Allosteric Modulation of Glycine Receptors

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Summary

Inhibitory (or strychnine-sensitive) glycine receptors (GlyRs) are anion-selective transmitter-gated ion channels of the cys-loop superfamily, which includes among others also the inhibitory γ-aminobutyric acid receptors ($GABA_A$ receptors). While GABA mediates fast inhibitory neurotransmission throughout the CNS, the action of glycine as a fast inhibitory neurotransmitter is more restricted. This probably explains why $GABA_A$ receptors constitute a group of extremely successful drug targets in the treatment of a wide variety of CNS diseases, including anxiety, sleep disorders, and epilepsy, while drugs specifically targeting glycine receptors are virtually lacking. However, the spatially more restricted distribution of glycinergic inhibition may be advantageous in situations when a more localized enhancement of inhibition is sought. Inhibitory GlyRs are particularly relevant for the control of excitability in the mammalian spinal cord, brain stem, and a few selected brain areas, such as the cerebellum and the retina. At these sites, GlyRs regulate important physiological functions, including respiratory rhythms, motor control, muscle tone and sensory as well as pain processing. In the hippocampus, RNA-edited high affinity extrasynaptic GlyRs may contribute to the pathology of temporal lobe epilepsy. Although specific modulators have not yet been identified, GlyRs still possess sites for allosteric modulation by a number of structurally diverse molecules, including alcohols, neurosteroids, cannabinoids, tropeines, general anaesthetics, certain neurotransmitters and cations. This review summarizes the present knowledge about this modulation and the molecular bases of the interactions involved.

Keywords: allosteric modulation, cannabinoid, glycine, endocannabinoid, ethanol, glycine receptor, neurosteroids, tropeines, zinc

Non standard abbreviations: 3α,5α-THPROG, 3α,5α-tetrahydroprogesterone, allopregnanolone; 5β-pregnan-3α-ol-20-one, 3α,5β-THPROG, pregnanolone;, 2-AG, 2-arachidonyl-glycerol; AEA, N-arachidonoyl ethanol amide; CB receptor, cannabinoid receptor; ECD, extracellular domain; GABA, γ-aminobutyric acid; GlyR, glycine receptor; IL, intracellular loop; NA-glycine, N-arachidonoyl glycine; TM, transmembrane;

Introduction

Strychnine-sensitive GlyRs are pentameric anion channels. Five GlyR subunits have been cloned from mammalian tissue and designated α1-α4 and β (Lynch, 2004). Each GlyR subunit contains an amino-terminal extracellular domain (ECD), four transmembrane domains (TM) and a large intracellular loop (IL) between TM3 and TM4, which configure the ligand binding region, the ion channel pore, and sites for intracellular modulation, respectively (Baenziger and Corringer, 2011; Lynch, 2004; Sine and Engel, 2006).

In the adult, most GlyRs are composed of α1 and β subunits probably in a 2(α1)/3β stoichiometry (Grudzinska *et al*., 2005). This GlyR subtype is the main mediator of glycinergic inhibition in the adult CNS. Many of these GlyRs colocalize with the postsynaptic scaffolding protein gephyrin (Todd *et al*., 1995; Waldvogel *et al*., 2010). Early in development most GlyRs are α2 homomers, which become replaced around postnatal day 14 by α1β heteromers in most CNS areas (Lynch, 2004; Malosio *et al.*, 1991). In a few selected areas, such as the retina, α2 persist however into adulthood. Like α1 subunits, α3 subunits are mainly found in the adult but their expression is spatially much more restricted. Immunohistochemistry and quantitative RT-PCR studies in mice have shown that α3-GlyR subunits are predominantly expressed in the superficial laminae of the dorsal horn (Anderson *et al.*, 2009; Harvey *et al.*, 2004) and in the respiratory network of the brain stem (Manzke *et al.*, 2010). RNA edited α2 and α3-GlyRs may serve a peculiar function as extrasynaptic high affinity GlyRs in the hippocampus (Legendre *et al.*, 2009; Meier *et al.*, 2005). The gene encoding for the GlyR α4 subunit is a pseudogene in humans due to the presence of a premature stop codon (Simon *et al*., 2004).

