Are nicotinic acetylcholine receptors coupled to G proteins?

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It was, until recently, accepted that the two classes of acetylcholine (ACh) receptors are distinct in an important sense: muscarinic ACh receptors signal via heterotrimeric GTP binding proteins (G proteins), whereas nicotinic ACh receptors (nAChRs) open to allow flux of Na^+, Ca^{2+}, and K^+ ions into the cell after activation. Here we present evidence of direct coupling between G proteins and nAChRs in neurons. Based on proteomic, biophysical, and functional evidence, we hypothesize that binding to G proteins modulates the activity and signaling of nAChRs in cells. It is important to note that while this hypothesis is new for the nAChR, it is consistent with known interactions between G proteins and structurally related ligand-gated ion channels. Therefore, it underscores an evolutionarily conserved metabotropic mechanism of G protein signaling via nAChR channels.

Keywords:
acetylcholine; G protein coupling; intracellular loop; ligand-gated ion channel; loop modeling; protein interaction; signal transduction

Introduction

It is often said that two main types of neurotransmitter receptors exist – ionotropic ligand-gated ion channels (LGICs), which permit rapid ion flow directly across the cell membrane, and metabotropic receptors, which set in motion a slower chemical signaling cascade via the binding and activation of heterotrimeric GTP binding proteins (G proteins) following ligand activation [1]. Neuronal nicotinic acetylcholine receptors (nAChRs) are a subdivision of LGICs widely distributed in nervous tissue and contribute to processes such as neurotransmitter release and synaptic plasticity [2, 3]. Mutations within nAChR genes are implicated in a number of human disorders including drug addiction and schizophrenia [4].

Nicotinic receptors belong to an evolutionarily conserved class of cys-loop containing receptor channels that includes $GABA_A$, glycine, and $5HT_3$ receptors as well as two newly discovered channels: a zinc-activated channel and an invertebrate GABA-gated cation channel [5]. In mammals, genes encoding neuronal nAChR subunits have been identified and labeled α (α1–α10) and β (β1–β4). Functional nAChRs are derived from an arrangement of five subunits into heteromeric or homomeric receptors [6] (Fig. 1A). The activity of nAChRs also appears driven by direct protein-protein associations with molecules such as receptor kinases, scaffolds, and signaling effectors [7]. A growing list of proteins has emerged as components of the nAChR signaling network. This list includes scaffold proteins such as 14–3–3, and the calcium sensor visinin like protein-1 [8, 9]. In this article, we discuss findings on associations between nAChRs and G proteins. These findings support the hypothesis that nAChRs couple to G proteins at the plasma membrane.

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Abbreviations:
ACh, acetylcholine; **GPCR,** G protein coupled receptor; **LGIC,** ligand-gated ion channel; **nAChR,** nicotinic acetylcholine receptor; **TM,** transmembrane.

Evolutionary emergence of an intracellular protein-protein interaction domain in nicotinic receptors

Nicotinic receptor subunits share a topology that consists of a large

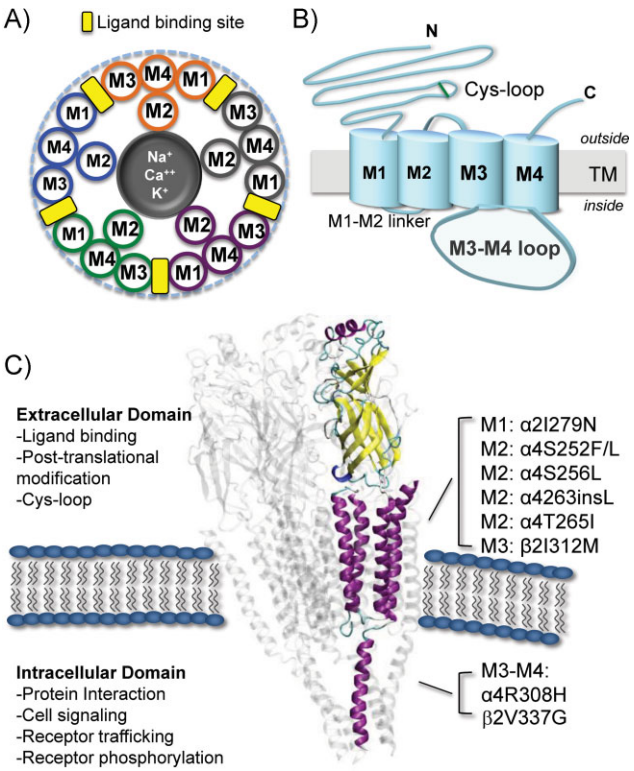

Figure 1. nAChR Structure and function. **A**: The nAChR as viewed from above shows five subunits arranged around a central cation-conducting pore. A ligand-binding site is formed at the interface of two subunits. **B**: An illustration of a single nAChR subunit embedded in the membrane. **C**: The protein structure of the pentameric nAChR obtained from *T. marmorata* (PDB 2BG9) in the plasma membrane. Shown are the location and function of the major receptor domains [13]. A single subunit is highlighted in purple using visual molecular dynamics (VMD) [89]. Mutations in the membrane and intracellular regions of the human nAChR are shown [90].

extracellular N-terminal domain, four transmembrane (TM) domains, and a single large intracellular loop located between TM domains 3 and 4 (M3–M4; Fig. 1B). In most nAChRs, the M3–M4 loop contains ≈100 amino acid residues and shares low sequence homology with other nAChRs [6]. Based on site directed mutagenesis studies, the M3–M4 loop is found to mediate important receptor properties such as export from the endoplasmic reticulum (ER) and trafficking to the plasma membrane (Fig. 1C) [10]. In contrast, the acetylcholine (ACh) binding site is highly conserved and is formed extracellularly at the interface of two α subunits or one α and one β subunit (Fig. 1A). Extracellular binding of ligands, agonists or antagonists, and allosteric modulators determines the conformation of the nAChR: basal, active, or desensitized [11].

