to 250 μM, while similar concentrations inhibited responses by α3β4 and α4β2 receptors [35].

Naturally synthesized steroids (i.e. progesterone, testosterone, estradiol, corticosterone) were shown to exert allosteric effects on heteromeric α4-containing and α3-containing nAChR, the effects however, are generally inhibitory at low μM concentrations [36–39]. One exception is 17β-estradiol, which has been found to potentiate human, but not rat, α4β2 and α4β4 responses about 3–4-fold with no potentiation of responses by α3β2 or α3β4 receptors [39–41]. Single-channel recordings from potentiated α4β2 receptors suggest that 17β-estradiol increases the frequency of channel openings, with little effect on unitary conductance [41]. Chimeric α4 subunits and site-directed mutagenesis were used to identify a C-terminal region of the α4 subunit as critical for potentiation by estradiols [40, 41].

The protein lynx-1, which contains remarkable structural similarity to snake neurotoxins, is expressed by cerebellar, cortical, hippocampal neurons and was found to co-localize with neuronal nAChR and modestly increase peak responses of α4β2 receptors when applied in soluble form [42], but was shown to increase macroscopic current decay rates, reduce rate of recovery from desensitization, and decrease agonist potency when lynx-1 was co-expressed with α4β2 receptors in *Xenopus* oocytes and mammalian cells [43], and appears to be a mechanism used to negatively modulate α4β2 containing receptors [44]. In general, the modulatory properties of divalent cations, proteins, and steroids for nAChRs support the hypothesis that allosteric modulation is a natural mechanism controlling the function of endogenous nAChRs [45].

The anthelmintic agents levamisole and morantel are full agonists at nematode nAChR, but have been found to allosterically potentiate human α3-containing receptors expressed in *Xenopus* oocytes [46, 47]. Micromolar concentrations of levamisole were found to potentiate agonist-evoked responses of α3β2 and α3β4 receptors about 4-fold, while millimolar concentrations significantly inhibited responses, likely due to open channel block [46]. Low micromolar concentrations (≤10 μM) of morantel potentiated ACh-evoked responses on α3β2 up to 8-fold primarily by increasing the frequency of single-channel openings and the duration of bursts, rather than increasing the unitary conductance or altering macroscopic response decay kinetics [47]. Although both levamisole and morantel produce weak activation of human α3-containing receptors, they appear to bind away from the orthosteric site since inhibition by DHβE is noncompetitive [46, 47]. Evidence that morantel binds in the N-terminal extracellular region at subunit interfaces not containing orthosteric sites was provided by experiments utilizing site-directed cysteine mutations and reactive sulfhydryl reagents [48].

The acetylcholinesterase inhibitor galantamine has been shown to potentiate α4β2, α3β4, and α6β4 nAChRs at concentrations between 0.1–1 μM, and at concentrations >10 μM galantamine was found to inhibit responses [49]. However, it has recently been reported that galantamine potentiation of α4β2* nAChR required the presence of α5 subunits [50]. At low concentrations, galantamine was shown to increase the frequency of single-channel openings of nAChR receptors expressed in PC12 cells [51]. The maximal potentiation produced by galantamine on heteromeric receptors is modest. Nonetheless, some have speculated that the therapeutic actions of galantamine are primarily produced through the compound's action on nAChRs, since the cholinesterase activity of galantamine is also weak compared to other known cholinesterase inhibitors [49].

Three (2-amino-5-keto)thiazole compounds (LY-2087101, LY-1078733, LY-2087133) were identified in a high throughput screen as PAMs of nAChRs [52]. Although non-selective for a single nAChR subtype, the compounds do show good selectivity against other ligand- and voltage-gated ion channels. Agonist-evoked peak responses of α2β4, α4β2, α4β4, and α7, but not α1β1γδ, α3β2, or α3β4, were potentiated from ~2-fold up to ~20-fold with concentrations < 10 μM, and inhibited with concentrations > 10 μM. These compounds showed the greatest potentiation of α4β2 mediated responses from receptors expressed in *Xenopus* oocytes. All three compounds were unable to displace [^{3}H]epibatidine from α4β2, α4β4, and α3β4 receptors, and potentiation was unaffected by blockade of a number of intracellular second messenger systems, suggesting these compounds work directly on the receptors through allosteric mechanisms [52]. In the ventral tegmental area, LY-2087101 enhanced nicotine-dependent increases of dopamine neuron firing in a DHβE sensitive manner, suggesting the effect was mediated through potentiation of α4β2 nAChR [53].

Desformylflustrabromine (dFBr) was identified as a PAM of α4β2 nAChR from screens of compounds isolated from *Flustra foliacea*, a bryozoan commonly found in the North Sea. Low micromolar (< 10 μM) concentrations of dFBr modestly potentiate (~2–3 fold) agonist-evoked currents from human α4β2 expressed in *Xenopus* oocytes, while higher concentrations inhibit responses, likely through open-channel block [54, 55]. No potentiation was observed for α7 or α3β4 receptors [56], and in fact dFBr was shown to inhibit α7 responses with an IC_{50} of 44 μM [55]. Recordings of single-channel currents reveal that dFBr potentiates responses of α4β2 by increasing the frequency of openings and modestly increasing the open durations. In addition, dFBr was reported to activate desensitized receptors [54]. dFBr is one of the few known compounds capable of discriminating α4β2 from other nAChR subtypes to produce selective potentiation of responses. Initial screens of a series of carbamates [57] and a series of piperidines [58] have

suggested the discovery of other novel molecules that selectively potentiate α4β2 mediated responses with good potency, but these compounds have not yet been fully characterized.

4. Positive allosteric modulation of α7 nAChR

Much of the preclinical data with nicotinic ligands provide positive support for selective stimulation of α7 nAChR as a therapeutic strategy to treat cognitive deficits in Alzheimer's disease and schizophrenia [59–61]. In addition, the α7 gene has been linked to sensory gating deficits observed in schizophrenia [62, 63], and α7 agonists have been shown to normalize sensory gating deficits in animal models (for example, [64, 65]), as well as in schizophrenic humans [66].

Positive allosteric modulators selective for α7 share some of the putative therapeutic activities ascribed to α7 agonists, including enhancement of memory-related behavioral performance in the eight-arm radial maze, social recognition, and Morris water maze tasks [67, 68], and normalization of sensory gating deficits [67, 69–72]. As noted above, PAMs of α7 have special appeal because they overcome the limitations associated with the intrinsically low P_{open} and rapid desensitization of α7 nAChR, potentially with significant benefit for processes that depend on channel activation [73].

Calcium ions were the first known allosteric potentiators of α7 responses [74], followed by the discovery of the antiparasitic agent ivermectin [75], the serotonin metabolite 5-hydroxyindole (5-HI; [76, 77]), the acetylcholinesterase inhibitor galantamine [78], serum albumin peptides [79], and secreted mammalian Ly-6/uPAR related protein 1 (SLURP-1 [80]). In addition, the tyrosine kinase inhibitor genistein augments α7 responses, and some evidence suggests it functions directly on α7 as a PAM [25, 81], but other evidence suggests an indirect mechanism through kinase inhibition [82]. Unfortunately, these "first generation" α7 PAMs lack desirable potency, efficacy, and/or selectivity profiles. For example, ivermectin is non-selective [83–86]; galantamine exhibits poor efficacy as a PAM, is non-selective [49, 87], and works through a duel mechanism as an acetylcholinesterase inhibitor; and 5-HI has low potency and also potentiates 5-hydroxytryptamine$_3$ (5-HT_3) receptors [88].