The subunit composition of the ion channel complex and the arrangement of the different subunits within this complex determine its pharmacological profile. According to recent data, the glycine binding site of α/β heteromeric GlyRs is jointly formed by α and β subunits (Grudzinska *et al*., 2005), while β subunits interact with gephyrin and thereby mediate synaptic clustering of GlyRs ((Kim *et al.*, 2006); Pfeiffer *et al.*, 1982; Schmitt *et al.*, 1987). Although very few established drugs primarily act through inhibitory GlyRs (Laube *et al.*, 2002), a number of endogenous messenger molecules and some drugs do modulate GlyRs function (Fig. 1). Such compounds include different cations, in particular zinc, cannabinoids, neuroactive steroids, tropeines, alcohols, avermectins, butyrolactones and general anaesthetics. Most of these molecules do not directly interact with the glycine binding sites but rather bind to allosteric sites within the GlyR complex (Fig. 2). It is at present not yet established to what extent these interactions contribute to physiology or to drug actions *in vivo*, but their existence clearly establishes a possibility for specific pharmacological intervention. The analysis of the molecular bases of the interaction of these compounds with GlyRs should hence foster the development of specific GlyR modulators.

Possible indications of glycine receptor as a therapeutic target

Early knowledge about possible roles of glycinergic neurotransmission in physiological functions or in diseases has mainly been obtained through pharmacological blockade of GlyR with the rodent poison strychnine (for a review see Callister and Graham, 2010). These early studies have identified a critical role of GlyRs in the control of muscle tone. Severe cramps of the skeletal musculature are the leading symptom of strychnine poisoning. Apart from these motor symptoms altered sensory perception such as an increased sensitivity to acoustic or tactile stimuli has also frequently been observed. Further evidence in particular for the involvement of altered glycinergic inhibition has come from studies in several strains of GlyR mutant mice (*spasmodic*, *oscillator*, and *spastic*) which carry mutations in the GlyR α1 subunit (*spasmodic*, *oscillator*) or in the β subunit (*spastic*) (Buckwalter *et al*., 1994; Mülhardt *et al*., 1994; Ryan *et al*., 1994). These mice are not only exhibit increased muscle tone but also show a strong hyperekplexic phenotype, very much reminiscent of human startle disease (Koch *et al*., 1996). In fact, mutations in GlyR subunit genes are frequently found in human patients suffering from hyperekplexia/startle disease (Rees *et al*., 2001).

There is also evidence that part of the spinal component of inflammatory hyperalgesia (i.e. an increased sensitivity to painful stimuli) comes from diminished glycinergic inhibition caused by the phosphorylation and inhibition of α3-GlyRs (Ahmadi *et al*., 2002; Harvey *et al*., 2004; Reinold *et al*., 2005). In the spinal cord, these GlyRs are largely confined to the superficial dorsal horn, the main termination area of nociceptive afferent nerve fibers. This result fits nicely to early reports showing that exaggerated nociceptive responses can be triggered by intrathecal injection of strychnine in rats (Beyer *et al*., 1985; Yaksh, 1989). Very recent evidence indicates that α3-GlyRs also serve an important function in brainstem respiratory control where their dephosphorylation through serotonin 5-HT1A receptor activation antagonizes opioid-induced respiratory depression (Manzke *et al.*, 2010).

Although glycinergic innervation is largely confined to the spinal cord, brain stem and cerebellum, GlyRs are widely expressed also in the forebrain, where they might become activated by ambient glycine. The affinity to glycine of un-edited receptors is normally too low for activation by ambient glycine. However, high affinity receptors can be generated through cytidine deamination of GlyR transcripts (RNA editing) (Meier *et al.*, 2005). This RNA editing gives rise to novel isoforms of α2 and α3-GlyRs carrying a proline to leucine point mutation (α2[P192L] and α3[P185L]) (Legendre *et al.*, 2009; Meier *et al.*, 2005). Recent evidence suggests that such high affinity extrasynaptic GlyRs contribute to pathological changes in temporal lobe epilepsy through the silencing of hippocampal neurons (Eichler *et al.*, 2008).