Fast, ionotropic neurotransmission mediated by LGICs is essential for survival responses in multicellular organisms [12]. Among the penatmeric LGICs, cys-loop receptors make up a subfamily previously thought only to exist in eukaryotes, but which was recently found in prokaryotes [13]. Studies on the prokaryotic origin of cys-loop receptor channels reveal that a functional, cation-conducting nAChR homolog exists in several bacterial species and an *archaea* genus [14]. The prokaryotic homolog, *Gloeobacter violaceus* LGIC (GLIC), binds extracellular protons instead of ACh, but maintains most of the membrane sensitive structural and biophysical properties of the eukaryotic nAChR [14, 15]. Electron microscopy and protein cross-linking experiments confirm a homopentameric organization of the GLIC protein. However, sequence analysis reveals a key difference between prokaryotic and eukaryotic channel composition at the intracellular domain. Notably, the marked absence of the M3–M4 loop in GLIC suggests a difference in the cellular activity between GLIC and mammalian nAChRs (Fig. 2A) [13]. Without an M3–M4 loop the GLIC channel is likely unable to participate in the myriad of intracellular interactions characteristic of eukaryotic nAChRs.

Protein interactions and post-translational modifications of the M3–M4 loop are now established functional features of nAChR function in cells. For example, specific residues in the M3–M4 loop of the α4β2 nAChR play a vital role in trafficking the receptor to the cell surface (Fig. 2B) [16]. Other residues in the M3–M4 loop target the nAChR to functional domains such as axons and dendrites [17, 18]. Lastly, a number of serine/threonine and tyrosine residues throughout the M3–M4 loop contribute to receptor kinetics and gating when phosphorylated [19]. It seems likely that most, if not all, of the intracellular protein binding of the nAChR evolved through the emergence of an M3–M4 loop in eukaryotes.

The absence of an M3–M4 loop in the recently discovered GLIC protein crystal structure [13] leaves a knowledge gap in our structural understanding of nAChR intracellular protein interactions. In Fig. 1C, we present a structural model of the nAChR based on the available crystal structure of the muscle nAChR from *Torpedo marmorata* [20]. This model is obtained through the I-TASSER structure prediction server [21] and allows us to computationally estimate a conformation for the M3–M4 loop that was absent in the reported crystal structure [20]. In Fig. 2A, we use LoopyTM [22] and JACKAL software [23] to propose a more probable structure for the human α7 nAChR. This type of analysis generated a series of energetically favorable M3–M4 loop structures for the human nAChR. One such structure is presented in Fig. 2A. It is not unlikely that this M3–M4 loop exists in several structural conformations, all of which were found to extend into the cytoplasm of the cell. Based on this structural modeling evidence,

