Modulating Inhibitory Ligand-Gated Ion Channels

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ABSTRACT

The glycine and γ -aminobutyric acid receptors (GlyR and GABA_AR, respectively) are the major inhibitory neurotransmitter-gated receptors in the central nervous system of animals. Given the important role of these receptors in neuronal inhibition, they are prime targets of many therapeutic agents and are the object of intense studies aimed at correlating their structure and function. In this review, the structure and dynamics of these and other homologous members of the nicotinicoid superfamily are described. The modulatory actions of the major biological macromolecules that bind and allosterically affect these receptors are also discussed.

KEYWORDS: Glycinereceptor, GABA_Areceptor, nicotinicoid, anesthetics, therapeutics

INTRODUCTION

The nicotinicoid receptors are a superfamily of ligand-gated ion channels that are essential mediators of fast neurotransmission at neuronal and neuromuscular synapses. In vertebrates, the major members of this ionotropic superfamily include the inhibitory receptors gated by glycine or γ -aminobutyric acid (GlyR and GABA_AR, respectively) and the excitatory receptors gated by acetylcholine or serotonin (nAChR and 5HT₃R, respectively). These receptors are assemblies arranged about a central pore that transiently opens upon the binding of agonist, allowing the passive diffusion of anions (GlyR and GABA_AR) or cations (nAChR and 5HT₃R).^{1,2} Each pentameric receptor may be comprised of different gene products (eg, GlyR is composed of α and β subunits) and/or splice variants, mixed at different stoichiometries. The specific conductance properties of all channel types are defined by their composition, posttranslational modifications, and interaction with different modulatory ligands. Although this review focuses on the inhibitory receptors, some discussion of homologous excitatory recep-

tors is unavoidable since the structure of a soluble acetylcholine binding protein (AChBP) that is a homolog of the extracellular domain (ECD) of nicotinicoid receptors has been resolved in crystallographic studies,³ and the structure of isolated nAChR from *Torpedo* has been resolved by cryomicroscopy to 4 Å.⁴ These structures provide an appropriate template for modeling all nicotinicoid receptors and have been extensively used by investigators to interpret and refine our understanding of these receptors.⁵

Both GlyR and GABA_AR are channels whose role in the central nervous system (CNS) of adults is to mediate inhibitory neurotransmission by facilitating Cl⁻ influx into neurons.^{6,7} Each receptor type has a distinctive tissue distribution, with GlyRs being the primary target for inhibitory neurotransmission in the brainstem and spinal cord and GABA_ARs being more widely distributed throughout the CNS. However, mixed GABA-Gly synapses have been observed in many cell types.⁸⁻¹¹ For example, both receptor types are found in the retina, where they function in rod photoreceptor development.¹² While the specific subunit composition of each receptor type may vary spatially and temporally, the inhibitory activities of the subtypes of both receptor classes are broadly similar. Typically, the multiple binding of agonists results in rapid channel opening, and prolonged ligand occupancy gives rise to receptor desensitization, a relatively long-lived state that is refractory to channel reactivation. Complex functional profiles of the receptors are generated by differential expression of subtypes and altered activities via subunit-specific allosteric modulation of the receptors (described below). Hence, each receptor subtype has distinct kinetics, conductance, and sensitivities to ligands, giving rise to receptors' exquisite and differential sensitivities and activities.

GlyR and GABA_AR are generally described as inhibitory neuroreceptors, because channel opening typically results in a hyperpolarizing inward Cl⁻ flux. However, in embryonic neurons, the higher intracellular Cl⁻ concentration results in Cl⁻ efflux upon GlyR and GABA_AR activation and a strong excitatory suprathreshold depolarization that gates Ca²⁺ influx and is hypothesized to guide neuronal development.¹³ GABA is particularly important, as the hyperpolarizing activity of GABA_AR in embryonic neurons provides most of the excitatory drive in early development.¹⁴ Both glycinergic and GABAergic pathways have been shown to