In the disease states discussed above, GlyR function is affected through inherited mutations, RNA editing, or posttranslational modifications such as phosphorylation. Patients suffering from diseases caused by diminished inhibition would probably benefit most from facilitated

glycinergic inhibition e.g. through positive allosteric GlyR modulators, while in temporal lobe epilepsy an inhibition of GlyRs might be desirable. The following sections address the mechanisms of and the molecular sites for such a positive allosteric modulation.

Cannabinoid ligands

A series of reports published starting in 2005 focused on a possible role of endocannabinoids and structurally or functionally related molecules as GlyR modulators. Endocannabinoids are endogenous activators of G-protein coupled cannabinoid receptors (CB1 and CB2 receptors) (Piomelli, 2003). N-arachidonoyl ethanol amide (AEA, also known as anandamide) was the first endocannabinoid discovered, followed by 2-arachidonyl-glycerol (2-AG). Both are lipid signalling molecules, structurally related to arachidonic acid. Although many lines of evidence indicate that G-protein coupled cannabinoid receptors are the primary targets of 2-AG and AEA, several studies showed that they interact with additional targets including several ion channels (Oz, 2006).

A direct modulation of GlyR by 2-AG and AEA was first reported in hippocampal neurons where both endocannabinoids reduced the amplitude of glycinergic membrane currents and altered their rise time, desensitization, and deactivation kinetics in a concentration-dependent manner (Lozovaya *et al*., 2005). This modulation was insensitive to CB1 receptor antagonists (SR141716A) and remained intact when the recorded cell was perfused with the ubiquitous G protein inhibitor GDP-β-S (Lozovaya *et al*., 2005). Direct modulation of GlyR by AEA has also been found in oocytes expressing recombinant α1-GlyRs (Hejazi *et al*., 2006). Neither SR141716A nor the cannabinoid reuptake inhibitor AM404 prevented the potentiating actions of AEA, again indicating that modulation occurred independent of CB1 receptors.

Following the identification of the two endocannabinoids 2-AG and AEA, additional, structurally related endogenous molecules were discovered (Huang *et al.*, 2001). Several of these, such as N-arachidonoyl glycine (NA-Gly) and N-arachidonoyl serine (NA-Ser), bind CB1 receptors only very weakly, but still modulate GlyRs or other ion channels (Barbara *et al.*, 2009; Guo *et al.*, 2008); Yang *et al.*, 2008). Other molecules with agonistic activity at CB1 or CB2 receptors but structurally unrelated to endocannabinoids also modulate GlyRs. Among these are some ingredients of the *Cannabis sativa* plant (Δ^9-tetrahydrocannabinol [Δ^9-THC], cannabidiol) and several synthetic CB_1 and/or CB_2 receptor ligands (HU-210, WIN 55,212-2), which either potentiate or inhibit GlyR currents, sometimes in a subunit-specific manner (compare Tab. 1). Although a consistent picture has yet to emerge, these data suggest that different molecular determinants exist in the target protein for CB receptor activation and GlyR modulation.

The studies discussed above consistently found that most of the cannabinoid related compounds did not directly activate GlyRs but in most cases caused a leftward shift of the

glycine concentration response curve. Another important aspect is that native $GABA_A$ receptors (in rat ventral tegmental area neurons) and recombinant $\alpha2\beta3\gamma2$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes were not modulated by AEA (Hejazi *et al*., 2006).