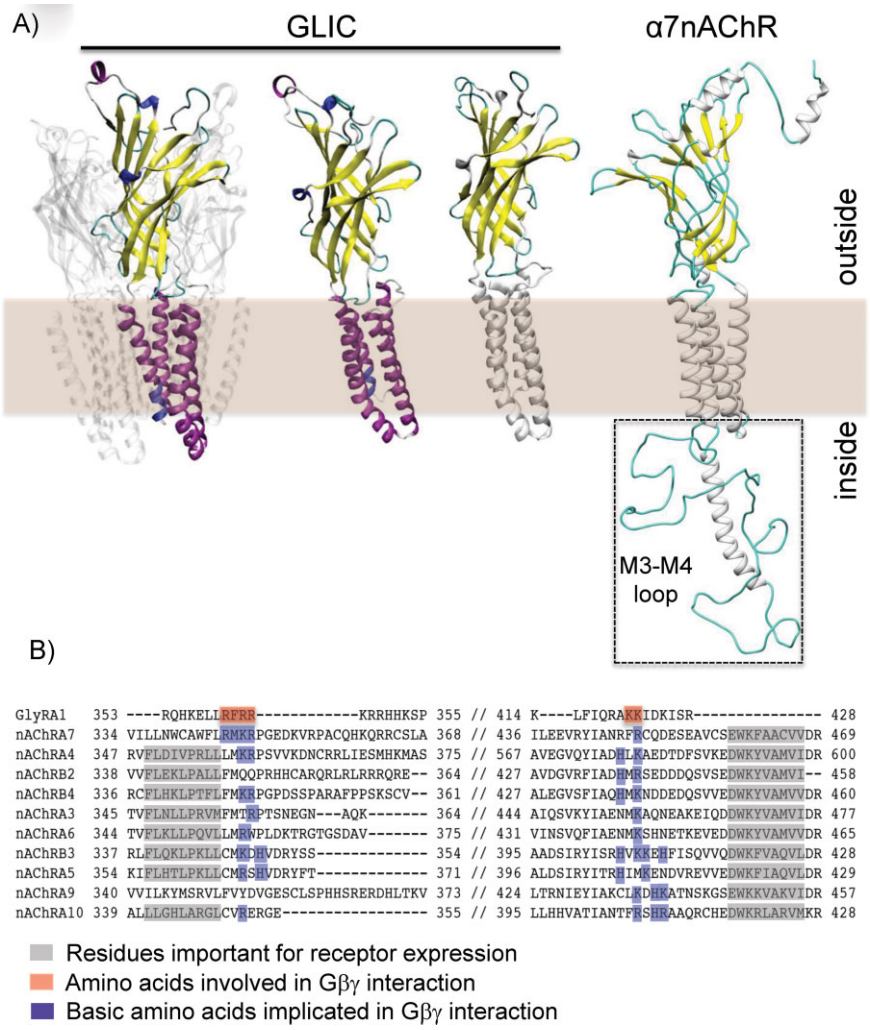

Figure 2. Evolution and structure of the nAChR. **A**: From left to right: First image, the structure of the crystallized prokaryotic GLIC channel protein [13] within the membrane. Second image, a single subunit within the GLIC channel protein is shown in purple. Third image, homology modeling using I-TASSER was used to confirm the structure of a GLIC subunit. Fourth image, homology modeling using I-TASSER was used to predict the structure of the human α7 nAChR (NCBI: NP_000737.1 was used as sequence template) [21, 89]. This computational model suggests an intracellular M3–M4 loop structure for the human α7 nAChR (dotted box). The loop conformation was specifically modeled de novo using the LoopyTM loop software [22]. Of the 100 possible conformations, the top 15 candidate loop structures were identified using Dfire followed by JACKAL softwares [23, 91, 92]. A representative M3–M4 loop conformation in the human α7 nAChR is presented. **B**: A multiple sequence alignment of the M3–M4 loop for various nAChRs as well as the human GlyR. Amino acids that contribute to Gβγ binding to the GlyR are shown [75]. Conserved and similar residues are indicated.

we predict an evolutionary emergence of an intracellular protein-binding interface at the M3–M4 loop of the nAChR. The M3–M4 loop region of eukaryotic nAChRs may also contain secondary structures formed by associations of the individual M3–M4 loops in the channel pentamer (Fig. 1C). This protein-binding interface is a focal point for interaction between nAChRs and the signaling elements of the cell.

G proteins regulate the signaling of various receptors at the plasma membrane

Heterotrimeric G proteins (G proteins) serve as molecular switches for various GPCRs and a growing list of ion channels at the plasma membrane [24]. The genome of *Homo sapiens* contains 23 α, 5 β, and 12 γ subunit G proteins. The structurally diverse α subunits are grouped into functional families on the basis of how they signal: $G\alpha_s$ increases cAMP synthesis, $G\alpha_i$ inhibits it, $G\alpha_q$ and $G\alpha_{11/12}$ couple to phospholipase C to release IP_3 and diacylglycerol, and $G\alpha_{12/13}$ signals via Rho kinases. Diversity within βγ subunits appears to have arisen later in evolution [25].

Based on early studies using non-hydrolyzable GTP analogs, cholera toxin and α mutants, it was originally thought that heterotrimeric G protein activity required a conformational change that led to a physical dissociation of the α subunit from the βγ subunits. However recent work using FRET imaging and chemical crosslinking shows that dissociation of the subunits may not be needed for signaling [26]. The βγ complex may remain associated with the α subunit and still allow for G protein signaling in the cell [27]. Indeed, a trimeric G protein appears able to bind and modulate effector targets just as well [28].

How G proteins recognize their cellular partners is not well understood. Compartmentalization of G proteins within membrane regions such as lipid rafts and focal adhesions plays an important role in localizing the G protein in the vicinity of its targets [29]. Mutagenesis studies also show that amino acids in a protein give information for the binding of specific G proteins [30]. An important body of literature exists on the regulation of G protein interaction with GPCRs. The evidence indicates that specific residues in the GPCR intracellular loops are critical for G protein binding and recognition [31]. In the example of D2 dopamine receptors, the $G\alpha_i$ activation site is found near the plasma membrane and can also bind calmodulin, suggesting that G protein-receptor interactions are influenced by association with other proteins [32].

G protein binding has also been studied in a number of ion channels. In voltage-gated calcium channels such as Ca_v2 channels, up to two distinct interaction sites for $G\alpha_q$ and $G\alpha_{i/o}$ and several binding sites for Gβγ have been discovered in a single channel [33]. A consensus QXXER and a G protein interaction domain (GID) sequence appears to dictate G protein interaction