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be crucially involved in establishing early inhibitory networks.¹⁵ In addition to their roles in neuronal development, both receptor types have been implicated in many channelopathies, as dysfunction of these receptors may lead to disease.¹⁶ GlyR mutations play a role in diseases associated with a lack of motor control, such as spasticity and startle diseases.¹⁷⁻¹⁹ Dysfunction of GABA_AR-mediated inhibition has been implicated in many neurological and psychiatric conditions, including depression, affective disorders, and epilepsy.^{20,21}

GlyR was the first neurotransmitter-gated ion channel isolated from mammals and was originally isolated by affinity chromatography from rats as an assembly of 48 kD α 1 and 58 kD β subunits.²² While α subunits can form homooligomers, the stoichiometry of hetero-oligomers has recently been shown to be 2 α ₁:3 β .²³ To date, 4 different α subunits (α ₁₋₄) and a single β subunit have been identified. The subunits comprising GABA_ARs are more numerous, with 19 different gene products among the 8 constituent subunit families, α ₁₋₆, β ₁₋₃, γ ₁₋₃, δ , ϵ , π , θ , and ρ ₁₋₃ (historically, GABA_ARs comprising ρ subunits have been referred to as GABA_CRs), described to date (for more information, including sequence alignments, see the Cys-loop Ligand-Gated Ion Channel database at <http://www.ebi.ac.uk/compneur-srv/LGICdb/LGICdb/cys-loop.php>²⁴). The majority of GABA_ARs contain α , β , and γ subunits with a probable stoichiometry of 2 α :2 β :1 γ , with the α and β subunits necessary for GABA activation and the γ subunits required for benzodiazepine sensitivity.²⁵

NICOTINICOID RECEPTOR TOPOLOGY AND STRUCTURE

For both types of channels, further structural and functional diversity is introduced by splice variants. Regardless of composition, all members of the nicotinicoid superfamily, including the excitatory nAChR and 5HT₃R cation channels, share a common topology. The pore domain of each subunit contains 4 transmembrane (TM) segments, M1 to M4, and their connecting loops, with the 5 M2 amphiphilic helices defining the central pore. All 4 TM segments of each subunit are observed as membrane-spanning helices in electron crystallographic studies of nAChR isolated from *Torpedo* ray⁴ (for an interactive display of this structure, see <http://www.pdb.org/pdb/explore.do?structureId=2BG9>). The large ECD of each pentameric receptor contains the binding site for its preferred agonist at specific interfaces between adjacent subunits. These domains also contain a conserved Cys-disulfide separated by 13 residues (giving rise to the alternate nomenclature of “Cys-loop” superfamily). As mentioned above, the structure of AChBP has been determined³ (for an interactive display of this structure, see <http://www.pdb.org/pdb/explore.do?structureId=1I9B>), and

its structural homology to the ECD of nAChR was confirmed in lysine scanning mutagenic studies,²⁶ suggesting that each ECD comprises a sandwich of β -sheets with very little α -helix. Furthermore, a chimera containing the AChBP and the pore domain of a 5HT_{3A}R retained activity,²⁷ showing that the AChBP structure can function as a modular ECD for the holoreceptor. The original AChBP crystals were grown in 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer, and HEPES was observed to bind to the nicotinamide binding site. The molecular details of HEPES binding were consistent with the agonist binding sites of homologous nicotinicoid receptors suggested by experimental studies. This structural determination of a homolog of the ECD of nicotinicoid receptors has provided an essential template to allow investigators to rationally model the ligand binding domains of these receptors²⁸⁻³² (K. Speranskyi, M. Cascio, and M. Kurnikova, unpublished data, 2006).