First analyses of possible molecular sites for these allosteric effects were performed by Hejazi and coworkers, who found that the sensitivity to AEA was similar in homomeric $\alpha1$ and heteromeric $\alpha1\beta$-GlyRs indicating that α subunits were sufficient for this modulation (Hejazi *et al*., 2006). They next investigated the influence of a serine to glutamine amino acid exchange in $\alpha1$ at position 267 (S267Q), which was previously shown to abolish the potentiation of GlyRs by ethanol and general anaesthetics (Mihic *et al*., 1997). No change in the potentiating action of AEA or Δ^9-THC was found in this mutant. However, mutation of the serine 267 into an isoleucine (I), which also abolishes GlyR potentiation by ethanol (Mihic *et al*., 1997), prevented potentiation by three molecules structurally-related to Δ^9-THC (cannabidiol, HU210 and ajulemic acid) (Foadi *et al.*, 2010) suggesting a role of TM2 residues for the actions of these cannabinoids ligands. More recently, Xiong and coworkers demonstrated that potentiation of $\alpha1$ and $\alpha3$ GlyRs by Δ^9-THC involves a TM3 serine residue (S296 on $\alpha1$ or S307 on $\alpha3$ GlyRs), which likely contributes to a direct interaction of Δ^9-THC *via* hydrogen bonds (Xiong *et al.*, 2011, see also Fig. 2).

Over the last several years, convincing evidence has accumulated for a direct modulatory action of cannabinoid-related compounds on recombinant GlyRs. Data supporting a significant contribution of these effects to the *in vivo* actions of (endo-)cannabinoids were however lacking until recently. The report by (Xiong *et al.*, 2011) provides the first evidence in support of an *in vivo* relevance showing that mice lacking $\alpha3$-GlyRs exhibit a pronounced reduction in Δ^9-THC-induced analgesia. An important piece of information which is still missing in the puzzle is data demonstrating a direct amplification or prolongation of glycinergic synaptic currents by (endo-)cannabinoids.

A second issue which is particularly relevant, when cannabinoid-related molecules are considered as lead structures for the development of GlyR modulators, is their lack of specificity. Almost all of these molecules also interfere with the function of other ion channels (Oz 2006, see also Tab. 2) and many of them also exhibit activity at CB1 or CB2 receptors. Again, the report by (Xiong *et al.*, 2011) provides new insights. Introduction of slight chemical modifications to the Δ^9-THC molecule significantly decreased affinity to CB1 receptors while fully retaining activity at GlyRs. Although comprehensive analyses of the molecular determinants are definitely still needed, the recent studies indicate that some of the cannabinoid-related molecules discussed above may constitute interesting lead compounds for the development of GlyR modulators.

Ethanol

Evidence from biochemical and electrophysiological experiments consistently indicates that GlyR currents are potentiated by ethanol at concentrations reached in humans after moderate ethanol intake. This potentiation originates from a decrease in the glycine EC_{50} without a change in maximal currents (Aguayo *et al.*, 1996; Mihic, 1999). Potentiation of GlyRs by ethanol is apparently not restricted to certain areas but occurs in neurons throughout many parts of the CNS, including spinal cord, hippocampus, hypoglossal nucleus and ventral tegmental area (Aguayo *et al.*, 1996; Eggers and Berger, 2004; Jiang and Ye, 2003). Experiments performed in motoneurons from brain stem and spinal cord slices have shown that ethanol increases the amplitude of glycinergic postsynaptic currents suggesting that modulation of synaptic GlyRs by ethanol could potentially explain some of the alterations caused by ethanol in motor control and respiratory rhythms (Eggers and Berger, 2004; Gibson and Berger, 2000; Ziskind-Conhaim *et al.*, 2003).

Homomeric α1-GlyRs are more sensitive to ethanol than α2-GlyRs especially at concentrations below 100 mM (Mascia *et al.*, 1996b; Perkins *et al.*, 2008; Yevenes *et al.*, 2010). This differential sensitivity correlates well with data from neuronal preparations, in which neonatal GlyRs (mostly α2-GlyRs) were less sensitive to ethanol than mature (α1) GlyRs (Eggers *et al.*, 2000; Sebe *et al.*, 2003; Tapia and Aguayo, 1998).