Since the discovery of the first α7 modulators, small molecule PAMs with diverse potency, efficacy and/or selectivity profiles have emerged (Figure 2, Table 1). These include 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea (PNU-120596) [69, 89], 2-(amino-5-keto)thiazole compounds (e.g. LY-2087101) [52], 1-(5-chloro-2-hydroxy-phenyl)-3-(2-chloro-5-trifluoromethyl-phenyl)-urea (NS-1738) [68], N-(4-chlorophenyl)-alpha-[[(4-chloro-phenyl)amino]methylene]-3-methyl-5-isoxazoleacet-amide (compound 6, also known as CCMI) [67], 4-naphthalene-1-yl-3a,4,5,9b-tetrahydro-3-H-cyclopenta[c]quinoline-8-sulfonic acid amide (TQS) [81], 3,5-dihydro-5-methyl-N-3-pyridinylbenzo [1,2-b:4,5-b′]-di pyrrole-1(2H)-carboxamide (SB-206553) [71], 4-(5-(4-chlorophenyl)-2-methyl-3-propionyl-1H-pyrrol-1-yl)benzenesulfonamide (A-867744) [70, 90], 2-[[4-fluoro-3-(trifluoromethyl)phenyl]amino]-4-(4-pyridinyl)-5-thiazolemethanol (JNJ-1930942) [72], and a series of amide derivatives (compounds 2–4) [91]. While these "second generation" PAMs generally provide a range of potencies and efficacies useful to consider for therapeutic development, some still suffer from lack of pure α7-selectivity. For example, CCMI was discovered from a library of $GABA_A$ PAMs [67], SB-206553 is a 5-$HT_{2B/C}$ receptor antagonist [71], and LY-2087101 (and analogues) potentiates responses at several nAChR subtypes [52]. In addition, NS-1738 [68], TQS [81], SB-206553 [71], A-867744 [90], and compounds 2–4 [91] have been shown to inhibit responses of α3β4 and α4β2 nAChR.

The identified α7 PAMs have considerable diversity, ranging from proteins to small molecules. Even the small molecule PAMs vary significantly in structure, as well as in

properties and probably mechanisms of the modulation [92]. Gronlien *et al*., 2007 proposed that α7 PAMs be divided into two classes, type I and type II, based on the functional properties of modulation. Following the rapid application of agonists, all PAMs appear to increase receptor sensitivity to agonists, current magnitudes, and empirical Hill coefficients; the type I PAMs (for example 5-HI, NS-1738, or CCMI) do so with little or no effect on the basic onset and decay kinetics, or shape, of the response, while the type II PAMs (for example PNU-120596, TQS, or A-867744) markedly slow response decay kinetics and can even activate receptors that have been desensitized by applications of high agonist concentrations or by application of agonists like GTS-21 (3-(2,4-dimethoxybenzylidene)-anabaseine) that produce residual inhibition or desensitization [14, 67, 69, 81, 90, 93]. An observation yet to be explained that is sometimes seen with responses potentiated by type II PAMs is an initial biphasic current which rapidly rises and decays, followed by the emergence of a distinct secondary component. Low concentrations of PNU-120596 and TQS produce such responses, but at higher PAM concentrations the two components merge [81, 90, 94]. Responses potentiated by A-867744 apparently do not show the secondary component at low or high concentrations [90]. Since the two classes of α7 modulators were proposed, additional PAMs (Figure 2) have been identified that display properties intermediate to the type I and type II classes [71, 72, 94].

4.1. Structures, binding sites, and mechanisms of α7 PAMs

As noted above, the potential mechanisms for α7 PAMs include the same mechanisms that might apply to the activation of heteromeric nAChR (Figure 1B) and, additionally, unique effects associated with the special (PAM-sensitive) D_s state of α7 nAChR. Specifically, modulation of the α7 D_s state appears to underlie the extremely effective modulation produced by type II PAMs such as PNU-120596. One question that arises is whether type II PAMs stabilize functional states intrinsic to the receptor or create new conducting states. PNU-120596 has no effect on ion selectivity and little, if any, effect on channel conductance [69], supporting the interpretation that PNU-120596 stabilizes intrinsic states of the channel.

In the absence of modulators, it may be the case that wild-type α7 receptors can only activate in a manner similar to heteromeric receptors with a single occupied binding site, that is, primarily to the short-lived O* state. We hypothesize that an effect of type II PAMs is to convert the D_s state of α7 nAChR to a functional homolog of the O′ state of heteromeric nAChR (Figure 1C). While the cooperative effects of a second binding event help to promote additional conformational change associated with the long-lived O′ state in heteromeric receptors, the analogous cooperative effects may be negative rather than positive in α7, effectively promoting the unique D_s desensitized state [27]. It is interesting to note that the α7 L248T (also known as L9′T) gain-of-function mutation has also been reported to slow or eliminate the unique form of α7 desensitization. The change in structure related to the binding of a type II PAM or to the L248T mutation in the transmembrane (TM) domain, may have the effect of reversing the negative cooperativity related to the effect of multiple ligand binding in α7.

As shown in Figure 2, the α7 PAMs are constituted of several aromatic rings, hydrogen bonding donors or hydrogen bonding acceptors, which are oriented differently in space. All of the PAMs are neutral (uncharged) under physiological pH except galantamine, consistent with the hypothesis that this molecule has a unique binding site near the orthosteric site for agonists [95]. Several of the known α7 PAMs contain unsymmetrical aryl ureas or amides (CCMI, NS-1738, PNU-120596, and SB-206553), a commonality which has been applied in pharmacophore *in silico* screening methods to identify novel PAMs [96].

Although the specific orientation of the pharmacophore is likely a key deciding element of a modulator’s potentiation profile, there appear to be no strict governing rules. Even the type I

modulators CCMI, 5-HI, and LY-2087101 may interact with the receptor differently, solely because of the different hydrogen bonding and hydrophobic group orientation, as shown in a solvent density guided docking study [97]. However, this study evaluated PAM binding in the ligand binding domain, which is an unlikely binding site for most allosteric modulators. Although pharmacophore orientations among individual PAMs appear variable, there are some common elements shared between the two classes of of PAMs on the electrostatic potential surface. For example, the type I modulator LY-2087101 and the typeI/typeII modulator JNJ-1930942 share the 2-(4-flurophenylamino)-thiozolyl group. Perhaps, the differences in their effects on the α7 desensitized states may arise from the dissimilar orientations of the misaligned aromatic rings (shown in Figure 3A). As shown in Figure 3B, the type I PAM NS-1738 has a unique electron deficit on the hydroxyl group (typical of hydrogen bond donors), while PNU-120596 features the electron rich isoxazole ring (suggesting function as a hydrogen bond acceptor or having a role in dipole-dipole interactions). We speculate that type I PAMs prefer hydrogen-bonding donors on aromatic rings, while type II PAMs favor electron-rich aromatic rings without hydrogen-bonding donors.