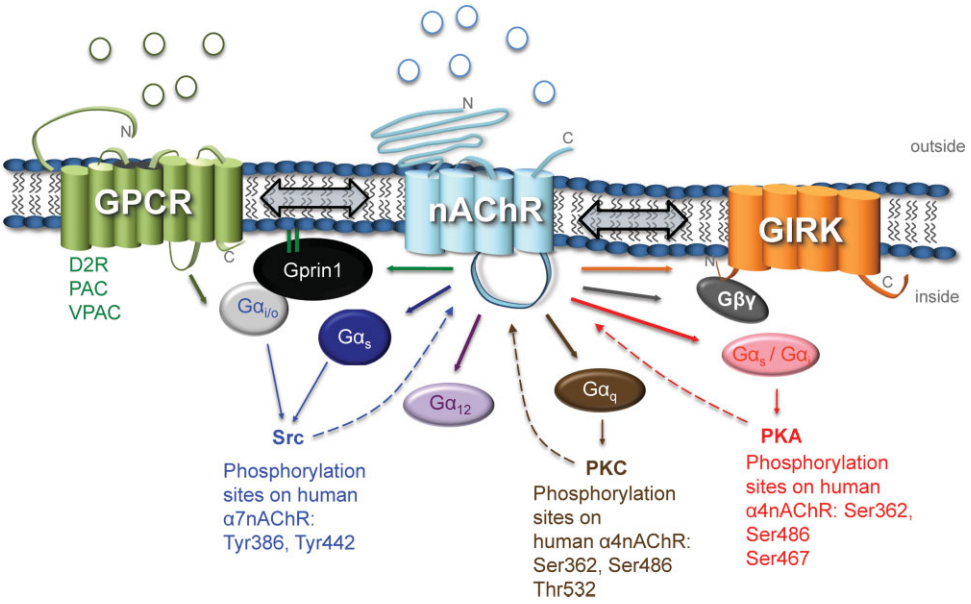

Figure 3. G protein pathways regulate nAChR function. A summary of the major interactions between nAChRs and G protein signaling pathways in neural cells. In addition to binding G proteins, nAChRs can associate with GPCRs and G protein-gated ion channels such as GIRK1 at the plasma membrane. A series of residues within the M3–M4 loop of the nAChR confer channel regulation by various G protein kinases (dotted lines).

with the calcium channel [33]. An integrated view of G protein-target interactions based on studies of ion channels and GPCRs suggests that G protein interaction is influenced by several factors: (1) structural preservation and molecular access to a G protein-binding pocket or domain; (2) a conducive proximity between the G protein and the target receptor; (3) simultaneous association with a mutual scaffold or adaptor that facilitates the formation of the G protein complex (GPC) in the cell.

Hypothesis: Nicotinic receptors couple to G proteins

Nicotinic receptors are regulated by G protein activated kinases

Conformational changes in the structure of the receptor as a result of phosphorylation or dephosphorylation affect its location in the cell, ability to bind proteins, and degradation [34]. Most nAChRs contain at least one phosphorylation site within the M3–M4 loop [19]. A cohort of kinases linked to G protein signaling phosphorylates and directly regulates nAChRs. To date, this includes Abelson family kinases (AFK), cAMP activated protein kinase A (PKA), protein kinase C (PKC) [35], and the Src family kinase (Src) [36–39].

$G\alpha_s$ and $G\alpha_i$ proteins are major activators of signaling pathways for the non-receptor tyrosine kinase Src in the cell. In addition to directly binding G proteins, this class of enzymes is increasingly recognized for its ability to modulate the function of LGICs such as nAChRs. Studies show that Src-family kinases bind and phosphorylate tyrosine residues in the M3–M4 loop region of several nAChRs (Fig. 3) [40–42]. In bovine adrenal chromaffin cells, Src has been found to form large multimeric complexes with nAChRs [43] and regulate ACh- and nicotine-induced catecholamine secretion [44, 45]. Wang et al. [39] demonstrate that α3β4α5 nAChRs in chromaffin cells and α3β4α5 nAChRs in HEK cells can be phosphorylated via c-Src. Several Src-family kinases including c-Src, Fyn, and Lyn positively regulate α3β4 nAChRs [46] and negatively regulate α7 nAChRs (see Box 1) [36, 47]. In light of these findings, it is interesting to consider the role of G proteins in Src-family kinase mediated regulation of nAChRs (Fig. 3).

Nicotinic receptors interact with GPCRs

G protein coupled receptors (GPCRs) are an important gene superfamily making up >4% of the human genome [48]. This family of receptors can transmit information from numerous extracellular ligands into the cell via a G protein chemical signaling cascade [49, 50]. A newer notion in receptor biology is that GPCRs assemble into higher order receptor networks (multimers) consisting of GPCRs and their functional partners [51, 52]. According to this theory, GPCRs not only bind to receptors, but can also associate with structurally diverse ion channels including LGICs [53, 54]. In this regard, coupling to GPCRs may facilitate interactions between ion channels and G proteins in the cell.

Functional and biophysical associations between GPCRs and LGICs in neurons are well documented [55]. In hippocampal neurons, a $GABA_A$/D5 dopamine receptor dimer is formed through the binding of the second intracellular loop of the $GABA_A$ channel with the carboxy terminal tail of the D5 dopamine receptor. This $GABA_A$/D5 dimer is found to direct dopaminergic modulation of inhibitory transmission

Box 1

Hippocampal neurons

- The α7 nAChR is phosphorylatd by c-Src at residues Y386 and Y442 within the M3–M4 loop. This mechanism alters the trafficking and the expression of the nAChR at the cell surface [47].