Most of the knowledge about the molecular mechanisms underlying the modulation of GlyRs by ethanol originally came from the electrophysiological analysis of a set of chimeric GlyR α1 / $GABA_A\rho$1 receptors (Mihic *et al.*, 1997). This seminal report identified residues in TM2 (S267) and TM3 domains (A288) which abolish the ethanol sensitivity of α1-GlyRs. Subsequent experiments combining site specific mutagenesis, molecular modelling, and covalent binding of alcohol analogues to cysteine mutants consistently determined that TM2 and TM3 residues jointly shape a water-filled cavity serving as an ethanol-binding pocket (Mascia *et al.*, 2000; Ye *et al.*, 1998). Other studies showed that the extracellular loop 2 and TM1 residues also play a role in the alcohol modulation of GlyRs, although it is not clear if they shape additional binding pockets (Crawford *et al*., 2008; Lobo *et al*., 2008). These studies clearly demonstrate that both ethanol binding pockets and regulatory elements for the ethanol actions are within the TM domains of GlyRs. However, ethanol sensitivity can also be effectively controlled by intracellular signalling, possibly suggesting that part of the ethanol actions occur indirectly through other ethanol-sensitive proteins. For instance, the ethanol-induced potentiation of recombinant and native GlyRs is attenuated by PKC inhibitors (Jiang and Ye, 2003; Mascia *et al.*, 1998) and by ct-GRK2, a specific G protein $\beta\gamma$ sequester peptide (Yevenes *et al*., 2008). The importance of the $G\beta\gamma$ signalling for the alcohol effects on GlyRs also has been demonstrated recently using $G\beta\gamma$-insensitive α1-GlyRs, in which the mutation of two intracellular residues (KK385-386) attenuated ethanol

effects without altering potentiation induced by general anaesthetics (Yevenes *et al*., 2008). Additionally, a recent report also showed that the differential ethanol sensitivity of α1 and α2-GlyRs can be better explained by a selective Gβγ modulation rather than by specific TM ethanol-binding pockets, which are conserved between these isoforms (Yevenes *et al.*, 2010). It is still a matter of debate whether direct or indirect actions are more relevant, but conceivably both the direct binding of ethanol to the receptor (reviewed in Harris *et al*., 2008) and the indirect modulation of signalling components by ethanol (reviewed in Morrow *et al*., 2004) could be equally important and act cooperatively to elicit the final effects on GlyRs.

The physiological importance of the molecular sites for the ethanol actions *in vivo* has been investigated through genetic approaches in mice carrying the ethanol-insensitive S267Q mutation in the α1-GlyR gene. Transgenic expression of S267Q mutated GlyR in mice decreased ethanol sensitivity in behavioural assays without inducing apparent behavioural changes in the absence of alcohol (Findlay *et al*., 2002). Although these results support the importance of this GlyR site for alcohol actions *in vivo*, they should be interpreted cautiously as a subsequent publication of the same group investigating S267Q point mutated ("knock-in") mice has yielded different results. Mice homozygous for the S267 point mutation exhibited spontaneous seizures and died three weeks after birth. Heterozygous mice survived but still displayed a severe increase in the acoustic startle responses (Findlay *et al*., 2003). I*n vitro* experiments demonstrated that the S267Q mutation in α1-GlyR significantly reduced the glycine-evoked chloride uptake in spinal cord synaptoneurosomes from heterozygous knock-in mice and dramatically disrupted receptor function at the single channel level (Findlay *et al*., 2003).

