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Commentary

Associated proteins: The universal toolbox controlling ligand gated ion channel function

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ABSTRACT

Ligand gated ion channels are integral multimeric membrane proteins that can detect with high sensitivity the presence of a specific transmitter in the extracellular space and transduce this signal into an ion flux. While these receptors are widely expressed in the nervous system, their expression is not limited to neurons or their postsynaptic targets but extends to non-neuronal cells where they participate in many physiological responses. Cells have developed complex regulatory mechanisms allowing for the precise control and modulation of ligand gated ion channels. In this overview the roles of accessory subunits and associated proteins in these regulatory mechanisms are reviewed and their relevance illustrated by examples at different ligand gated ion channel types, with emphasis on nicotinic acetylcholine receptors. Dysfunction of ligand gated ion channels can result in neuromuscular, neurological or psychiatric disorders. A better understanding of the precise function of associated proteins and how they impact on ligand gated ion channels will provide new therapeutic opportunities for clinical intervention.

1. Introduction

Cells can be considered as discrete entities separated from the outside milieu by their plasma membrane. Pluricellular organisms have evolved multiple and redundant systems to ensure communication between cells and transmission of information. One means of achieving fast communication between cells is through ligand gated ion channels (LGICs), integral membrane proteins that have progressively evolved for this purpose. In mammals, LGICs are widely expressed throughout the body and are highly represented in the central nervous system. These ion channel receptors are specific for a given transmitter and accomplish the major task of detecting transmitter molecules and changing the membrane permeability as a consequence of this recognition event. This can modify the membrane potential of the cell, and/or alter concentrations of second messengers, e.g., Ca^{2+}, triggering a variety of short and longer term cellular events.

LGICs are subdivided according to their ionic permeabilities and the neurotransmitter that they are able to detect with high affinity. There are two classes of vertebrate LGICs permeable to anions: the γ-amino butyric acid receptors ($GABA_AR$) and glycine receptors (GlyR). Cation-selective receptors include glutamate (GluR), purinergic (P2X), serotoninergic ($5HT_3$) and nicotinic acetylcholine receptors (nAChRs). LGICs are multimeric proteins that define 3 distinct gene families. The best characterized is the cys-loop family of pentameric receptors that includes GlyR, $GABA_AR$, $5HT_3R$ and nAChR [1]. The GluR gene family comprises tetrameric ionotrophic receptors defined by their specificity for AMPA, kainate or N-methyl-D-aspartate (NMDA) [2]. Finally, P2X receptors constitute a distinct family of trimeric cation channels gated by ATP [3,4]. For each LGIC class, multiple subunit-encoding genes have been identified in the human genome; for example, sixteen nAChR subunit genes have been identified to date [5]. Different subunit combinations generate multiple subtypes of each receptor.

LGICs are important therapeutic targets and functional defects in LGICs can cause channelopathies with neurological manifestations. For example, mutations in neuronal nAChR or $GABA_AR$ are associated with some isoforms of genetic epilepsy [6]. Mutations in the GlyR give raise to hyperekplexia, also known as "stiff baby syndrome" or "startle disease" [7]. Anti-NMDA receptor encephalitis is an autoimmune condition associated with profound neurological symptoms including psychosis and seizures [8,9].

Abbreviations: LGICs, ligand gated ion channels; GluR, glutamate receptor; GlyR, glycine receptor; $GABA_AR$, γ-amino butyric acid receptor; P2X, purinergic; $5HT_3R$, 5-hydroxytryptamine 3 receptor; nAChR, nicotinic acetylcholine receptor; NMDA, N-methyl-D-aspartate; SLURP, secreted Ly-6/uPAR-related protein.

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Although much has been learnt over the past two decades regarding LGIC structure and function, less is known about how cells regulate LGIC activity. The aim of this commentary is to discuss, in the light of the most recent findings, the diverse mechanisms so far identified that cells utilize to modulate receptor activity, directly and indirectly, and to tailor their physiological response to a given stimulus. We highlight common mechanisms or unique strategies associated with different members of the LGIC family.

2. Associated proteins

The tenet of this article is that receptor function is regulated via physically associated proteins. We define associated proteins as either permanent or transient, depending on the duration of their interactions. Permanently associated proteins are typically incorporated in the receptor complex during its synthesis or assembly whereas transiently associated proteins interact only temporarily and reversibly with the receptor complex.

A schematic representation of these two types of association is shown in Fig. 1. Permanently associated proteins are typically integral receptor subunits including those that modify receptor function or regulation without participating in the formation of the ligand-binding site. For nAChR, such subunits have been termed "accessory subunits" [10]. Heteromeric pentameric neuronal nAChR require 4 subunits (2 α and 2 β) to contribute ACh binding sites at the α/β subunit interfaces plus a fifth subunit to complete the ion channel. This fifth (accessory) subunit can influence many features of the receptor, including agonist sensitivity, channel kinetics, Ca^{2+} permeability, receptor assembly and subcellular targeting. α5 and β3 neuronal nAChR subunits are thus "obligate accessory subunits", in that they are incapable of forming ACh binding sites [10,11].