While the mechanisms of action of PAMs remain unknown, some progress is being made through introduction of point mutations, use of α7-5HT$_3$ chimeras, and molecular docking simulations to identifying locations on the receptor that are important for PAM function (Figure 3C–D). It is important to appreciate that alteration of PAM function by point mutations does not necessarily indicate the site of ligand interaction. The use of the *Caenorhabditis elegans* α7 homolog ACR-16, which is insensitive to potentiation by ivermectin, 5-HI, genistein, NS-1738, and PNU-120596, has been suggested as a complementary approach to use of α7/5-HT$_3$ chimeras to identify α7 domains that are critical for allosteric potentiation [98].

In general, PAMs have little to no effect on equilibrium binding of radiolabeled α7 competitive antagonists or high-affinity agonists in membrane preparations from rodent brain or heterologous expression systems [67, 68, 71, 72, 90, 91, 99], supporting the concept that PAMs bind in a non-competitive manner away from the traditional agonist binding site. However, while A-867744 was unable to displace [^{3}H]methylcaconitine (MLA) binding, it displaced the high affinity α7 agonist [^{3}H]A-585539 ([(1S,4S)-2,2-Dimethyl-5-(6-phenylpyridazin-3-yl)-5-aza-2-azoniabicyclo[2.2.1]heptane]) from rat brain membranes (K_i ~23 nM), while PNU-120596, TQS, and 5-HI did not displace either ligand under the same conditions [90]. The ability of A-867744 to displace [^{3}H]A-585539 binding was lost in an α7/5-HT$_3$ chimera, suggesting A-867744 may interact away from the extracellular regions of α7 and has the ability to induce or stabilize significant conformational changes in the α7 orthosteric site from a distance [90]. Further support that modulators influence protein conformation is provided in a study which compared effects of ACh and PNU-120596 on thiol modification rates at cysteine residues introduced in the inner beta sheet, transition zone, and agonist binding site [100]. Within the (non-conservative) L248T mutant background, ACh and PNU-120596 were found to produce similar changes in cysteine accessibility, suggesting that PNU-120596 may induce or stabilize conformational changes that are similar to those of the channel gating process. This study also exploited a mutant receptor, α7C116A/L248T/W149C, which responded to PNU-120596 alone, to show that sulfhydryl modification at W149C greatly reduced ACh-evoked currents with little effect on PNU-120596 evoked currents, providing further support that PNU-120596 binds at a non-orthosteric site [100]. A similar study provides evidence that divalent cation potentiators also produce conformational changes via modulatory allosteric sites [101].

Putative allosteric modulator binding sites of GABA$_A$ receptors have been identified in the N-terminal extracellular domains at subunit interfaces not forming orthosteric agonist

binding sites (benzodiazepine binding site) and in the transmembrane region (barbiturate, alcohol, neurosteroid, general anesthetic binding sites [102–106]). This information, along with the differing functional profiles of type I and type II PAMs may be seen as consistent with the hypothesis that multiple allosteric sites also exist on α7. The site where calcium binds to potentiate responses on α7 was localized to the N-terminal extracellular domain [74, 107]. In addition, the crystal structure of galantamine bound to acetylcholine binding protein [95], mutagenesis studies [108], and computer docking simulations [97, 109] provide further evidence for nAChR allosteric sites in the extracellular domain, some of which may be related to benzodiazepine binding sites on $GABA_A$ receptors [95, 110]. Using α7/5-HT_3 chimeras, the N-terminal extracellular domain of α7 has been shown to be sufficient for potentiation by the type I PAMs NS-1738 [111] and 5-HI [112], but not the type II PAM PNU-120596 [111]. In addition, PNU-120596, but not NS-1738, potentiated currents (ACh-evoked) in a reverse 5-HT_3/α7 chimera containing α7 TM regions [111]. These studies also provide evidence that the extracellular α7 M2–M3 loop is implicated in potentiation by genestein and NS-1738 [111, 112].

Other studies using α7/5-HT_3 chimeras suggest that TM1–TM3 regions are critical for potentiation by PNU-120596 and the type I modulators LY-2087101 and ivermectin [113, 114]. Furthermore, mutations at several α7 amino acid residues, which are hypothesized to contribute to an intrasubunit cavity within the four transmembrane domains, significantly reduced potentiation by PNU-120596, LY-2087101 [113], and ivermectin [114]. Through site-directed mutagenesis, these studies together identify potentially important differences in modulator interactions with the receptors. For example, the A225D mutation reduced potentiation by PNU-120596 significantly more than it reduced potentiation by LY-2087101 [113] and the S276V mutation had no effect on potentiation by PNU-120596 [113], but conferred inhibitory properties to ivermectin [114]. Based on their work with α7/5-HT_3 chimeras, mutagenesis studies, blind computer docking simulations, and evidence that an intrasubunit transmembrane site appears to be important for potentiation of glycine and GABA receptors [103, 115–118], Young *et al*., 2008 proposed the intrasubunit cavity is a highly conserved modulatory site of Cys-loop ion channels [113], a hypothesis which has been supported by others [98, 103]. The fact that CCMI was discovered from a library of $GABA_A$ PAMs (that bind away from the benzodiazepine site), provides further support for the existence of conserved allosteric sites and/or mechanisms [67]. The findings that the extracellular domain appears to be sufficient for potentiation by some type I PAMs (NS-1738 and 5-HI), but not others (ivermectin and LY-2087101) suggest that more than one mechanism may produce the type I potentiation profile, and perhaps the same applies to the type II profile.

Kinetic models of ligand-gated ion channels and MWC allosteric theory predict that binding of agonist and channel conformational state (i.e. gating) are tightly coupled and exercise mutual influence through the principle of reciprocity [12]. Although by definition, PAMs generally have no intrinsic ability to open the ion channel, they do alter the distribution of receptors across conformational states [90, 100, 101, 103, 112], and basic allosteric theory predicts that binding of agonists at orthosteric sites facilitates binding of PAMs at allosteric sites, and vice-versa [103, 119]. Some experimental evidence is beginning to emerge that is consistent with this prediction. For example, the potency of the agonist PNU-282987 (N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide) to evoke calcium responses was increased with higher concentrations of PNU-120596, and, vice-versa, the potency of PNU-120596 to potentiate responses was increased with higher PNU-282987 concentrations in IMR-32 cells [99]. In our own studies, we have observed that the onset of potentiation by PNU-120596 is dependent on agonist concentration for both α7 nAChR-expressing *Xenopus* oocytes and outside-out patches from cultured mammalian cells (manuscript in preparation). In addition, computer docking simulations predicted that binding of either PNU-120596 or

LY-2087101 to the proposed TM intrasubunit cavity has lower free energy (higher affinity) in an “open channel” model than in a “closed channel” model [113].