General anaesthetics

Many studies on recombinant GlyRs consistently demonstrate that volatile anaesthetics, such as isoflurane, enflurane, halothane and sevoflurane potentiate homomeric α1-GlyR currents at anaesthetic concentrations (Downie *et al.*, 1996; Krasowski and Harrison, 1999; Mascia *et al.*, 1996a; Yamakura *et al.*, 2001). This potentiation is not specific for α1-GlyRs, as homomeric α2-GlyRs are also sensitive to isoflurane (Harrison *et al*., 1993), while α3-GlyRs remain to be investigated. The anaesthetics studied were unable to activate GlyRs by themselves (Downie *et al.*, 1996; Harris *et al.*, 1995; Krasowski and Harrison, 1999), but rather caused a leftward shift of the glycine concentration response curve. These effects have been reproduced in native receptors. Isoflurane potentiated the glycine-activated currents in rat medullary neurons (Downie *et al*., 1996), and prolonged the decay kinetics and increased the frequency of mIPSCs in rat trigeminal nucleus and spinal motoneurons (Cheng and Kendig, 2002; Yamashita *et al.*, 2001). Because glycinergic inhibition is largely confined to the hindbrain and spinal cord, it is unlikely that the loss of consciousness by volatile

anaesthetics is caused via an interaction with GlyRs. However, immobility is an action of volatile anaesthetics, which is much more likely related to interactions with GlyR (Rudolph and Antkowiak, 2004; Sonner *et al.*, 2003). In line with this idea, isoflurane, enflurane and sevoflurane indeed significantly reduced spontaneous action potential firing in neurons recorded in organotypic slice cultures of the rat ventral horn (Grasshoff and Antkowiak, 2006 and 2004). An interaction with GlyRs might also be relevant for sensory processing at the spinal dorsal horn level. Extracellular recordings from wide-dynamic range (WDR) neurons in intact rats have shown that halothane induced depression in the responses to thermal and mechanical noxious stimuli and that this depression was partially reversed by strychnine at doses which had no per se effect on WDR neuron firing (Yamauchi *et al*., 2002). In line with these studies, *in vivo* experiments performed in rats demonstrated that spinal GlyRs are important, although not the only, mediators of the isoflurane-induced immobility (Zhang *et al.*, 2003).

Intravenous anaesthetics may also have some effect on GlyR function, but these actions are more controversial. Propofol displayed significant modulatory activity at GlyR, but the degree of this modulation was much less than that of volatile anaesthetics in particular at clinically relevant concentrations (Krasowski and Harrison, 1999; Pistis *et al.*, 1997). Homomeric α1-GlyRs, α1/β heteromers, and homomeric α2-GlyRs are similarly sensitive to propofol (Mascia *et al*., 1996b; Pistis *et al*., 1997), while at least α1-GlyRs appeared to be insensitive to etomidate (Mascia *et al*., 1996a; Pistis *et al*., 1997). Despite the lack of information on the sensitivity of other GlyR subunits or subunit combinations, available evidence suggests that GlyRs are unlikely to play a major role in the *in vivo* effects of the intravenous anaesthetics (but see Nguyen *et al*., 2009). This is also supported by reports, which showed that propofol-induced immobility was produced exclusively via spinal $GABA_A$ receptors (Grasshoff and Antkowiak, 2004; Sonner *et al.*, 2003).

The molecular determinants of GlyR modulation by anaesthetics have been worked out hand-in-hand with those of $GABA_A$ receptors. In fact, pioneering studies performed in the late 1990s found sites for volatile anaesthetics within TM domains of GlyRs through the analysis of chimeric receptors between the enflurane-sensitive α1-GlyRs and enflurane-insensitive ρ1-$GABA_A$ receptors (Mihic *et al*., 1997). This and other studies have consistently shown that specific residues within TM2 and TM3 domains of α1-GlyRs potentially shape an intra-subunit cavity which serves as a general anaesthetic binding pocket and which also acts as an acceptor for ethanol and other *n*-alcohols with longer carbon chains (reviewed in Krasowski and Harrison, 1999; Lobo and Harris, 2005). Unfortunately, the knowledge regarding the molecular sites for anaesthetics on GlyRs has not yet been translated into genetic mouse models. This would be necessary in order to address the role of GlyRs on the general anaesthetic actions *in vivo*.