Transiently associated proteins are those that reversibly interact with a receptor (Fig. 1B); they comprise a wide range of proteins and include enzymes that catalyze post-transcriptional and post-translational modifications. Other subgroups of transiently associated proteins include chaperones, e.g., RIC-3 [12,13] and the Ca^{2+} sensor VILIP1 [14,15] that participate in the maturation of functional receptors and their expression at the cell surface, and scaffold proteins like the PDZ-domain proteins

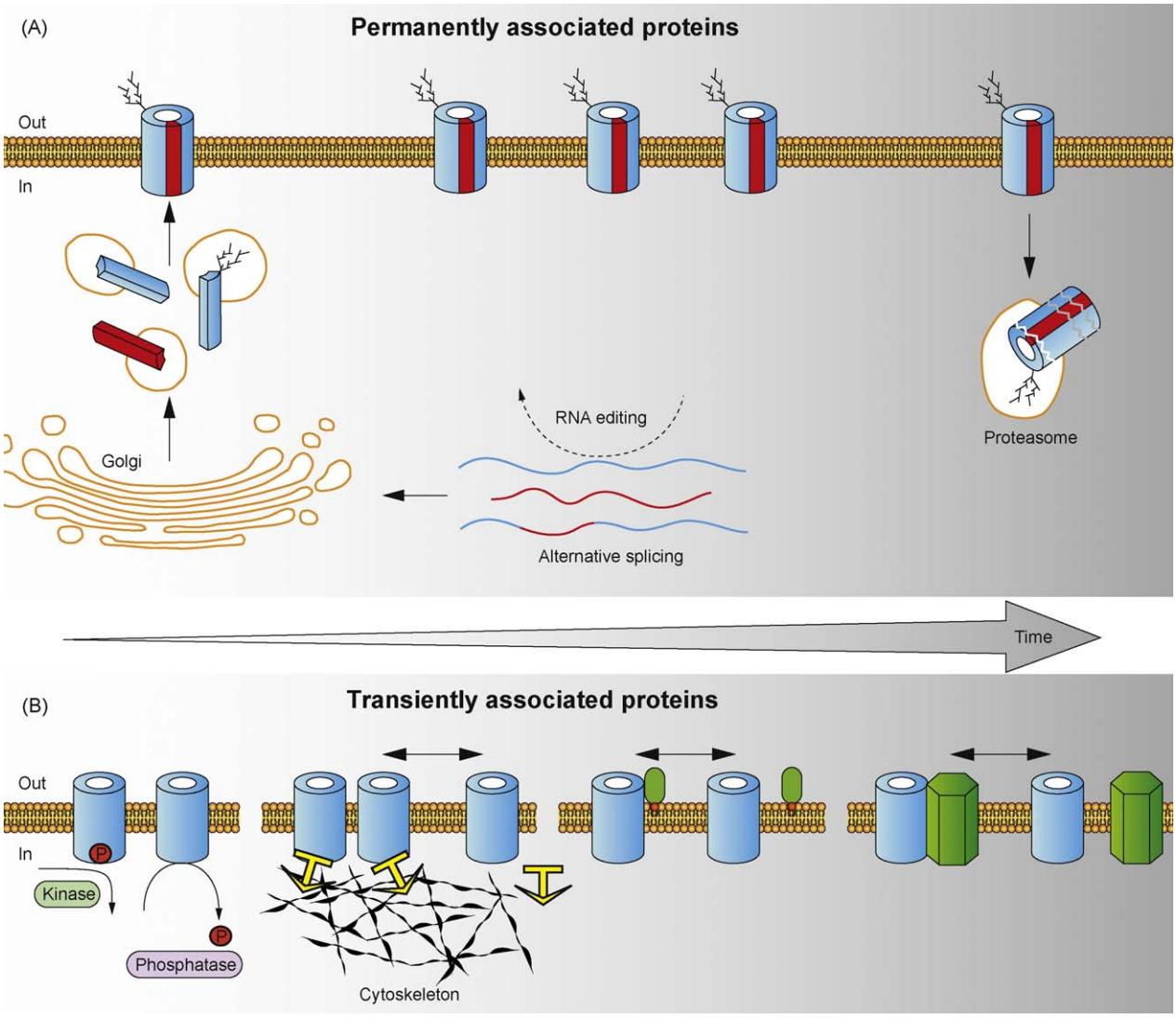

Fig. 1. Schematic representation of LGICs and associated protein interactions. The (A) panel summarizes permanent association possibilities, reflected in the diversity of integral subunits. Genes coding for LGIC subunits are transcribed in the nucleus in response to different stimuli. After export to the cytoplasm, mRNA may undergo post-transcriptional modification (mRNA editing, alternative splicing), increasing the diversity of gene products. Translated in the endoplasmic reticulum, subunits are assembled into multimeric receptors in the Golgi, where some post-translational modifications may occur, and the newly synthesized receptors are exported to the plasma membrane. The (B) panel summarizes the principal transient associations with interacting proteins. Interactions represented from left to right are: phosphorylation, anchoring to the cytoskeleton, interaction with extracellular proteins (such as GPI-anchored Lynx-1) and transmembrane proteins.

[16] that stabilize receptors at pre- or postsynaptic sites. Transiently associated proteins are not exclusively intracellular. Transmembrane proteins such as the TARPs [17] and cornichon [18] are auxillary subunits that accompany AMPA receptors in the synaptic membrane, and the secreted Ly-6/uPAR-related protein SLURP-1 acts extracellularly as a powerful allosteric modulator of α7 nAChR [19].