Parasympathetic ganglion neurons

- VIP potentiation of the α7 nAChR is directly blocked by the application of the $G\alpha_{i/o}$ blocker pertussis toxin as well as the inactive GDP substrate GDP-β-S [67].
- Antibodies selective for $G\alpha_o$, $G\alpha_i$, and $G\beta\gamma$ indicate that blocking the activity of $G\alpha_o$ and $G\beta\gamma$ is sufficient to entirely abolish the VIP and PACAP induced potentiation of the nAChR response [68]
- Application of GTP-γ-S was found to directly increase whole cell current amplitudes of the nAChR in the presence of ACh or nicotine. In inside-out patch recordings, the same application of GTP-γ-S resulted in reversible fourfold increase in the open probability of the nAChR channel [58].
- The addition of $G\beta\gamma$ resulted in a fivefold increase in the open probability of the nAChR channel, whereas the addition of $G\alpha_o$ alone had little to no effect on channel kinetics [58].

in the hippocampus [53]. A similar functional dimer between the D1 dopamine receptor and the NR1 subunit of the NMDA glutamate receptor channel has been identified [55]. In hippocampal neurons, formation of an NMDA/D1 dopamine receptor complex is critical for glutamatergic transmission underlying synaptic plasticity and learning [56].

Coupling between D2 dopamine receptors and nAChRs has been observed at presynaptic terminals of the ventral striatum (Fig. 3 and Table 1). This β2 nAChR/D2 dopamine receptor dimer regulates dopamine release in the striatum and plays a role in nicotine mediated reward behavior in rodents [57]. Co-immunoprecipitation experiments confirmed the interaction between β2 nAChRs and D2 dopamine receptors in cells and brain tissue. Complexes of the β2 nAChR and the D2 dopamine receptor were also found to contain Gα proteins [58]. However, it is not clear whether these G proteins associate with the nAChR or with the dopamine receptor or possibly both. Recent work by our laboratory and others support the possibility of interaction between G proteins and nAChRs independent of GPCRs [7, 59, 60].

Functional interactions between nicotinic receptors and G proteins

Functional interactions between nAChRs, GPCRs, and G proteins occur in a number of systems. Experiments conducted in mammalian parasympathetic neurons demonstrate a functional role for nAChR interactions with pituitary adenylate cyclase-activating (PAC) and vasoactive intestinal peptide (VIP) (VPAC) receptors and their associated G protein pathways. PAC_1 receptors bind pituitary adenylate

Table 1. G protein interactions of the nAChR.

Nicotinic receptor	G protein interaction	Cell and tissue source	Protein interaction detection method	Reference
α7	$G\alpha_{i/o}$[a]	Whole brain tissue of C57BL6 mice	α-Bgtx pulldown, Western blot, LC-ESI MS	[59]
		Cortical neurons and PC12 cells from rat	IP, Western blot, LC-ESI MS	[60]
	$G\alpha_{q/12}$	Whole brain tissue of C57BL6 mice	α-Bgtx pulldown, Western blot, LC-ESI MS	[59]
	Gβγ	Cortical neurons and PC12 cells from rat	IP, Western blot, LC-ESI MS	[60]
	Gprin1	Cortical neurons and PC12 cells from rat	IP, Western blot, LC-ESI MS	[60]
α4β2	$G\alpha_o$	Intrinsic cardiac ganglia from rat	GST–M3–M4 loop pulldown, IP, Western blot	[58]
	Gβγ	Intrinsic cardiac ganglia from rat	GST–M3–M4 loop pulldown, IP, Western blot	[58]
	$G\alpha_{i/o}$	Whole brain tissue of C57BL6 mice	IP, MALDI-TOF MS	[7]
	Gprin1	Whole brain tissue of C57BL6 mice	IP, MALDI-TOF MS	[7]
	GIRK1	Whole brain tissue of C57BL6 mice	IP, MALDI-TOF MS	[7]
	D2R	Striatal neurons from rat and HEK cells	Transfection, IP, Western blot	[57]
α3β4α5	$G\alpha_o$	Intrinsic cardiac ganglia from rat	GST–M3–M4 loop pulldown, IP, Western blot	[58]
	Gβγ		GST–M3–M4 loop pulldown, IP, Western blot	[58]

[a]Interaction between α7 nAChRs and $G\alpha_{i/o}$ were not detected in Fischer et al. [58].

cyclase-activating polypeptide (PACAP) with high affinity. $VPAC_{1/2}$, on the other hand, are activated by the neuropeptides VIP and PACAP [61, 62]. In chick ciliary ganglion cells that express α7- and α3-containing nAChRs, PACAP only inhibits α7 containing nAChRs [63]. VIP, on the other hand, can direct the recruitment of both nAChRs to the cell surface in a G protein dependent manner [63–65]. Similar work by Nörenberg et al. [66] demonstrated that neuropeptide Y inhibits the release of catecholamines via G protein activation of PKA and phosphorylation of nAChRs.

An understanding of the mechanisms of nAChR regulation by G proteins in rat parasympathetic neurons came from several studies using whole cell patch clamp recordings. The findings of these experiments indicate that VIP and PACAP potentiate nAChR mediated currents in a G protein dependent manner (Box 1). Experiments using the broad G protein agonist GDP-γ-S confirm the role of G proteins in the potentiation of ACh-evoked currents in neurons (Box 1) [68]. Taken together, these studies underscore an important mechanism of G protein interaction and regulation of nAChRs in neurons. The evidence also suggests that nAChR interaction with G proteins is *direct* and *selective* and is consistent with G protein binding to other LGICs. Because each pentameric nAChR has five potential G protein (Gβγ) binding sites, cooperative binding could be responsible for the fivefold increase in open channel probability observed in response to Gβγ application [58]. In future studies it will be important to determine the stoichiometry of Gβγ binding to the nAChR.