However, 10 μM JNJ-1930942 had no effect on the equilibrium binding of the high-affinity α7 agonist [^{3}H]A-585539 to membranes from GH4C1 cells expressing human α7 [72]. In addition, JNJ-1930942 does not appear to significantly increase choline affinity, as measured by shifts in the IC_{50} of MLA in the absence (~1 nM) or presence (~2.3 nM) of 5 μM JNJ-1930942 [72]. Likewise, there were no significant effects of PNU-120596 on the binding of the α7 agonist ABT-107 or the antagonist MLA as revealed by the displacement of [^{3}H]A-585539 in rat cortical homogenates [120]. Conclusions regarding changes in agonist affinity are hard to interpret in these experiments since binding is determined only at equilibrium. The degree to which antagonist affinity is affected by modulator is unknown, and the degree to which antagonists are purely competitive is unknown.

4.2. Demonstrated effects of PAMs with relevance to biological systems

Positive allosteric modulators have been demonstrated to potentiate many types of α7-mediated responses that may be important within biological systems. For example, extracellular signal-related kinase-1 and -2 (ERK1/2) phosphorylation was enhanced in PC12 cells by A-867744 [120] and PNU-120596 [120–122] with several structurally diverse agonists. Several studies have provided evidence that activation of ERK1/2, the prototypical mitogen activated kinase, is important for α7-mediated protection from death in PC12 cells [121–125] and have implicated ERK1/2 in cognitive functions [125–129].

Several PAMs, including 5-HI, PNU-120596, SB-206553, and A-867744, enhance agonist-evoked α7 responses from hippocampal CA1 stratum radiatum interneurons [69, 71, 90, 130, 131] and glial cells [132] in acute brain slices. Spontaneous and choline- or ACh-induced increases in GABAergic inhibitory post synaptic currents were enhanced by 5-HI [133, 134], PNU-120596 [69], LY-2087101 [53], and A-867744 [90] in hippocampal neurons. JNJ-1930942 increased the amplitude of excitatory post-synaptic potentials in hippocampal dentate gyrus [72]. In cerebellar slices, 5-HI enhanced ACh-induced frequency increases of excitatory post-synaptic currents mediated by glutamate [77]. PNU-120596 (10 μM) potentiated α7 induced increases in [^{3}H]-D-aspartate release from prefrontal cortex synaptosomes, as well as [^{3}H]-dopamine release from prefrontal cortex in vitro and *in vivo* [135]. The nicotinic facilitation of long-term potentiation was enhanced by PNU-120596 [136] and JNJ-1930942 [72] in rat dentate gyrus. Physiological concentrations of choline (~10 μM) and 1–5 μM PNU-120596 activated α7-containing receptors in tuberomammillary neurons and hippocampal CA1 pyramidal neurons sufficiently to depolarize a cell and facilitate the firing of action potentials [137, 138].

Supporting the potential therapeutic significance of these in vitro assays, NS-1738 [68] and CCMI [67] have been shown to enhance performance in behavioral measures of cognitive function; and CCMI [67], PNU-120596 [69, 70], SB-206553 [71], A-867744 [70], and JNJ-1930942 [72] have been shown to reverse auditory gating deficits in drug-induced or DBA/2 models with systemic administration to rodents, indicating that these PAMs have sufficient pharmacokinetic properties to modulate brain α7 *in vivo* and that a sufficient level of endogenous agonist is present in relevant brain regions. Pharmacokinetic studies generally suggest that brain concentrations of PAMs are low compared to the EC_{50} for potentiation determined *in vitro* [67, 68, 70, 72]. However, since α7 has an intrinsically low P_{open}, even modest potentiation of α7-mediated signals may be sufficient to produce significant in vivo effects.

A recent study has provided evidence that simultaneous modulation of α5 $GABA_A$ and α7 nicotinic receptors may function to synergistically enhance long-term potentiation in rodent

brain slices and memory-related behavior *in vivo* [139]. This study provides proof-of-concept evidence that molecules engineered to modulate multiple targets might provide an optimized approach for specific therapeutic purposes.

4.3. Limiting factors of allosteric modulation as a therapeutic approach

Despite having identified and initially characterized several α7 PAMs, our current knowledge of the limiting factors for PAM-based therapy and the most desirable functional characteristics of PAMs as therapeutic agents is incomplete and many questions persist unanswered. For example, are there different conditions when either a type I or type II PAM might be most advantageous? To what degree might a type II PAM disturb native temporal characteristics of a neuronal circuit? Can a PAM alter the native channel kinetics and still provide an acceptable therapeutic index? Are the most efficacious PAMs most desirable? Can α7 PAMs induce cytotoxicity due to high levels of channel-mediated calcium flux? What are the limiting factors of allosteric potentiation, and under what condition is the potentiation optimized? Does the ability of a PAM to modulate responses change over significant amounts of time? Are the known α7 PAMs sufficiently selective to avoid undesired effects? Some of these questions have begun to be answered, but clearly, our search for understanding and identifying characteristics of the ideal α7 PAM continues.

If PAM therapy is going to work, obviously the endogenous agonist must be present in sufficient concentrations and able to evoke responses that the allosteric modulator can potentiate. Under conditions of degeneration in the cholinergic system, as in Alzheimer's disease, the question of whether sufficient agonist levels are present for effective modulation arises. Alternatively, under conditions of trauma, choline and possibly ACh concentrations may become unregulated [140, 141] potentially resulting in over-stimulation if PAMs were present. There are some instances when stimulation of α7, and especially potentiation, may be undesirable. For example, activation of α7, particularly by nicotine, has been associated with cell proliferation, angiogenesis, and inhibition of apoptosis in some cancers, including non-small cell lung cancer and malignant pleural mesothelioma [142–144]. In addition, α7 has been shown to modulate aspects of immune system function, [145–147], and in immuno-compromised individuals potentiated activation of α7 may be more harmful than beneficial. Another cautionary perspective may come from the consideration that *in vitro* characterizations of PAMs performed at room temperature may not be entirely predictive of the behavior of the drugs *in vivo*. Despite several observations that α7 PAMs are effective *in vivo* [67–72] it has been suggested that the modulatory effects of NS-1738, PNU-120596, and SB-206553 may actually be reduced at physiological temperatures (John Dunlop, personal communication, also published in abstract form [148]).

Numerous articles in the literature state that type II PAMs reverse or eliminate desensitization, commonly citing references that show modulators both activate previously desensitized receptors and produce non-decaying currents in the presence of relatively high agonist throughout relatively prolonged applications [67, 69, 81, 90]. In addition, we have used large non-decaying steady-state currents generated in *Xenopus* oocytes with 60 μM choline and 10 μM PNU-120596 to demonstrate channel block by agonists [93] and to study properties of antagonists [149]. Interestingly, applications of the type II PAM TQS [81] or A-867744 [90] were indeed shown to re-activate receptors that had been desensitized by a high concentration of agonist and produced a non-decaying current on the time scale of the experiments. However, the peak of the potentiated current in the presence of either TQS or A-867744 was only about 50% of the peak response recorded in the absence of PAM, indicating that only a fraction of the receptor population was actually conducting current at any given moment. These and a few other published observations, along with our own unpublished observations (manuscript in preparation) indicate the existence of desensitized states which are nonconducting in the presence of type II PAMs, meaning that at least two

forms of desensitized states exist: type II modulator sensitive (D_s) and insensitive (D_i). On one hand, the existence of D_i states may limit the efficacy of PAMs to potentiate receptor-mediated responses, but on the other hand might actually provide an intrinsic safety mechanism against the dramatic potentiation produced by some modulators (see below).