It is important to note that permanent and transient protein interactions are not mutually exclusive but can combine to generate more complex patterns of receptor modulation. Insertion of a given subunit in a receptor complex will introduce its own repertoire of transient protein interactions. An example of such combined effects is provided by the $GABA_AR$: their sensitivity to benzodiazepines requires the presence of the γ2 subunit (defined here as a permanent protein interaction), which presents novel phosphorylation sites and also permits a transient protein interaction with the dopamine D5 receptor [20–22]. This is discussed further below.

3. Modes of receptor regulation

3.1. Receptor structure

LGICs are multi-domain proteins. They comprise an extracellular domain that contains specific recognition sites for transmitter, and may have additional sites for allosteric regulators; in the case of GluR, there is a distinct N-terminal domain for putative regulatory interactions [2]. The transmembrane segments of LGICs constitute the ion channel, and a conformational transition couples agonist binding to channel opening. A cytoplasmic domain facilitates interactions with intracellular scaffolds and modulators.

3.2. The concept of receptor activation and desensitization

First identified at the neuromuscular junction, nAChR are the prototype LGIC [23]. Electrophysiological recordings carried out at the muscle end plate revealed that exposure to the agonist (ACh) causes a rapid inward current (activation) followed by a progressive decline upon sustained agonist exposure (desensitization). This led to the concept that nAChRs have at least three states: receptors are closed at rest, open during activation and closed by desensitization. Recordings of single channel activity at the neuromuscular junction marked another step in understanding the receptor processes with the finding that single receptors display only two discernable states, open or closed [24]. Thus, activation and desensitization must reflect the probability of opening and closing of the channels.

The simplest and most robust model to explain this behavior of nAChRs, and LGICs in general, is the allosteric model, derived from the theory of allosteric regulation developed by Monod et al. [25,26]. This assumes that the receptor has multiple conformational states. In the absence of ligand, the receptor has a certain probability to occupy any of these states. Exposure to a ligand preferentially stabilizes the receptor in a given state. The minimal allosteric model comprises three states: the resting R (closed), the active A (open) and the desensitized D (closed) state (Fig. 2A). A refinement of this model is the incorporation of a series of slowly interconverting inactive states (reviewed in Ref. [26]). The binding of agonists favors the open state, whereas the binding of

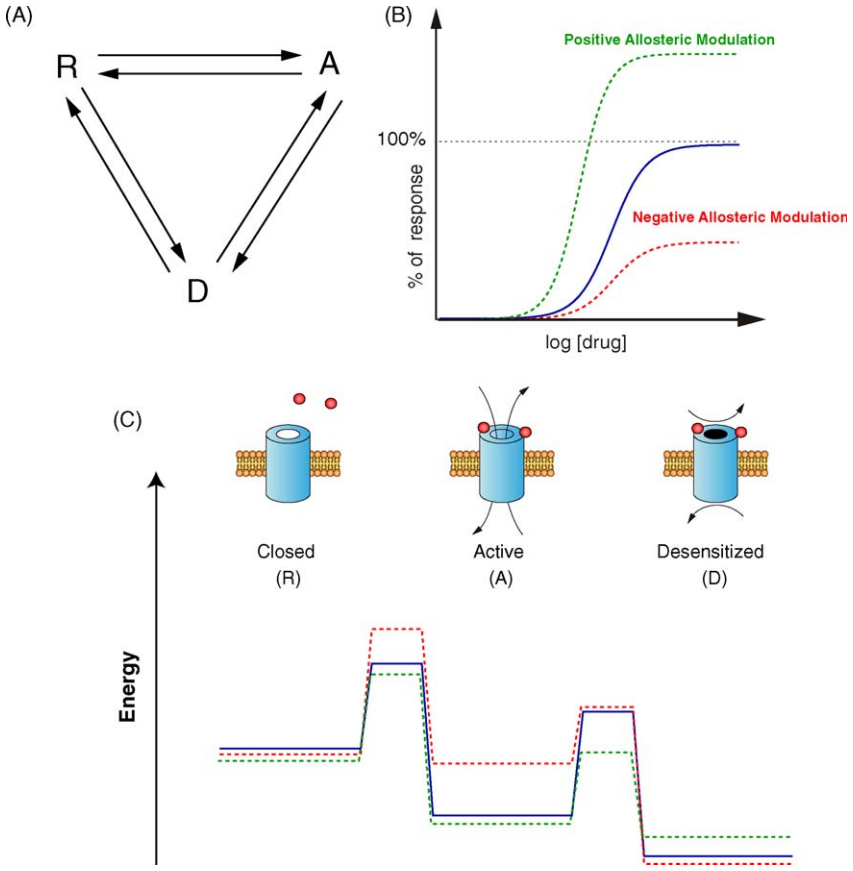

Fig. 2. Modeling LGICs properties with the allosteric concept. (A) Minimal three state model required for the description of receptor activation and desensitization, such as proposed by Monod et al. [25]. (B) Schematic representation of concentration activation curves illustrating the effects caused by the presence of a positive or negative allosteric modulator. (C) Effects of an allosteric modulator on the transition energy barriers.

competitive antagonists preferentially stabilizes either the closed or desensitized state. Partial agonists exhibit a low efficiency of channel opening while allosteric modulators act at sites distinct from the agonist binding site to influence the transitions between states (Fig. 2B and C).