Proteomic discovery of a nicotinic receptor/G protein-signaling complex

Isolation and characterization of protein complexes from cultured cell or native tissue has become common using methods of immunoaffinity-, ligand-, and tag-based chromatography followed by mass spectrometry (MS) for proteomic analysis [73]. Recently, matrix assisted laser desorption/ionization time of flight (MALDI-TOF) MS identified components of an immunoprecipitated nAChR complex from the adult mouse brain. A single interaction network of the β2 subunit was found to contain over 20 new receptor interacting proteins [7]. Evident among the interactions was a cohort of G protein pathway molecules including $G\alpha_{i/o}$ and the Gα interacting protein G protein regulated inducer of neurite outgrowth 1 (Gprin1). In addition, β2 nAChRs associated with the G protein coupled, inwardly rectifying, K^+ channel GIRK1 [7].

A similar proteomic screen of the α7 subunit uncovered more than 50 new proteins that bound α7 nAChRs in the cortex of mice [59]. This screen revealed a number of shared α7 and β2 nAChR interactions and highlighted a difference in the interaction network between the two subunits. Common to both α7 and β2 subunits was the ability to physically associate with several G proteins including $G\alpha_{12}$, $G\alpha_i$, and $G\alpha_o$ subunits as well as $G\alpha_q$ and Gβγ in the brain [59]. These findings strongly support our hypothesis that nAChRs interact with G proteins in neural cells.

Evidence on direct coupling between G proteins and nAChRs also comes from protein pull-down experiments using a glutathione S-transferase (GST) fusion protein of the M3–M4 loop of several nAChRs. These pull-down studies clearly show that several Gα as well as Gβγ subunits can bind the M3–M4 loop segment [58]. Interactions between nAChRs and G proteins are summarized in Table 1. As shown in Table 1, the findings are not entirely conclusive and based on a limited number of published observations. For example, $G\alpha_o$ appears to interact with α3, α4, α5, α7, β2, and β3 subunits [58–60], but Gβγ can only bind α3, α4, α5, and β2, and not β3, β4, or α7 subunits [58]. Binding inconsistencies between nAChRs and G proteins may stem from important experimental variables within the M3–M4 pulldown assay. First, a number of studies are based on recombinant expression of the GST M3–M4 loop fusion protein within *E. coli*, in which post-translational modification and protein editing will not be similar to that in mammalian cells. These changes may explain a significant amount of variability in specificity and binding affinity between G proteins and the M3–M4 loop of the nAChR. Second, it is tempting to speculate that the minimal structure for G protein binding exists not within one but multiple M3–M4 loop segments, which are rendered accessible only in the fully formed pentameric receptor. This notion is supported by our observation of the ability to detect $G\alpha_{i/o}$ interaction in an immunoprecipitation assay of the endogenous β2 nAChR, but not in a pull-down experiment of the M3–M4 loop of the β2 subunit [7].

Identification of a G protein-binding site within the nicotinic receptor

Cys-loop LGIC proteins are differentially regulated by G proteins [74]. Of note is the glycine receptor (GlyR) because G proteins directly modulate them. An important study by Yevenes et al. [75] shows a role for Gβγ in regulating the amplitude of the GlyR in cells. The effect of Gβγ on the GlyR was phosphorylation-independent but highly sensitive to pertussis toxin. The same study reveals that Gβγ enhances GlyR function by increasing the apparent affinity of the receptor for glycine, as measured by increased channel open probability (Fig. 4A) [75]. Exposure to GTP-γ-S or overexpression of Gβγ in the cell can strongly potentiate the receptor, which is consistent with the effect of Gβγ on other ion channels at the plasma membrane [75].

Basic amino acids such as arginine (R), histidine (H), and lysine (K) mediate G protein binding to targets such as β adrenergic kinases, GIRK channels, Ca^{2+} channels, and phospholipase C [76–80]. Based on structural mutagenesis, a series of basic residues in the M3–M4 loop of the GlyR have been found to accommodate Gβγ interaction [81]. As shown in Fig. 2B, two separate amino acid sequences (RFRRK and KK) in the M3–M4 loop of the α1 subunit of the GlyR appear necessary for Gβγ binding [81]. Mutations at these residues not only inhibit Gβγ interaction with the GlyR, but also change receptor potentiation by ethanol [82].