For GlyR and GABA_AR, the binding sites for agonists and antagonists reside at the interface between adjacent subunits, and this binding site is modeled as being defined by residues on 3 loops of 1 subunit and 3 loops from its neighbor.^{23,28} Phylogenetic analysis of animal nicotinicoid receptors and their prokaryotic homologs (with unknown agonist specificity) indicated that at least 1 aromatic residue is involved in ligand binding, with cation- π interactions implicated in binding interactions.³³ GlyRs are gated by glycine, and β -alanine and taurine are considered partial agonists. Strychnine is a selective competitive antagonist exhibiting the greatest potency toward most GlyR isoforms. For GABA_AR, the preferred agonist is GABA, but the receptors are also activated by muscimol and antagonized by benzodiazepines (described in more detail below). Both receptors may be gated by β -alanine and taurine,³⁴ and taurine plays an important role as agonist for either receptor in regions where both are coexpressed.¹² The selectivity of agonists for specific subunit composition may be exploited therapeutically. For example, the δ -containing GABA_AR is highly expressed extrasynaptically in the thalamus and has a high affinity for gaboxadol, which has efficacy as a treatment for insomnia.³⁵ Given the homology between GlyR and GABA_AR, Schmieden et al³⁶ substituted GABA_AR residues for GlyR residues in the putative binding pocket of GlyR and successfully generated recombinant forms of GlyR that exhibited GABA-like agonist preferences. These studies indicated that the ECDs of these receptors share a common structure and elucidated some of the molecular details involved in the ligand binding pocket, as well as foreshadowing the capability wherein one could exploit knowledge of receptor structure and function to therapeutically target specific native and/or mutant receptor complexes for modulation.

The function of the inhibitory receptors may also be modulated by ions that interact with the ECD of the receptors. For

the GlyR, Zn^{2+} has biphasic activity in that it potentiates activity of the receptor at submicromolar concentrations but is inhibitory at higher micromolar concentrations.³⁷ These concentration ranges are physiologically relevant, as zinc may be released into the synaptic cleft at fairly high levels. The inhibitory site was the first to be identified,³⁸ and subsequent studies identified this inhibitory site as residing at subunit interfaces, with His107 of one subunit and His109 of the neighboring subunit forming a Zn^{2+} coordination site.³⁰ Recently, the distinct site responsible for the potentiating Zn^{2+} site in the ECD of GlyR has been elucidated.³⁹ Zn^{2+} also has several discrete binding sites on the ECD of GABA_ARs that inhibit channel activity.⁴⁰ Extracellular pH also appears to modulate GlyR and GABA_AR function via redox-sensitive sites in the ECD⁴¹ and TM regions.^{42,43} These studies show that the binding of small agonists and ions at sites in the ECD activate or modulate receptor function.

CHANNEL GATING AND ALLOSTERY

One of the controversies in nicotinicoid receptor (or, for that matter, any gated neuroreceptor) research is the molecular details of activation and desensitization: how does binding of an agonist at a distinct distant site in the subunit interface of the ECD transiently open a central conducting pore? While many models for channel gating have been proposed and some details have been elucidated,⁴⁴⁻⁵⁰ the full molecular details remain unresolved. With respect to GlyR and GABA_AR, allosteric coupling of binding and gating is apparently effected through interactions of residues on the membrane-proximal loops in the ECD with residues on the pre-TM1 and TM2-TM3 linker.⁵⁰⁻⁵⁴ Recently, a chimera in which the ECD of $\alpha 7$ nAChR subunit was fused to the pore-forming TM domain of $\alpha 1$ was shown to retain activity, albeit with altered kinetics such that the receptor opened slowly and did not desensitize to any great degree.³³ As discussed by the authors of this study, the functional coupling of distinct modular domains encompassing the ECD of the cationic nAChR with the TM domain of the inhibitory GlyR suggests a gating mechanism that is common to the entire nicotinicoid superfamily. Homologs of the nicotinicoid receptors have been identified in prokaryotes, and it appears that the coupling of the ECD with the pore domain, and the channel-gating mechanisms of this receptor type, have been remarkably well conserved evolutionarily.⁵⁵