Within the same family, different receptor subtypes may display significantly different properties, for example with respect to agonist sensitivity and propensity to desensitize. In the nAChR family, the heteromeric receptors at the neuromuscular junction show a sustained response to ACh, whereas the homomeric neuronal α7 nAChR exhibit a very transient response with a fast desensitization. Moreover, α7 nAChR have much greater relative permeability to Ca^{2+} than muscle nAChR. Similar functional differences can be found between kainate and NMDA receptors in the GluR family or between homomeric $P2X_2$ and $P2X_3$ receptors in the purinergic family [2,27].

3.3. Intrinsic versus extrinsic regulation

Cellular responses to transmitter actions are shaped by the properties of individual receptors. These properties may be modified by permanent associated proteins and post-translational modifications. The magnitude and impact of cellular responses will also reflect other factors such as the density and localization of receptors within the cell. These aspects are determined by assembly, trafficking, stabilizing interactions and internalization events, governed by specific transient protein associations that can be considered extrinsic to receptor function at the molecular level. The dynamics of these processes are critical for neuronal plasticity. Crosstalk through physical association with other signaling proteins that adds another level of complexity has yet to be fully appreciated and certainly necessitates caution in interpreting data obtained using non-native receptor constructs, e.g., those in which the receptor is expressed in a cell line lacking many of the ancillary proteins that are required for physiological function. A further corollary is that disease states associated with receptor dysfunction may not always involve the receptor per se but differences in the ancillary protein repertoire of the dysfunctional tissue.

4. How associated proteins modulate receptor function

4.1. The importance of receptor subunit composition

The activation and desensitization properties of a given receptor depend upon the thermodynamic properties of the receptor complex (Fig. 2C). Implicit in the allosteric model, the transition from the resting to the active state is governed by the energy barrier that exists between these two states. The simplest form of a LGIC is composed of identical subunits as seen, for example, with the homomeric α7 nAChR, GlyR, $5HT_3$ or $P2X_4$ receptors. Only a restricted number of subunits are able to assemble as homomeric complexes; of these some preferentially associate with other subunits to form heteromeric receptors. For example the α9 nAChR subunit associates with the α10 to form a functional nAChR in cochlear hair cells [28], the $5HT_{3A}$ subunit can associate with $5HT_{3B}$ [29], and the GluN1 subunit associates with the GluN2A and/or GluN2B subunits in native NMDA receptors [30,31]. Incorporation of a subunit to form a heteromeric receptor complex represents the prototype of a permanent association as this "associated protein" modifies both the physiological and pharmacological properties of the receptor.

Firstly, let us consider the effect caused by the associated protein in a heteromeric receptor composed of only two subunit types. Remembering that the agonist binding site resides at the subunit interface of cys-loop receptors, it is easy to understand that the amino acids that compose the ligand-binding site will change when subunits of different types are incorporated into the receptor complex. This is exemplified by an nAChR composed of α3 and β2 subunits versus one formed by α3 and β4 subunits: these two receptors differ in their sensitivity to ligands and desensitization [32], and this is reflected in their different physiological roles, in the central and autonomic nervous systems, respectively. Native nAChR can be more complex; addition of the α5 subunit increases the sensitivity to ACh when associated with α3 and β2 subunits but not α3 and β4 subunits, but enhances the calcium permeability of both subtypes [33].

As the α5 nAChR subunit is believed to be an accessory subunit (discussed above), it does not alter receptor properties by contributing directly to the agonist binding sites. Associated subunits can also confer changes in the energy barrier or create additional, modulatory sites. This is illustrated by the requirement for the incorporation of the γ2 subunit into functional $GABA_A$ receptors for benzodiazepine sensitivity [34]. This is readily explained by the finding that the benzodiazepine binding site resides at the γ/α interface, in contrast to GABA binding sites at the α/β subunit interfaces [20]. The NMDA receptor family provides an interesting example of an obligate heterotetramer of agonist binding and modulatory subunits. Two GluN2 subunits contribute glutamate binding sites whereas the GluN1 or GluN3 subunits that they associate with confer binding sites for the essential co-agonist glycine [2].

While it is beyond the scope of this review to catalog the multiple examples for the different receptor families, the important point to note is that association of different subunits determines the properties of a receptor and hence vastly increases the repertoire of receptors, fine-tuned for distinct tasks. A good example of tailoring properties to physiological needs is given by extrasynaptic $GABA_A$ receptors that display a high sensitivity to agonist and very slow desensitization properties that aid the detection of tonic levels of neurotransmitter. These properties are conferred by incorporation of β2 and δ subunits in extrasynaptic $GABA_A$ receptors [35]. Similarly, functional distinctions are seen between embryonic and mature forms of nAChR in skeletal muscle, with substitution of the ε subunit for the γ subunit in early development [36].

Interestingly, major differences between subunits within a given receptor family are found in the amino acid segments that face the cytoplasm. These intracellular loops or terminal sequences facilitate interactions with other associated proteins, including anchoring proteins, enzymes and other receptors or membrane proteins. These interactions are dictated by the subunit composition of a receptor and will be discussed in Sections 4.4 and 4.5.