The extent to which type II modulators are capable of allowing otherwise desensitized receptors to conduct current appears to depend on both agonist and modulator concentrations as well as duration of applications, with high concentrations and prolonged applications producing more D_i. When agonist concentrations are increased in the presence of a fixed PAM concentration, responses are generally enhanced over the full agonist concentration ranges, but the magnitude of potentiation often tends to peak at intermediate concentrations and actually decrease at the higher agonist concentrations [69, 72, 81], meaning that in these cases, the maximal increases in efficacy do not appear to reach a true plateau. A similar phenomenon is sometimes observed when modulator concentrations are increased in the presence of a constant agonist concentration [68, 71, 72, 77, 94]. To date, published studies involving α7 PAMs generally have utilized protocols that make short duration applications with long interstimulus intervals, a procedure which does not strongly induce D_i states [69], and may account for the lack of articles reporting type II modulator-insensitive desensitization. The only published example to our knowledge of agonist applications made with short interstimulus intervals [67] in the continued presence of PAMs does indeed show decreased responses when several agonist pulses are delivered in succession, indicative of the induction of modulator-insensitive states. In this study, D_i states appeared to accumulate in the presence of the type I PAM CCMI, as well as the type II PAM PNU-120596 [67]. Even though a seven-second application is not particularly long in duration, Dinklo *et al*., 2010 provide an example of a slowly decaying current despite the continued presence of agonist and modulator, indicating a conversion of receptors to JNJ-1930942-resistant states [72]. When 60 μM ACh was applied every three minutes on top of 60 μM choline and 10 μM PNU-120596 in *Xenopus* oocytes expressing α7, only the initial ACh response was greatly potentiated (~130-fold), with much less potentiation observed with subsequent ACh applications (< 20-fold), relative to the response to 60 μM ACh alone. After several minutes of no 60 μM ACh stimulation (application of choline and PNU-120596 continued), subsequent responses to 60 μM ACh (immediately after choline and PNU-120596 application stopped) were again greatly potentiated (~80-fold), indicating receptor recovery from the D_i states induced by ACh, choline, and PNU-120596 stimulation [93].

Additional evidence of D_i states is found in a recent study measuring calcium currents evoked by nicotine, PNU-282987, and PNU-120596 in bovine chromaffin cells [150]. In this study, the authors were only able to detect an α-bungarotoxin (α-btx)-sensitive and PNU-120596-dependent increase in calcium response when low (1 μM) concentrations of nicotine were used to stimulate the cells. At concentrations of ≥ 3 μM nicotine, neither PNU-120596 nor α-btx had an effect on the response, indicating that the α7 component was lost by the higher nicotine concentrations [150]. To isolate and more closely examine the disappearing α7 component, varying concentrations of the α7 agonist PNU-282987 with 1 μM PNU-120596 were applied for 5 minutes to evoke a calcium response. Any fluorescence increases that were insensitive to blockade by α-btx were subtracted from the total response. Interestingly, as stimulating agonist (in this case PNU-282987) concentrations increased, the α7-dependent calcium responses decreased, indicating greater D_i as agonist concentrations increased in the presence of a fixed concentration of PNU-120596 [150].

Two modes of potentiation, analogous to the two modes of activation proposed for α7 [27], may exist. For example, relatively low occupancy of agonist and PAM binding sites may generate an equilibrium condition between open, D_s, and D_i states that allows for large

prolonged steady-state currents, whereas higher occupancy of agonist and PAM binding sites promote D_i states, which ultimately result in biphasic and reduced steady-state currents. The potentiation of α7 responses likely depends on a complex interplay of many factors, including the effective agonist and modulator concentrations, nature of agonist/modulator combinations, and duration of applications [67, 81, 131].

Activation of α7 can have either protective or toxic effects depending on the mode of stimulation [151]. Positive allosteric modulators have been identified which increase the open probability of α7 by several orders of magnitude, inviting the question of whether PAMs may take the activation of this receptor subtype, which has high permeability to the natural catalytic ion calcium, to dangerously high levels. Calcium-mediated toxicity has been reported in SH-SY5Y cells and mice expressing mutant forms of α7 that display dramatically prolonged responses after stimulation [152–154]. *In vivo* toxicity profiles for PAMs are lacking, but there are some *in vitro* experiments that suggest type I and type II PAMs may have different profiles. Ng *et al*., 2007 showed that 24-hour exposure to the type I PAM CCMI did not reduce the viability of SH-SY5Y cells, whereas the type II PAM PNU-120596 did under the same conditions and in a MLA-sensitive manner [67]. Prior to the toxicity assays the authors tested the cells to verify the presence of functional receptors and successful modulation by PNU-120596 and CCMI. A similar experiment with stable α7-expressing GH4C1 cells showed that the type II PAM PNU-120596 was toxic, while JNJ-1930942 was not toxic in the same paradigm [72]. However, Hu *et al*. 2009 failed to detect toxic effects in undifferentiated PC12 cells and cortical neurons after treatment with the type I PAM CCMI, but they also did not detect any toxic effects with the type II PAMs PNU-120596 or A-867744 [155]. The reason for the contradictory results is unclear, but two shortcomings of this study are that functional α7 expression and potentiation were indirectly evaluated, and only one agonist, PNU-282987, was tested at only one concentration, 10 μM. The result of this study, in light of the existence of D_i states discussed above, invites consideration of the interesting possibility that D_i states provide an intrinsic safety mechanism that shuts the channel down with strong activation. In IMR-32 cells, PNU-282987 was reported as the most potent of several agonists tested in the presence of 10 μM PNU-120596 to induce calcium responses, with an EC_{50} of 0.1 ± 0.1 μM [99]. In *Xenopus* oocytes, the EC_{50} of PNU-282987 was reported as approximately 200 nM in the presence of 3 μM PNU [81]. Given that 24-hour applications of 10 μM PNU-282987 and PAM is expected to be a strong stimulus, the possibility that sufficient amounts of D_i were induced to protect cells from toxicity seems plausible. In addition, del Barrio *et al*., 2011 demonstrated that D_i states are induced with responses evoked by PNU-282987 in the presence of 1μM PNU-120596 in adrenal chromaffin cells, which are related to PC12 cells [150]. In contrast, the toxicity studies of both Ng *et al*., 2007 and Dinklo *et al*., 2010 used the weakly potent agonist choline at concentrations of ~100 μM.