An alignment of residues in the M3–M4 loop of nAChRs reveals similar

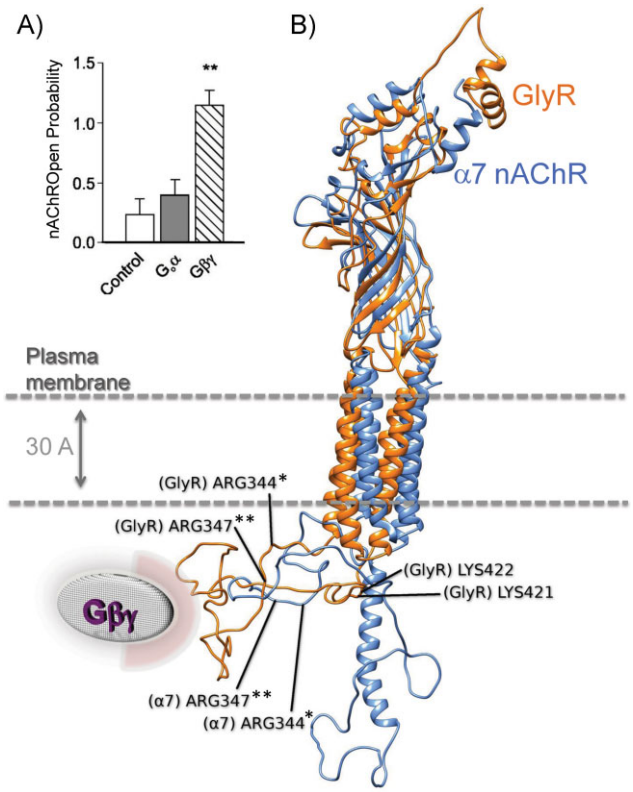

Figure 4. Structural model of a G protein-binding site within GlyR and α7 nAChR. **A:** Effect of $G\alpha_o$ and Gβγ on nAChR channel open probability in rat intrinsic cardiac ganglia neurons (mean ± SEM; $^{**}p < 0.01$). (Adapted from [58, 68]). **B**: A structural model of a subunit for the human GlyR and α7 nAChR. For the GlyR, NCBI: NP_001139512.1 was used as a query sequence, whereas the sequence NCBI: NP_000737.1 was used for the α7 nAChR in addition to PDB: 2BG9 chain A as a template. *C*-scores of −1.00 and −2.20 were obtained for the best GlyR and α7 nAChR I-TASSER models, respectively. The loop segments were generated using LoopyTM [22] and then energetically filtered to the top 10 candidates using Dfire [91]. A top conformation is presented. The nAChR and GlyR structures show proximity of ARG residues 344 (*16.1 Å) and 347 (**15.8 Å), which are known to be involved in the Gβγ binding of the GlyR [75]. LYS residues 421 and 422 within the GlyR are also known to contribute to Gβγ binding.

basic amino acids in nAChRs (Fig. 2B). In particular two positively charged amino acids (K and R) in the M3–M4 loop of the nAChR and GlyR indicate sequence conservation at the G protein-binding site. Structural modeling of the α7 nAChR and the GlyR through LoopyTM [22] and JACKAL software [23] suggests that these putative G protein binding residues within the M3–M4 loop are near the plasma membrane (Fig. 4B) [21, 83]. In particular, amino acids R344 and R347, which mediate GlyR interaction with Gβγ [75], appear conserved and similarly oriented to the plasma membrane in the α7 nAChR and GlyR structures. Residues K421 and K422 which also contribute to GlyR association with Gβγ are also near the plasma membrane and in the vicinity of R344 and R347 in both the α7 nAChR and the GlyR. Structural alignment of the α7 nAChR and the GlyR subunits using TM-Align [83] confirms the structural homology between the two receptor subunits. The structural data support our hypothesis and suggest that nAChRs and GlyRs bind Gβγ via similar features of the M3–M4 loop.

At this point, however, it is not clear whether these residues contribute to G protein binding. This question can be addressed directly in future studies using site-directed mutagenesis to alter the nAChR peptide sequence at these specific sites. If the residues contribute to Gβγ interaction with the nAChR, a next step would be to investigate if each receptor subunit can bind a Gβγ or do multiple nAChR subunits contribute to Gβγ association. Studies of Gβγ interactions with GIRK channels suggest that only one Gβγ binds to the tetrameric GIRK channel [84]. If this is also the case for the nAChR, it may explain the incremental potentiation of the nAChR initiated by adding Gβγ in patch clamp experiments [85].

Nicotinic receptor association with G proteins regulates neurite growth

While nAChRs are an important class of ion channels that modulate neuronal activity, evidence now suggests that they function by also turning on and off longer-lived cellular signaling events. This notion of metabotropic signaling through an ion channel surpasses the limited view that these receptor channels operate solely through ligand driven ion conduction. In non-neuronal cells such as immune cells, nAChRs can regulate inflammatory responses in the absence of a measured electrochemical signal [71]. Binding to the cellular signaling machinery is a fundamental new perspective on the function and regulation of nAChRs in neurons and other cell types.

In a recent study, we demonstrated the existence of an α7 nAChR/GPC comprising the scaffold protein Gprin1, $G\alpha_o$ and growth associated protein 43 (GAP-43) in developing neural cells [60]. Using protein cross-linking, proteomic analysis, and immunoprecipitation methods, we isolated and characterized the functional dynamics of the α7 nAChR/GPC complex. We also identified that α7 nAChR receptor activation (by ACh as well as nicotine) is associated with receptor interaction with $G\alpha_o$ and Gprin1 (Fig. 5). In the ligand activated state, the α7 nAChR receptor is preferentially bound to $G\alpha_o$[GDP], whereas in the inactivate state, the receptor associates with $G\alpha_o$[GTP]. Experiments using the $G\alpha_o$ activator mastaporan and the $G\alpha_o$ inhibitor pertussis toxin confirm an effect of Gα signaling on neurite growth (Fig. 5) [60].