4.2. RNA editing and alternative splicing

We have already seen that subunit heterogeneity generates receptor diversity by affecting the ligand-binding site or by changing the energy barriers between transitions to alter the functional properties of receptors. Receptor subunit isoforms are normally encoded by distinct genes but additional variants of a given subunit can be generated by alternative splicing and/or post-transcriptional modifications. The formation of receptors by association of such modified subunits with non-modified subunits enriches the panoply of physiological and pharmacological properties.

RNA editing allows genetically encoded amino acid sequence to be altered at the RNA level, by deamination of adenosine (A) or cytosine (C) residues to generate inosine (I; read as guanosine) or uracil (U), respectively. This is achieved by deaminases, enzymes that transiently associate with receptor subunit RNAs that function as substrates. Depending upon the codon where substitution occurs, this can result in amino acid substitution. Well-documented examples of A to I RNA editing are found in AMPA and kainate

GluRs. A glutamine to arginine (CAG to CIG, Q607R) substitution in the pore-lining domain (M2) of GluA2 subunits causes a marked reduction in calcium permeability [37]. This is a critical event in development and prevention of editing results in severe epilepsy [38]. This editing event also reduces sensitivity to intracellular polyamines [39] and allows faster recovery of the receptor from desensitization. Similar glutamine to arginine RNA editing occurs during development in GluK1 and GluK2 subunits of the kainate receptor, with comparable changes in calcium permeability [40].

More recently, developmentally important A to I RNA editing of the $GABA_AR$ α3 subunit has been demonstrated [41]. The resultant isoleucine to methionine switch (I342M) in the third transmembrane domain is proposed to decrease assembly of the developmentally abundant α3-containing $GABA_AR$ in favor of the α1-containing subtype that predominates in adults [42]. GlyR α subunits undergo C to U RNA editing that generates a single amino acid substitution (proline to leucine) in the N-terminal domain (P185L). This increases GlyR affinity for glycine without altering desensitization kinetics [43,44].

The process of mRNA maturation consists of removing the intronic (non-coding) sequences and stitching together exons. For genes presenting several exons, alternative splicing can generate multiple isoforms of mature mRNAs, depending upon the inclusion or removal of one or more exons. As a consequence, alternative splicing yields proteins with distinctive amino acid sequences. Perhaps the best characterized example within the LGIC families occurs in the two splice variants of AMPA receptor subunits, termed "flip" and "flop". These isoforms differ by 38 amino acids located between the third and fourth transmembrane domain, a region that contributes to the agonist binding domain and may influence channel gating [45]. The "flop" variant of GluA2 and GluA3 (but not GluA1) displays shorter channel open time than the "flip" variant, which is reflected in the whole cell current by an increased rate of desensitization [46,47]. Comparison of the gene sequences encoding GluA1–4 reveals that GluA2 differs in having a shorter DNA segment separating the RNA editing site from the exon 13–14 flip/flop splicing site. This proximity has led to the suggestion that mutual interactions between RNA editing and alternative splicing probably occur and could be coordinated [48].

Alternative splicing may be a widespread mode of generating additional receptor diversity. Splice variants of NMDA and P2X receptors have also been reported [49,50]. A well-documented example within the cys-loop family of LGICs is the presence or absence of an eight amino acid insertion in the cytoplasmic loop of the γ2 subunit, to generate γ2 long (L) and short (S) isoforms, respectively [51]. This insertion generates an additional phosphorylation site with numerous functional implications.

α7 nAChR in autonomic ganglia exhibit evidence of alternative splicing. α7 nAChR in rat superior cervical ganglia display a slow rate of desensitization and rapidly reversible blockade by the snake toxin α-bungarotoxin [52]. These properties were correlated with a variant subunit in which exon 4 is replaced by the novel exon 4a [53]. Several novel isoforms of α7 nAChR have been identified in brain tissues from schizophrenics and suggest the occurrence of multiple transcription-level modifications with corresponding pathological implications [54].

4.3. Receptor assembly and trafficking: association with chaperones

For each class of receptor, the diversity of subunits generated by multiple genes and post-transcriptional mechanisms poses a serious challenge for the regulated assembly of receptor subtypes of particular subunit composition and their correct disposition in the cell or subcellular compartment. Integral associated subunits (e.g., the "accessory" subunits α5 and β3 in nAChR) can influence these processes but chaperones play a determinant role in the folding, assembly and trafficking of complex proteins such as LGICs. Through specific physical interactions they can prevent subunits from aggregating into non-functional structures and are thought, for the assembly of multimers, to regulate the final subunit composition.

Genetic analysis in *Caenorhabditis elegans* identified RIC-3 (Resistant to Inhibitors of Cholinesterase), a protein important for the maturation of nAChR (folding, assembly and surface expression) [12,55] that is effective in facilitating expression of homomeric α7 nAChR [12]. Other groups however, report contradictory results concerning the influence of RIC-3 upon heteromeric nAChR subtypes, such as α4β2 [56,57]. Recent evidence indicates that these differences in activity may depend upon the host cell, suggesting the involvement of additional, cell-specific proteins [58]. While other well known chaperones such as the 14-3-3η protein [59], BiP or calnexin [60] associate with and/or influence trafficking of multiple LGICs, RIC-3 appears to show more restricted specificity, although it can also modulate the maturation of $5HT_3$ receptors [13].