In many cases, non-potentiated α7-mediated responses that are below or at the limits of experimental detection become easily measurable with the application of type II PAMs. For example, increases in ERK1/2 phosphorylation in PC12 cells that were evoked by agonists were detectable only in the presence of PNU-120596 [120–122, 155]. Similarly, agonist-evoked calcium responses were undetectable in PC12 cells [122, 155], IMR-32 cells [70, 90, 99, 120, 156], primary cortical neurons [99], chromaffin cells [150], and CHO-K1 cells [157] unless PAMs were co-applied. In these experiments, type I PAMs were generally unable to increase either ERK1/2 phosphorylation or calcium responses to detectable levels [99, 155, 157], however, calcium responses were significantly potentiated with 5-HI to detectable levels in GH4C1 cells stably expressing α7 [158]. In addition, in hippocampal CA1 pyramidal cells, PNU-120596 was shown to enhance α7-mediated responses evoked by physiologically relevant concentrations of choline, which are difficult to detect under normal conditions, to the point of depolarizing the cells and facilitating action potential firing [138].

Together these observations raise questions of whether PAMs (primarily type II) may alter the α7 component of neuronal systems sufficiently to interrupt natural synchrony and regulation of important signaling events. These results also bring to light technical challenges in high-throughput screening methods to identify type I PAMs: potentiated responses may still fall below experimental detection limits, and the sampling rate of data collection in commonly used assays is too slow to detect the rise and decay of an α7 response potentiated by a type I PAM.

5. Summary

The recent programs of drug development to discover and characterize nAChR PAMs, particularly ones which are selective for α7 nAChR, have created both new opportunities and challenges. We are at a crucial point in the testing of PAMs with unique attributes, and we must identify the correct model systems to determine whether they should be moved forward into clinical trials. We must also consider whether they will be safe from serious side effects, as they will reach into all of the many areas of α7 nAChR activity. An additional consideration is that evidence would suggest that PAMs would work primarily, if not exclusively, through increasing signal transduction mediated by α7 ion channel activation. Some studies have reported PAM enhancement of intracellular signal transduction pathways [120–122], and the tacit assumption is that in these cases, a PAM-sensitive, channel-mediated calcium influx is the essential connection between receptor activation and signal transduction. However, there are numerous cases, especially in non-neuronal cells, where there is important α7-mediated signal transduction under conditions when no ion channel activation can be detected [146, 159]. Likewise, we have learned from the development of α7 agonists and other drugs that the most efficacious compounds are not always the best leads to follow. DMXBA (GTS-21), one of the first α7-selective drugs to be identified [160], has been shown to have good activity in many model systems [161], including human trials [162], and yet it has very low efficacy as an ion channel activator [163, 164] and strongly stabilizes receptor desensitization [93]. Considering the extremely low P_{open} of α7 nAChR, especially in the presence of high concentrations of agonists, the common condition for these receptors is for them to be in non-conducting states. Experiments with PNU-120596 have shown that α7 nAChR have multiple non-conducting states that are invisibly and dynamically interconvertible. It has been shown that many intracellular proteins interact with α7 receptors [165], and it seems reasonable to hypothesize that the dynamics of the conformational changes associated with conversions among multiple non-conducting states may extend into the largely mysterious intracellular domain and thereby have channel-independent signal transducing effects.

The identification of α7-PAMs has opened up new avenues for therapeutic approaches, and taught us much about the mysteries of these important receptors. They also show that there are likely to be more mysteries to uncover and understand about α7 nAChR.

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Abbreviations

A-585539	[(1S,4S)-2,2-Dimethyl-5-(6-phenylpyridazin-3-yl)-5-aza-2-azoniabicyclo[2.2.1]heptane]

A-867744	4-(5-(4-chlorophenyl)-2-methyl-3-propionyl-1H-pyrrol-1-yl)benzenesulfonamide
5-HI	5-hydroxyindole
5-HT	5-hydroxytryptamine
α-btx	α-bungarotoxin
ACh	acetylcholine
CCMI	N-(4-chlorophenyl)-alpha-[[(4-chloro-phenyl)amino]methylene]-3-methyl-5-isoxazoleacet-amide
CNS	central nervous system
dFBr	desformylflustrabromine
D_i	type II modulator-insensitive desensitization
D_s	type II modulator-sensitive desensitization
ERK1/2	extracellular signal-related kinase-1 and -2
GTS-21	3-(2,4-dimethoxybenzylidene)-anabaseine
JNJ-1930942	2-[[4-fluoro-3-(trifluoromethyl)phenyl]amino]-4-(4-pyridinyl)-5-thiazolemethanol
LY-2087101	[2-(4-fluoro-phenylamino)-4-methyl-thiazol-5-yl]-thiophen-3-yl-methanone
nAChR	nicotinic acetylcholine receptor
MWC	Monod, Wyman, Changeux
NS-1738	1-(5-chloro-2-hydroxy-phenyl)-3-(2-chloro-5-trifluoromethyl-phenyl)-urea
PAM	positive allosteric modulator
PNU-120596	1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea
PNU-282987	N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide
SB-206553,	3,5-dihydro-5-methyl-N-3-pyridinylbenzo [1,2-b:4,5-b′]-di pyrrole-1(2H)-carboxamide
SLURP-1	secreted mammalian Ly-6/uPAR related protein 1
TM	transmembrane
TQS	4-naphthalene-1-yl-3a,4,5,9b-tetrahydro-3-H-cyclopenta[c]quinoline-8-sulfonic acid amide