As alluded to above in discussions of the multiple states of the receptors (resting, open, and desensitized), the receptors are dynamic complexes with different allosteric states.⁵⁶ With respect to GABA_AR and GlyR, the mobilities of distinct regions of these receptors are well documented in studies that examine the state-dependent accessibility of engineered sites of GABA_AR residues⁵⁷⁻⁶¹ or GlyR⁶¹⁻⁶⁸

residues to chemical modifications. Distinct structural rearrangements in GABA_AR upon activation and antagonism were also observed in simultaneous electrophysiological studies and fluorescence monitoring of site-specific labels.⁶⁹ Recently, spin labeling studies in which homomeric GlyR incorporating 5 symmetrically related nitroxides at a single site per subunit have also observed changes in the spin-spin distances in the resting and desensitized states (K.M. Stone, Z. Yang, M. Bonora, M. Cascio, and S. Saxena, unpublished data, 2006). In addition to agonists and competitive antagonists, the receptors also interact with a wide range of compounds that affect receptor function. The modulatory actions and allosteric effects of interactions with drugs, ligands, and other macromolecules are discussed in the following sections. These interactions are often complex and interdependent (eg, interactions of a receptor with one compound will alter the sensitivity and effects of interactions with other compounds).

LIPIDS, ANESTHETICS, AND NEUROSTERIODS

All membrane proteins are intimately associated with the lipid bilayer in which they reside. The effects of lipids on membrane proteins, and of proteins on bilayer structure, has always been appreciated but has only recently been the focus of intense investigation.^{70,71} While the modulatory effects of the lipid microenvironment on ion channel activity have been widely noted, they remain poorly understood.^{72,73} The roles of lipids in the function of nicotinicoid receptors have been most extensively characterized for nAChR,⁷⁴ where there is an absolute requirement for cholesterol and negatively charged phospholipids for activity. Investigators have noted the effects of lipid composition on nAChR structure and function⁷⁵⁻⁷⁷ and have mapped the contact regions of proteins with surrounding lipids.⁷⁸⁻⁸³ Given the homology of the nicotinicoid superfamily, many of the lipid-facing regions found for nAChR are postulated to be similar for GlyR and GABA_AR.

The cholesterol content in membranes profoundly affects the physicochemical properties of the bilayer, and cholesterol content's role in lipid organization and human disease is well documented.⁸⁴ As described above, the cholesterol content in membranes is essential for nAChR activity. While some of the effects of cholesterol on receptor function may be due to cholesterol's role in altering the properties of the membrane sites, distinct sites of direct interaction with the nAChR have been broadly mapped.^{81,85-87} The cholesterol content has been shown to modulate both GlyR activity⁸⁸ and GABA_AR activity.^{89,90} We found that purification of active recombinant $\alpha 1$ GlyR homopentamers requires cholesterol to be present in proteoliposomes/detergent mixtures during all steps of purification, suggesting that the interaction with cholesterol stabilizes the receptor.⁹¹

The activity of some anesthetics has been proposed to be attributable to their partitioning into the lipid bilayer and altering the physicochemical properties of the bilayer.^{92,93} However, the observed enantiomeric specificity of some anesthetics in modulating ion channel activity argues for specific binding sites on these proteins in the membrane, though this does not rule out the potential contribution of a nonspecific lipid-based mechanism of action.^{94,95} Volatile and intravenous anesthetics, as well as alcohols, modulate the activity of nicotinic receptors, potentiating GlyR and GABA_AR at concentrations consistent with those used pharmacologically.⁹⁶⁻⁹⁸ These compounds appear to bind in a discrete water-accessible cavity between the TM segments.⁹⁹ *n*-Alcohol potentiation of the receptors increases with increasing length of the alcohol until a cutoff is reached, and further increases give rise to reduced potentiation.¹⁰⁰ The location of the alcohol binding pocket was confirmed by mutagenesis studies wherein the volume of side chains in TM segments M1, M2, and M3 that putatively define the alcohol binding pocket was varied, altering the potency of the observed effects.^{65,101} Studies conducted during gating and alcohol modulation of GABA_AR also confirm dynamic changes in the accessibility of select residues thought to define this binding pocket.^{102,103} Residues on the TM helix M4 are also involved in the response to anesthetics, as scanning tryptophan mutagenesis studies¹⁰⁴ and accessibility studies⁶⁶ showed that select residues of M4 of GABA_AR play a role in this response. It has been hypothesized that a possible mechanism for anesthetic action is that the volume of the binding pocket expands and contracts during channel opening and closing, and this finding is supported by state-dependent changes in accessibility to this putative pocket⁶⁵; occupancy of this pocket by anesthetics stabilizes the open state, giving rise to their potentiating effects.