For LGICs the chaperone concept has been extended to receptor agonists; although these are not associated proteins, they merit attention alongside conventional chaperones. It is proposed that only functionally competent GluR can exit the endoplasmic reticulum and that the binding of glutamate provides the necessary "quality control" assessment [61,62]. However, the TARP, stargazin can interact with and promote AMPA receptor trafficking in the absence of bound glutamate so putative interactions between pharmacological and proteinaceous chaperones remain to be resolved.

Another example of ligand interaction is nicotine which has been proposed to act as an exogenous pharmacological chaperone for nAChR. This hypothesis is based on the observation that chronic exposure to nicotine causes the "upregulation" of nAChR by promoting the oligomerisation and maturation of high affinity receptors [63,64] leading to the introduction of the term SePhaChARNS, for "selective pharmacological chaperoning of acetylcholine receptor number and stoichiometry" [65]. It is presently unclear if there is an endogenous pharmacological chaperone for nAChR or how nicotine might interface with interacting proteins, such as those of the Lynx-1 family, that might also play a role in nAChR biosynthesis and maturation.

As well as promoting the assembly and trafficking of LGICs, associated proteins could also exert the opposite effect by acting as a negative regulator of receptor expression. This is exemplified by the action of HSP90 on $P2X_7$ receptors [66].

4.4. Receptor clustering and stabilization in the membrane

The aggregation of LGICs in the postsynaptic membrane is an important step in the development and stabilization of a synapse and has been particularly well studied for nAChR at the neuromuscular junction [67]. Although a specialized synapse, the muscle endplate illustrates some key aspects. On clustering at the muscle endplate, nAChR become less susceptible to disassembly during maturation and their lifetime in the membrane is increased from a few hours to several days. This was reflected in the longer retention of radioactive α-bungarotoxin at adult neuromuscular junctions compared with embryonic tissue [68]. Once established, the neuromuscular junction remains extremely stable throughout life. Motorneuron activity is required for endplate formation and secretion of ACh and the heparin proteoglycan, agrin act in concert to establish monosynaptic innervation of muscle. Agrin activates the transmembrane receptor tyrosine kinase MuSK (muscle-specific kinase) that leads to clustering of nAChR via rapsyn. This 43 kDa protein binds nAChR and may serve to tether the receptor to the cytoskeleton. This has been proposed to occur through ACF7 (also known as MACF), a

protein of the spectrin superfamily, that has multiple cytoskeleton-binding domains, including an N-terminal actin-binding domain that directly interacts with rapsyn [69]. Although rapsyn co-purifies with muscle nAChR, how these two proteins interact remains unclear. Recent studies suggest that rapsyn binds to the cytoplasmic loops of multiple nAChR subunits; MuSK-dependent phosphorylation of the β1 subunit may consolidate or stabilize clustering through its interaction with rapsyn [70].

The developmental switch in nAChR subunit composition from γ to ε subunit during maturation of neuromuscular junction might contribute to anchoring nAChR clusters to the cytoskeleton, in addition to altering the properties of the receptor [70]. As GluR, $GABA_AR$ and GlyR also exhibit developmental switches in subunit composition (see Sections 4.1 and 4.2) this could reflect a common mechanism in synapse maturation.

Anchoring LGICs to the cytoskeleton via interaction with specialized linker proteins, some of which bind F-actin filaments, is a common feature. At inhibitory synapses, the cytoplasmic protein, gephyrin has a role analogous to that of rapsyn. It was initially co-purified with the GlyR, and associates with the cytoplasmic domains of $GABA_AR$ and GlyR subunits, in particular interaction with the α2 $GABA_AR$ or β GlyR subunit is important for receptor trafficking/clustering [71]. Through auto-oligomerisation gephyrin fosters clustering by forming a scaffold that links the receptors to the cytoskeleton and other associated proteins [72]. For example, the ubiquitin-like protein, ubiquilin (or Plic-1) associates with $GABA_ARs$ at inhibitory synapses and has been shown to protect this complex from proteasomal degradation [73]. The lifetime of $GABA_ARs$ is prolonged by the presence of ubiquilin which participates in its stabilization in the plasma membrane. The co-localization of $GABA_A$ receptors, gephyrin and multiple associated proteins such as ubiquilin is important not only for the construction of inhibitory synapses but as determinants of the inhibitory strength of the synapse [74].

In contrast to rapsyn and gephyrin that lack PDZ domains, the PSD-95 family of PDZ scaffolding proteins is associated with the organization of glutamatergic synapses [16,75]. PSD-95 regulates the activity of LGICs by influencing their subunit composition, subcellular localization, surface expression, downstream signaling, endocytosis, and even intrinsic functional properties [16,76–78]. However, AMPA receptors are also indirectly linked to PSD-95 via stargazin. This difference may be important for transiently stabilizing laterally mobile AMPA receptors, a critical aspect of synaptic plasticity [79].

PSD-95 proteins also contribute to postsynaptic scaffolds at nicotinic synapses [80]. Directing receptors to the appropriate subcellular compartment and specialized synaptic area requires elaborate mechanisms that are poorly understood. In autonomic neurons, α7 and α3β4 nAChRs are inserted in a concerted manner with precise topological organization of α7 nAChRs on membrane cristae [81]. Using the microchimera approach in which the major intracellular loop (between TM3 and TM4) was exchanged between α3, α5 and α7 nAChR subunits, it was demonstrated that this region is critical for targeting α3-containing nAChR to synaptic domains whereas nAChR lacking this subunit or its intracellular loop has a peri-synaptic localization [82,83]. Factors responsible for targeting and localizing populations of LGICs to presynaptic domains are largely unknown; recently type III neuregulin-1 has been implicated in regulating the surface expression of α7 nAChRs in sensory axons [84].