Interestingly, binding to G proteins appears central for α7 nAChR mediated effects on neurite growth. This signaling

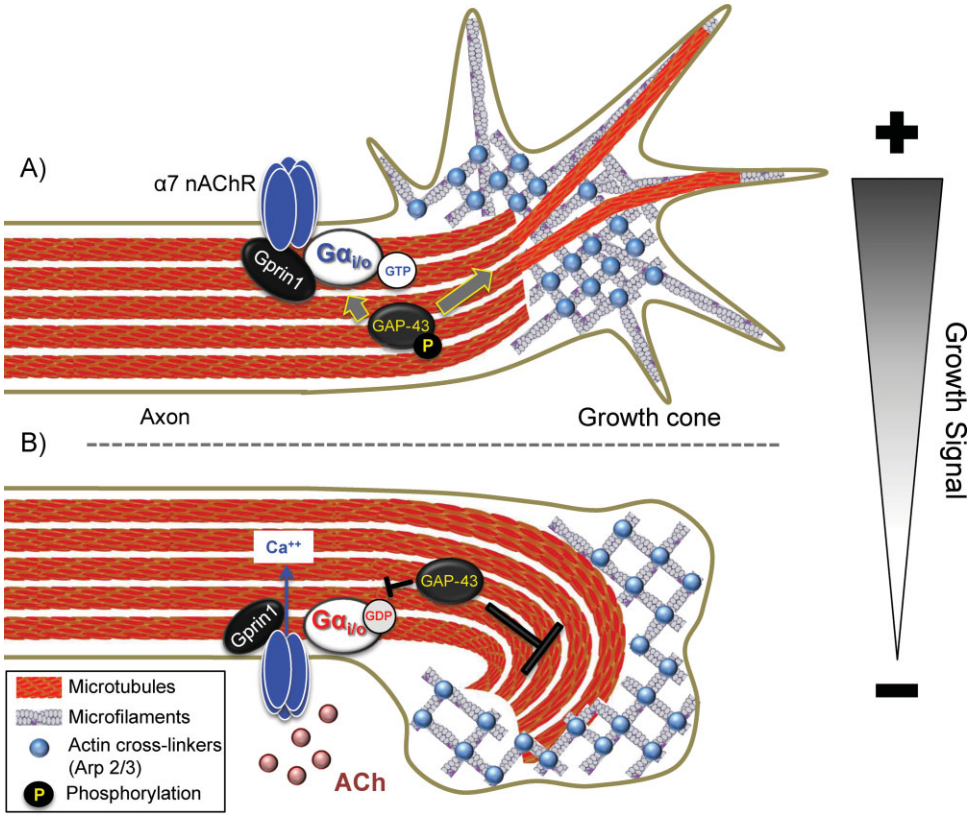

Figure 5. Interaction with G proteins mediates α7 nAChR signaling during axon growth. GAP-43 is a chief mediator of $G\alpha_o$ and cytoskeletal proteins actin and tubulin [60]. **A:** In the inactive state, the α7 nAChR associates with $G\alpha_o$ (GTP bound), which is associated with GAP-43 phosphorylation in the growth cone. This process drives cytoskeletal assembly and axon growth. **B:** Activation of the α7 nAChR, on the other hand, promotes the dephosphorylation of GAP-43 (by the calcium sensor PP2B) and an inhibition of $G\alpha_o$ (GDP bound). This leads to cytoskeletal disassembly and collapse of the growth cone [60].

pathway is driven via the ability of GAP-43 to regulate G proteins and the assembly/disassembly of the axon cytoskeleton. $G\alpha_o$ in particular is enriched in the growth cone [86]. Thus by directly coupling to G proteins, the α7 nAChR signals to regulate axon growth. While it is interesting to consider that G protein signaling via the α7 nAChR can occur simultaneously with ion conduction, the kinetics of α7 channel activation and deactivation are dramatically faster than those of the G protein signaling cycle [6]. Current data thus allows for an intracellular signaling mechanism of the nAChR *independent* of ion conduction, while suggesting that calcium influx through the open channel can also contribute to longer-lived G protein signaling. This is supported by the finding that the phosphorylation of GAP-43 by the calcium sensor calmodulin kinase II is at least in part driven by α7 nAChR calcium entry into the neurite [60].

Conclusion

The emergence of protein-protein interaction domains in various molecules is suggested to be one way in which evolution accommodates adaptations in cellular signaling [87]. For various nAChRs interaction with G proteins appears to be a functional metabotropic component of the channel response, alongside its ionotropic function. The evidence put forth here is compelling and provides a new testable framework for exploring G protein interaction with nAChRs. Future experiments based on the construction of nAChR mutants with specific site directed mutations of the proposed G protein binding residues and their analysis in electrophysiological and biochemical assays will provide information on the role of G proteins in nAChR function.

In the brain, nAChRs have been found in presynaptic terminals, postsynaptic compartments, and in various other non-synaptic regions of the cell [69, 70]. Pre-synaptic receptors regulate neurotransmitter release [71], while post-synaptic receptors contribute to plasticity and neuronal excitability [72]. While nAChR signaling capacity is influenced by subtype dependent desensitization to ACh [72], regulation by G proteins may modify receptor activity and critically amplify nAChR signaling within the cell. The computational models provided on the structure of the M3–M4 loop in the nAChR support intracellular loop localization but point to a structure capable of some degree of spatial mobility at equilibrium (Fig. 4). Because the presented models are based on the predicted conformation of an individual receptor subunit, the pentameric assembly of the nAChR may facilitate loop-loop interactions in

the final tertiary protein. Interestingly, binding to G proteins, and or being in proximity of other cellular binding partners, may also influence the conformation and function of the M3–M4 loop in the cell [88].

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