In addition to cholesterol, other sterols have been noted to affect the activity of the inhibitory receptors. It was first recognized that the general anesthetic effect of alphaxalone, a synthetic pregnane steroid, is via enhancing of GABA_AR function by promoting longer open durations.¹⁰⁵ This modulation was also dependent on the cholesterol content of the membrane.⁸⁹ The enantiomeric-selective response of the receptors to steroid compounds suggests that specific binding sites for these compounds are present on the receptor, but the exact site of interaction has not been fully resolved. The binding site of alphaxalone has been suggested to be in the N-terminal half of M2¹⁰⁶ and in the pre-M1 region of the β_2 subunit.¹⁰⁷ Another neurosteroid, pregnenolone, is a highly potent modulator of GABA_AR-mediated neurotransmission. At higher concentrations it is considered GABA-mimetic and directly activates the receptor. Pregnane and androstane steroids have also been shown to modulate GlyR in a subunit-specific manner.¹⁰⁸ Depending on dosage, neurosteroids can induce analgesic, anticonvulsant, anxio-

lytic, hypnotic, or sedative effects.^{109,110} However, sulfated neurosteroids such as pregnenolone sulfate inhibit GABA_AR activity, producing opposite effects.

OTHER DRUGS AND THERAPEUTICS

As described above, anesthetics, ethanol, and other *n*-alcohols potentiate GlyR and GABA_AR. The nicotinic receptors are also involved in long-term effects of alcohol abuse, as well as addiction to nicotine and barbiturates.¹¹¹⁻¹¹⁴ The role of GlyR in the development of alcohol addiction has recently been elucidated, as GlyRs in the nucleus accumbens appear to regulate extracellular dopamine levels.^{115,116} Other drugs of abuse also interact with the inhibitory receptors. For example, ethanol,¹¹⁷ cocaine,¹¹⁸ and inhaled solvents¹¹⁹ affect GlyR activity. GlyR and GABA_AR are also targeted and modulated by various narcotics (eg, barbiturates) and therapeutic agents for the treatment of a wide range of psychiatric disorders such as anxiety and drug dependence.^{32,96,100,120} In addition, GABA_AR is the target for some drugs used in treating epilepsy. Antiepileptic drugs often involve blockage of Na⁺ or K⁺ channels, but antiepileptic effects are also mediated via enhancement of the inhibitory activity of GABA_AR and/or by effects on glycine systems.^{121,122} For example, the barbiturate phenobarbital increases the channel mean open time of GABA_AR. Initially, barbiturates were used as anxiolytic and anticonvulsant agents, as well as intravenous anesthetics. However, because of the risk of dangerous respiratory depression, these barbiturates have been preferentially replaced with benzodiazepines. Among the many drugs that interact with glycinergic and GABAergic systems, the 2 drug classes that have been best characterized at a structural and functional level are the benzodiazepines and convulsant toxins such as picrotoxin. The interactions of these compounds with GABA_AR and GlyR are described briefly below.

Benzodiazepines are the most widely used hypnotic compounds (this family includes diazepam, more commonly known as valium) and bind to a site at the interface of α and γ subunits, distinct from the GABA site.¹²³ These compounds do not open the channel, but binding allosterically modulates ligand affinity and increases the opening frequency of GABA_AR, inducing anxiolytic and sedative effects.¹²⁴ Inverse agonists acting at the same binding site are drugs with anxiogenic and convulsant properties (eg, β -carbolines). While benzodiazepines are effective sedatives and have been used in the treatment of some forms of epilepsy, prolonged use leads to tolerance, and this class of compounds is addictive.¹¹³ In accessibility studies, the binding of diazepam in the ECD of GABA_AR altered the accessibility of select residues in the membrane-spanning M3 segment, suggesting that the allosteric modulation effected by benzodiazepine binding extends into the

membrane-spanning domain, giving rise to a conformational state that is distinct from the resting and open state of the receptor.⁵⁸