4.5. Receptor phosphorylation and other post-translational modifications

Attachment or removal of one or more phosphate groups, resulting from transient protein interaction with a kinase or a phosphatase, respectively, is schematized in Fig. 1B. Tight control of the phosphorylation status of target LGIC subunits (and associated interaction partners) may be facilitated by tethering the enzymes to the postsynaptic scaffold [85]. Protein phosphorylation is a rapidly reversible post-translational modification that can act as a switch to influence LGIC function (such as desensitization rate), subunit assembly, receptor aggregation and stability, and synaptic strength [86–90]. Consensus phosphorylation sites for serine/threonine or tyrosine kinases exist in the major intracellular loops of most, if not all, LGIC subunits. For example at the neuromuscular junction, nAChR clustering and stabilization involves phosphorylation of a tyrosine residue within the intracellular loop of the β and δ subunits by MuSK [86,91]. Tyrosine phosphorylation of the α7 nAChR by *src*-kinase was shown to limit receptor activity: the tyrosine kinase inhibitor genistein increased the acetylcholine-evoked responses and the tyrosine phosphatase inhibitor, pervanadate had the opposite effect [92]. Ablation of the putative tyrosine phosphorylation sites in the intracellular domain produced more active α7 nAChR that were insensitive to genistein and pervanadate. Tyrosine phosphorylation of the NMDA receptor GluN2A subunit was recently proposed to have a profound effect on behavior: mice homozygous for a mutated tyrosine 1325 in the GluN2A subunit displayed antidepressant-like behavior in the tail suspension and forced swim tests [93]. Serine phosphorylation of GluA1 subunits of heteromeric AMPA receptors regulates receptor trafficking to and from the plasma membrane and hence is a determinant of synaptic strength [90].

Other post-translational modifications have recently been recognized to also influence LGIC function. These include palmitoylation, sumoylation and ubiquitination, through association with the requisite enzymes [90,94–96].

4.6. Extracellular protein–protein interactions

LGICs are characterized by the presence of a large domain facing the extracellular space. This region is commonly N-glycosylated, a post-translational modification that aids stability by preventing attack by proteases. Agonists and other molecules bind in this region to alter or influence receptor function. An interesting example is that of the endogenous protein modulator. Lynx-1 that represents an evolutionary and functional relationship with elapid snake toxins. The snake toxin α-bungarotoxin binds to nAChR at the neuromuscular junction with very high affinity, blocking function. α-bungarotoxin and homologous snake toxins sharing the same cysteine-rich three finger backbone compete for the agonist binding site of various neuronal nAChRs [23,97]. Lynx-1 shares structural homology with these snake toxins [98,99]. However, it is a GPI-anchored protein that is co-localized with nAChR; through a physical interaction Lynx-1 modulates receptor affinity and desensitization [99].

The identification of SLURP (secreted mammalian Ly-6/urokinase plasminogen activator receptor-related protein)-1 and -2 as modulators of nAChR in non-neuronal tissues (keratinocytes, epithelial cells and immune cells) extends the family of "prototoxins", as these soluble proteins present a three finger-like structure similar to that of the snake toxins [19,100,101]. SLURP-1 is a potent allosteric potentiator of human α7 nAChR expressed in *Xenopus* oocytes [19] whereas SLURP-2 acts as a competitive antagonist of heteromeric α3-containing nAChR [101]. Thus the SLURPs have opposite actions, considered to be important in modulating the autocrine cholinergic regulation of cell proliferation.

These examples of endogenous protein–protein interactions involving the extracellular domain of LGICs raise the prospect of

modulation of other receptors by secreted or tethered extracellular proteins.

4.7. Transmembrane protein interactions

LGICs are also capable of interacting with transmembrane proteins that may communicate some extracellular signal to the intracellular milieu with concomitant modulation of LGIC function or reciprocal interaction. The transmembrane AMPA receptor proteins (TARPs) [17] provide a well defined example of such an auxiliary protein partner [90]. Stargazin, the prototypical TARP, was the first such protein to be discovered and is essential for functional AMPA receptors in cerebellar granule cells [102]. Although stargazin is co-precipitated with AMPA receptors the site(s) of physical interaction is not established. A recent proteomics study has identified transmembrane, cornichon proteins as alternative intrinsic auxiliary subunits associated with AMPA receptors [103] and we can be confident that more such proteins will emerge as novel partners for other LGICs.

Recently a different kind of association has been described for many LGICs: a direct functional interaction with other receptors, both LGICs and metabotropic receptors, introducing the concept of "horizontal" signaling between receptors in the plane of the membrane. For example, enteric neurons that control gastrointestinal function co-express P2X receptors and nAChR and simultaneous activation of both LGICs in these cells produces inward currents that are smaller than the sum of the individual currents elicited by their independent activation [104]. Functional interaction between $P2X_2$ receptors and $\alpha3\beta4$ nAChR is reproduced when recombinant receptors are expressed in heterologous systems [105,106]. Cross-inhibition also occurs in cell-free patches obtained from cultured neurons, ruling out an interaction via intracellular signaling mechanisms and implicating a direct molecular interaction between the two types of LGIC [107]. Indeed, the C-terminal tail of the $P2X_2$ receptor subtype mediates a physical interaction with $5HT_3R$ and $GABA_AR$ but the relationship with nAChR appears to be more promiscuous and $P2X_3$ and $P2X_4$ receptors also promote cross-inhibition with $\alpha3\beta4$ nAChRs [106,108,109]. Desensitized $P2X_2$ receptors or $\alpha3\beta4$ nAChRs can confer cross-inhibition, consistent with a physical interaction and this is reinforced by the application of elegant microscopy techniques to fluorescently tagged receptors, showing the two channels to be 80 Å apart, compatible with dimer formation [110].