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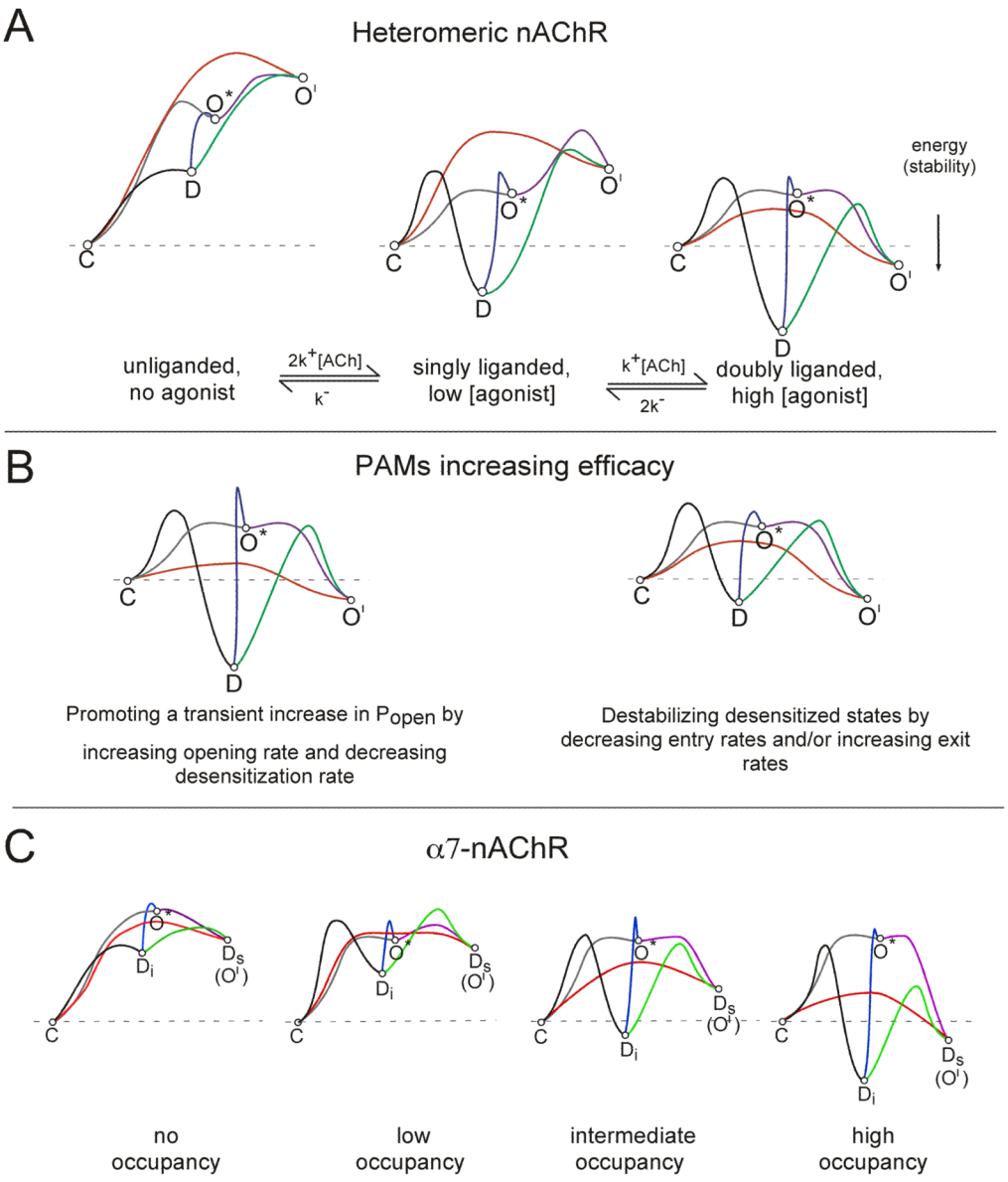

Figure 1.

The energy landscapes for nAChR state transitions. **A)** Hypothetical energy wells and barriers for the conformational states of heteromeric nAChR (e.g. muscle-type or neuronal α4*nAChR) as functions of the level of agonist occupancy (see description in text). Under equilibrium conditions, the distributions of receptors into the resting closed (C), brief open (O*), long-lived open (O′), and desensitized (D) states will be determined by the relative free energy of the states (represented by vertical displacements). Dynamically, the transition rates between the states will be inversely related to the log of the energy barriers between the states. **B)** Two ways in which PAMs may operate on the energy profile of receptors in the doubly liganded state to increase the probability of channel opening: either transiently, after a jump in agonist concentration (left schematic) or under steady-state conditions (right schematic). **C)** Hypothetical scheme representing state transitions of α7 nAChR under various levels of agonist occupancy. In the absence of PAMs, α7 nAChR do not exhibit a long-lived open state, but do show a unique desensitized state (D_s) that is preferentially

favored at higher levels of agonist occupancy. They also have an intrinsic desensitized state (D_i), which is analogous to the D state of heteromeric receptors (Figure 1A). Very efficacious type II PAMs, such as PNU-120596, appear to either destabilize the D_s state, or hypothetically may convert it into a conducting state similar to the O′ state of heteromeric receptors. This hypothesis is represented by (O′) symbols inserted into the panel.

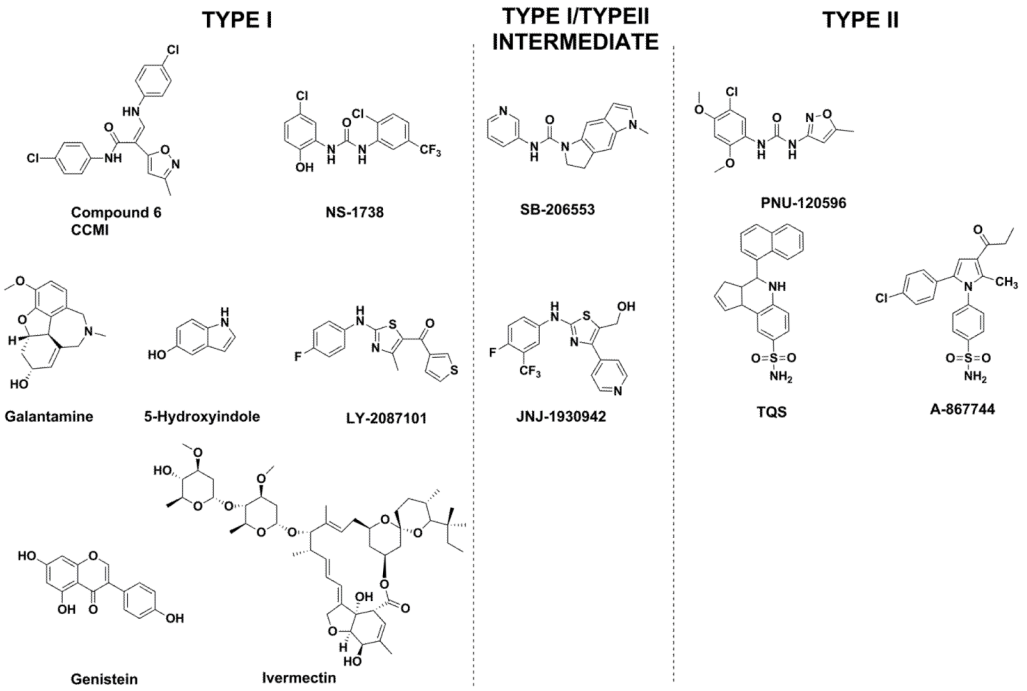

Figure 2.

Structures of α7-active PAMs. The PAMs classified as type I, which increase the amplitude but do not strongly alter the kinetics of a7-mediated agonist evoked responses, are shown on the left. They include: CCMI, N-(4-chlorophenyl)-alpha-[[(4-chloro-phenyl)amino]methylene]-3-methyl-5-isoxazoleacet-amide; NS-1738, 1-(5-chloro-2-hydroxy-phenyl)-3-(2-chloro-5-trifluoromethyl-phenyl)-urea; Galantamine; 5-HI, 5-hydroxyindole; LY-2087101, [2-(4-fluoro-phenylamino)-4-methyl-thiazol-5-yl]-thiophen-3-yl-methanone; Genistein; and Ivermectin. Type II PAMs, which appear to slow or reverse α7 desensitization are shown on the right and include: PNU-120596, 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea; TQS, 4-naphthalene-1-yl-3a,4,5,9b-tetrahydro-3-H-cyclopenta[c]quinoline-8-sulfonic acid amide; and A-867744, 4-(5-(4-chlorophenyl)-2-methyl-3-propionyl-1H-pyrrol-1-yl)benzenesulfonamide. PAMs proposed to be intermediate in their activity are shown in the center and include: JNJ-1930942, 2-[[4-fluoro-3-(trifluoromethyl)phenyl]amino]-4-(4-pyridinyl)-5-thiazolemethanol; and SB-206553, 3,5-dihydro-5-methyl-N-3-pyridinylbenzo [1,2-b:4,5-b′]-di pyrrole-1(2H)-carboxamide.