Glutamate

A recent publication (Liu *et al.*, 2010) provides strong evidence that glutamate, the principal fast excitatory neurotransmitter in the CNS, can act as a positive allosteric GlyR modulator. This potentiation was seen in spinal neurons in culture and in slices as well as in HEK293 cells transiently expressing GlyRs. Potentiation of GlyR currents manifested in increased single channel open probability and occurred not only by glutamate but also by NMDA, AP5, kainate, quisqualate, aspartate and kynurenic acid, while CNQX and NBQX inhibited GlyR currents indicating that the pharmacology of this modulation did not match with that of any known glutamate receptor. Experiments performed in isolated membrane patches showed that this facilitation most likely occurred through a direct binding of glutamate to the GlyR channel complex. Homomeric α1-GlyR currents were doubled by glutamate, while potentiation of α1/β heteromeric channels was in the rage of about 40-60%, possibly suggesting that the putative binding site resides on the GlyR α1 subunit. Potentiation of glycinergic inhibition by glutamate may provide an extremely fast feedback mechanism for the maintenance of balanced synaptic excitation and inhibition. Although the relevant binding sites have not yet been determined and because the findings certainly require independent verification, this previously unrecognized modulation may provide an additional possibility for therapeutic intervention with GlyRs.

Ivermectin

Avermectins are a family of macrocyclic lactones derived from the bacterium *Streptomyces avermitilis* and commonly used as anti-parasitic and insecticide agents. They act mainly through an allosteric modulation or a direct activation of glutamate-gated chloride channels (GluCls) expressed by nematodes and insects (Wolstenholme and Rogers, 2005). Ivermectin is one member of this group whose activity on invertebrate GluCls has been characterized in detail in recombinant systems (Arena *et al.*, 1992; Cully *et al.*, 1996; Cully *et al.*, 1994; Kane *et al.*, 2000). Notably, ivermectin also modulates cationic and anionic ligand-gated ion channels including GlyRs in vertebrate (see Table 2). Early electrophysiological studies showed that ivermectin influences recombinant homomeric α1 and heteromeric α1β GlyRs at submicromolar concentrations (Shan *et al.*, 2001). More recent studies revealed that different amino acid substitutions of the TM3 residue A288 differentially affected ivermectin's action on α1 GlyR currents. The mutation A288G increased the ivermectin sensitivity to the nanomolar range, whereas the A288F substitution completely abolished its agonistic actions (Lynagh and Lynch, 2010), Fig. 2). Equivalent mutations in the corresponding residue in a nematode GluCl ion channel showed a similar pattern of effects suggesting that the effects of avermectins on these receptors occurs through similar molecular sites (Lynagh and Lynch, 2010). Since homologous residues have been extensively characterized as binding sites for

ethanol and general anaesthetics on $GABA_A$ and GlyRs (reviewed in Krasowski and Harrison, 1999; Lobo and Harris, 2005; Yamakura *et al.*, 2001), it appears likely that avermectins modulate these receptors through similar mechanisms and binding sites. The direct activation of GlyRs by ivermectin makes this compound an interesting template to design GlyR ligands with agonistic activity. However, ivermectine is not specific for GlyRs (see Table 2) and could even inhibit GlyRs in some preparations (Dawson *et al.*, 2000). A better understanding of the mechanisms underlying the effects of ivermectin on GlyRs will be necessary to design new analogues with improved selectivity.