Functional receptor–receptor interactions at the plasma membrane also occur between LGICs and other types of receptor. $P2X_7$ receptors physically interact with Pannexin-1, a pore-forming protein, and together they are proposed to constitute the second, large conductance state associated with ATP responses, with permeability to organic cations including various dyes. Thus this model posits that prolonged stimulation of $P2X_7$ receptors results in activation of Pannexin-1 channels [111].

Interactions between LGICs and G-protein-coupled receptors (GPCRs) are exemplified by the numerous reports of associations with dopamine receptors. The dopamine D5 receptor binds directly, via its C-terminal domain, to the major intracellular loop of the $GABA_A$ receptor $\gamma2$ subunit. The effects of this physical association are reciprocal, producing mutual inhibition [22]. This modulation depends on the presence of the $\gamma2$ subunit so both permanent ($\gamma2$ subunit) and transient associated proteins (D5 receptor) collaborate to generate this regulation. Dopamine D1 receptors form heterodimers with NMDA receptors [112]. The cytoplasmic C-terminal extremity of the D1 receptor can interact via two distinct regions with GluN1 and GluN2A NMDA receptor subunits. The effect of this interaction is the inhibition of NMDA receptor mediated currents with implications in the hippocampus for LTP and working memory [113]. Presynaptic nAChR have also been proposed to physically couple to dopamine autoreceptors (D2/3), via the cytoplasmic loop of the $\beta2$ nAChR subunit [114]. As with the previous examples, the dopamine receptor exerts an inhibitory influence on nicotinic responses.

5. Associated proteins: novel targets or new inspiration for therapeutic approaches

The finding that permanently or transiently associated proteins can modulate LGIC properties presents a potential opportunity for enhancing or decreasing this modulation and hence LGIC function. Modulatory strategies, rather than agonist or antagonist actions, are attractive therapeutically. The flagship class of drugs representing this approach is the benzodiazepines, which interact with the associated $\gamma2$ subunit of $GABA_AR$ to potentiate responses to endogenous agonist. An alternative or complimentary approach would be to manipulate the incorporation of such permanently associated proteins. Sustained expression of the embryonic form of nAChR containing the γ subunit would be a potential treatment for congenital myasthenia gravis caused by mutations in the ε subunit, the subunit unique to the adult form of the receptor [115]. This will require decryption of the pathways regulating associated protein expression and assembly. Although the developmental switch at the neuromuscular junction from nAChR composed of $\alpha1,\beta1,\gamma$ and δ subunits to those containing $\alpha1,\beta1,\varepsilon$ and δ subunits provides the best understood example of the transcriptional regulation of receptor subunit expression, knowledge of the signals that trigger this switch is still lacking, making manipulation an aspiration for the future [67,116].

A more tractable approach is the generation of small molecules to target transiently associated proteins. For example, phosphorylation of LGIC subunits has many effects, as discussed above, and compounds directed at kinases or phosphatases could modify the impact of this post-translational modification. However, a caveat to this approach is that inhibitors of these enzymes are likely to generate many effects because they regulate the phosphorylation status of multiple targets. Most kinase inhibitors are panspecific and we have no idea what constitutes a physiological substrate and whether altered ATP levels as a result of tissue dysfuction may modulate kinase function. More specific protein targets are needed: SLURP-1, the allosteric modulator of $\alpha7$ nAChR appears to fulfill this requirement. Moreover, mutations in SLURP-1 that decrease its function are responsible for the pathogenesis of Mal de Meleda, a condition of hyperproliferative epithelium, notably the skin [19]. Normally SLURP-1 appears to promote $\alpha7$ nAChR-mediated apoptosis. Therefore a positive allosteric modulator of the $\alpha7$ nAChR could be administered to compensate for dysfunctional SLURP-1 in Mal de Meleda patients. Several positive allosteric modulators with specificity for $\alpha7$ nAChR have been identified and characterized and offer hope for the future [117]. The credibility of this approach is encouraged by the discovery of Lynx-1 and -2 proteins that are related endogenous positive allosteric modulators of nAChRs.

Heterodimeric receptor assemblies offer another novel therapeutic target. The physical interaction between NMDA receptors and dopamine D1 receptors has been identified as having potential for the treatment of major brain disorders such as schizophrenia and Parkinson's disease [118]. The subsynaptic scaffold supporting and regulating GluR at the synapse has also been mooted as a new target for innovative drug discovery with reference to schizophrenia [119].

The aim of this short review has been to illustrate the variety of protein–protein interactions made by LGICs at different levels throughout the cell and their impact on receptor function. The number and type of interactions has increased rapidly over the last

20 years. Each level arguably offers potential for intervention, although in most cases this is presently only a matter of speculation.

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