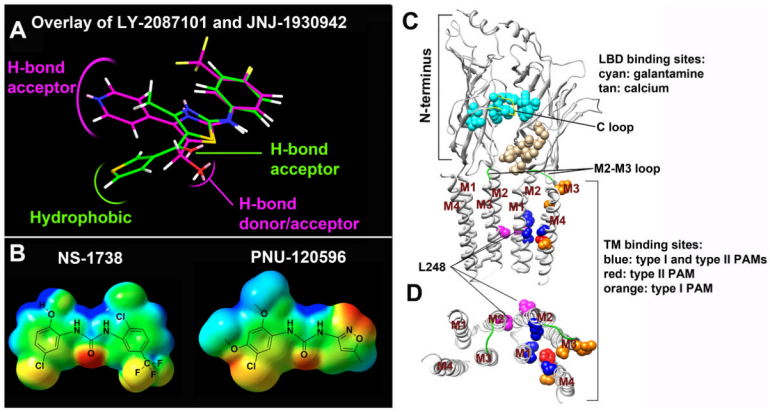

Figure 3.

α7 PAM structures and putative binding sites. **A)** Pharmacophore orientation comparison between LY-2087101 and JNJ-1930942. The structures of the two compounds were optimized using Chem 3D Pro v.12.0 (CambridgeSoft, Cambridge, MA): their energies were first minimized by molecular mechanics to minimum RMS gradient of 0.001 separately. Then LY-2087101 and JNJ-1930942 were overlaid on top of each other and shown in green and magenta, respectively. **B)** Molecular electrostatic potential surfaces of NS-1738 (left) and PNU-120595 (right). The structures of the two compounds were optimized using Gaussian09 (Gaussian, Inc., Wallingford, CT). The electrostatic potential on a total electron density isosurface (0.002 Bohr/Å^3) of the two compounds is displayed in GaussView (Gaussian, Inc., Wallingford, CT) with red color indicating the most negative electrostatic potential and blue color indicating the most positive electrostatic potential, respectively. The structures of the two compounds are displayed on top of each electrostatic potential surface. **C)** Structural domains of nAChR regulating PAM activity and/or binding. The key elements of the α7 receptor were modeled using the 2BG9 template for the transmembrane domain fused to the N-terminal extracellular domain made from the 2PGZ template. The C-loop is shown in yellow, and the M2 and M3 linker loop is shown in green. The view was made from the outside of the channel pore, and the four transmembrane helixes (M1 to M4) were lined clockwise as shown, putting the M2 helix toward the channel pore. Residues involved for different α7 PAMs potentiation are displayed as spheres of different colors as indicated: calcium, tan; galantamine, cyan; common residues for type I PAMs Ivermectin and LY-2087101 and the type II PAM PNU120596, blue; residues only for Ivermectin, orange; residues only for PNU-120596, red. Leucine 248 is also shown in magenta in both subunits. **D)** A close-up view of the transmembrane domain, made by rotating the view in part C 90° vertically away from the plane of the paper.

Table 1

Summary of published α7 PAM EC_{50} and maximal potentiation values*

PAM	EC_{50}, μM	max % or fold-increase	cell-type(species of α7)	stimulating agonist	PAM preapplication	measurement	reference
Ivermectin	6.8	3.5-fold	*X. laevis* oocytes (rat)	100 μM ACh	60 sec	peak current	Collins and Millar, 2010
BSA	10	6-fold	chick ciliary ganglion	20 μM nicotine	not specified	peak current	Conroy *et al*., 2003
Genestein	20	267%	*X. laevis* oocytes (human)	100 μM ACh	60 sec	peak current	Gronlien *et al*., 2007
	40	227%	*X. laevis* oocytes (human)	100 μM ACh	no	peak current	Gronlien *et al*., 2007
5-HI	2500	1208%	*X. laevis* oocytes (human)	60 μM ACh	no	peak current	Zwart *et al*., 2002
	631	541%	*X. laevis* oocytes (human)	100 μM ACh	60 sec	peak current	Gronlien *et al*., 2007
	1585	350%	*X. laevis* oocytes (human)	100 μM ACh	no	peak current	Gronlien *et al*., 2007
CCMI	0.7	45% of [EC_{100}] ACh response	*X. laevis* oocytes (human)	[EC_5] ACh	30 sec	peak current	Ng *et al*., 2007
NS-1738	3.4	322%	*X. laevis* oocytes (human)	100 μM ACh	60 sec	peak current	Timmermann *et al*., 2007
	1.6	1170%	GH4C1 (human)	300 μM ACh	60 sec	peak current	Timmerman *et al*., 2007
	12.5	12-fold	GH4C1 (rat)	300 μM ACh	150 sec	current; area	Friis *et al*., 2009
PNU-120596	0.2	400%	SH-EP1 (human)	100 μM ACh	120 sec	calcium transient	Hurst *et al*., 2005
	1.6	455%	*X. laevis* oocytes (human)	100 μM ACh	60 sec	peak current	Gronlien *et al*., 2007
	1.5	37-fold	*X. laevis* oocytes (rat)	50 μM ACh	duration not specified	peak current	Young *et al*., 2008
	0.1	203% of 10 μM nicotine response	GH4C1 (rat)	0.5 μM nicotine	10 min	calcium transient	Dunlop *et al*., 2009
	4.9	2500-fold	GH4C1 (rat)	300 μM ACh	150 sec	current; area	Friis *et al*., 2009
	5	not reported	IMR-32 (human)	10 nM PNU-282987	60 sec	calcium transient	Gopalakrishnan *et al*., 2011
	1	not reported	IMR-32 (human)	100 nM PNU-282987	60 sec	calcium transient	Gopalakrishnan *et al*., 2011
TQS	3.2	418%	*X. laevis* oocytes (human)	100 μM ACh	60 sec	peak current	Gronlien *et al*., 2007
	6.2	65-fold	*X. laevis* oocytes (human)	100 μM ACh	not specified	peak current	Gill *et al*., 2011
A-867744	1	52% of max potentiation by PNU-120596	IMR-32 (human)	10 μM PNU-282987	3–5 min	calcium transient	Faghih *et al*., 2009
	1.1	733%	*X. laevis* oocytes (human)	100 μM ACh	60 sec	peak current	Malysz *et al*., 2009
SB-206553	1.5	82% of 10 μM nicotine response	GH4C1 (rat)	0.5 μM nicotine	10 min	calcium transient	Dunlop *et al*., 2009
JNJ-1930942	1.9	21-fold	GH4C1 (human)	100 μM choline	duration not specified	calcium transient	Dinklo *et al*., 2011

*EC_{50} values for potentiation of α7-mediated responses by SLURP-1, galantamine, and LY-2087101 not found in literature