The convulsant plant alkaloid picrotoxin inhibits both GABA_AR and GlyR, but GABA_AR is generally more sensitive to this alkaloid, having sensitivities in the low micromolar range. Picrotoxin binds with lower affinity to the α/β heteromers of GlyR (as opposed to the α homomers of this channel, which have similar affinities as GABA_AR) and is thought to bind in the central pore of the receptors. The binding is of higher affinity to the closed channel form of the receptor, thereby decreasing the equilibrium constant for channel opening.¹²⁵ This is supported by recent studies, wherein binding of picrotoxin in the channel mediated allosteric conformational changes in the M2-M3 loop of GlyR.⁶⁴ These changes to loop accessibility could not be replicated in studies in the presence or absence of glycine, suggesting a complex global allosteric modulation of the receptor in response to picrotoxin binding and underscoring the critical role of the M2-M3 loop in coupling agonist binding to channel gating.

OTHER PROTEINS

In addition to interactions with small molecules that alter the activity of GlyR and GABA_AR, their activity may be directly or indirectly modulated by other proteins. These interactions are most typically mediated via interactions of other proteins with the large intracellular loop between the third and fourth TM segments. This cytoplasmic linking region between M3 and M4 is the least evolutionarily conserved region among nicotinic family members. This loop has been shown to be involved in interactions with other proteins that result in receptor crosstalk, subcellular targeting and clustering, and activity modulation.¹²⁶⁻¹²⁹ For example, GlyRs may be functionally modulated via interactions with G-protein $\beta\gamma$ subunits,¹³⁰ and GABA_AR and dopamine D5 receptors crosstalk via direct protein-protein interactions.¹³¹

One of the most common protein-protein interactions that affect receptor function is that of neuroreceptors with kinases or phosphatases to alter their phosphorylation state. The phosphorylation of neurotransmitter-gated channels can have short- and long-term effects and has been implicated in forms of synaptic plasticity. The effects of phosphorylation on GlyR and GABA_AR function are complex and depend on both the subunit composition and the specific site(s) of phosphorylation. To date, all phosphorylation sites of nicotinic receptors have been mapped to the M3-M4 linker region. Multiple isoforms of kinases and phosphatases have been implicated as affecting receptor activity, but these effects may be directly due to the modulatory effects attributable to phosphorylation on channel activity,

or be due to indirect effects, such as targeting receptor-associated proteins.^{132,133} GlyR activity is enhanced in vivo by cAMP-dependent protein kinase phosphorylation,¹³⁴ and tyrosine kinases enhance GlyR activity in rat hippocampal neurons.¹³⁵ Various protein kinase C isoforms have been shown to modulate GABA_AR conductance, their sensitivity to allosteric effectors, and their trafficking.¹³⁶ With respect to the direct effects of phosphorylation, protein kinase C phosphorylates the $\alpha 1$ subunit of GlyR at a single site, potentiating its activity.¹³⁷

One recent example of the profound effects of phosphorylation on receptor function is the inhibitory effects of phosphorylation of $\alpha 3$ GlyR subunits. Prostaglandin E₂, a key mediator of inflammatory pain sensitization, induces phosphorylation of $\alpha 3$ subunits of GlyR,¹³⁸ and this inhibition underlies central inflammatory pain sensitization. These studies also identify GlyR as a new therapeutic target for analgesia since $\alpha 3$ GlyR is in spinal cord dorsal horn, where nociceptive afferents terminate,¹³⁹ and underscore the utility in targeting inhibitory ion channels as therapeutic targets.^{140,141}

CONCLUSIONS

Given their role as essential mediators of fast inhibitory neurotransmission in the CNS, the structure, function, and pharmacology of GABA_AR and GlyR have been extensively studied. These channels are also the targets for many drugs of abuse, and dysfunctions of these receptors underlie many channelopathies. The recent determination of the homologous structures of the soluble AChBP and the full-length nAChR has provided researchers with the requisite tools for interpreting the large body of experimental data available and modeling GABA_AR and GlyR structure and dynamics. These models provide us with the potential to develop and exploit novel subunit-specific therapeutics to modulate the activity of targeted subsets of GABA_AR and GlyR in treating disease.

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