Neuroactive steroids

Endogenous neurosteroids are cholesterol metabolites produced locally in the CNS. They induce fast changes in neuronal excitability through a direct interaction with ion channels. Best established is the facilitating action on $GABA_A$ receptors by 5α-pregnan-3α-ol-20-one (3α,5α-tetrahydroprogesterone; 3α,5α-THPROG; allopregnanolone) and 5β-pregnan-3α-ol-20-one (3α,5β-THPROG; pregnanolone), collectively called 3α reduced neurosteroids. These neurosteroids neither directly activate nor potentiate GlyRs (Lambert *et al.*, 2001; Pistis *et al.*, 1997; Weir *et al.*, 2004). 3α,5β-THPROG in fact causes a small but significant inhibition of spinal GlyR currents (Fodor *et al.*, 2006; Wu *et al.*, 1997) and 3β-sulphates of pregnenolone have been shown to inhibit recombinant GlyRs expressed in oocytes (Maksay *et al.*, 2001).
By contrast, synthetic neurosteroids such as minaxolone, Org20599 and alphaxalone significantly enhanced homomeric α1 GlyR currents in recombinant systems (Ahrens *et al.*, 2008; Weir *et al.*, 2004). Another recent report has shown that two synthetic pregnanolone analogues potentiated homomeric α3 GlyR currents in a voltage-dependent fashion (Jin *et al.*, 2009). Although facilitation occurred with EC_{50} values approximately 10-fold higher than those required for $GABA_A$ receptors and with generally lower efficacies (Weir *et al.*, 2004), experiments on spinal dorsal horn neurons have shown that low micromolar concentrations of minaxolone prolong the decay time kinetics of glycinergic mIPSCs in lamina II neurons (Mitchell *et al.*, 2007). At 10 μM, minaxolone additionally increased the amplitude, but not the frequency of glycinergic mIPSCs. Tonic glycinergic currents found in the same dorsal horn neurons were insensitive to minaxolone (Mitchell *et al.*, 2007). The molecular sites involved in the modulation elicited by synthetic steroids on different GlyR subtypes are still not investigated in depth. The high sensitivity of $GABA_A$ receptors to neuroactive steroids suggests that their effects are mainly related to GABAergic inhibition.

Tropeines

Tropeines were originally identified as potent 5-HT_3 receptor antagonists. Tropisetron, also known as ICS-205,930, is one of the best-known compound of this group. It is used mainly

as an antiemetic following chemotherapy due to its ability to target 5-HT_3 receptors involved in vomiting reflexes. An increasing body of evidence has shown that tropeines also allosterically modulate GlyRs of different subunit composition. Pioneering electrophysiological recordings in cultured spinal neurons have revealed that two tropeines, MDL-72222 and tropisetron, were able to potentiate GlyR chloride currents at nanomolar concentrations (Chesnoy-Marchais, 1996). In contrast, higher micromolar concentrations caused inhibition. Subsequent studies determined that potentiation only occurred in the presence of GlyR agonists, depended on the agonist concentration, and was also present in outside-out patches (Chesnoy-Marchais, 1996; Supplisson and Chesnoy-Marchais, 2000; Yang *et al.*, 2007). The potentiation elicited by tropisetron remained unaltered in the presence of zinc, ethanol or propofol, suggesting different binding sites and mechanisms (Chesnoy-Marchais, 1999). Interestingly, tropisetron also displayed subunit-specificity. Studies in recombinant GlyRs showed that tropisetron potentiated homomeric α1 but inhibited homomeric α2-GlyRs. Furthermore the expression of β subunits significantly increased the potentiation sensitivity of α1 and switched α2-GlyR inhibition to potentiation. These results suggest that the tropeine potentiating site lies within the α-α or α-β interface (Supplisson and Chesnoy-Marchais, 2000). Other studies also found that α2-GlyR was more effectively inhibited by tropisetron than α1-GlyR, but in contrast, they did not find any potentiation even in the presence of β subunits (Maksay *et al.*, 1999). Despite these differences, the electrophysiological data correlated well with binding studies in recombinant and native membrane preparations. For example, several tropeines have been shown to inhibit ^{3}[H]strychnine binding to GlyRs with high nanomolar affinity. In addition they increase the glycine potency to displace ^{3}[H]strychnine, suggesting direct effects on glycine binding sites (Maksay, 1998; Maksay *et al.*, 2004). In general terms, structure-activity analysis suggests that the tropeine ring itself, the tropeine nitrogen, an aromatic ring and a carbonyl group are necessary for binding and functional potentiation (Chesnoy-Marchais *et al.*, 2000; Maksay, 1998; Maksay *et al.*, 2004). The tropeine ring, on the other hand, appears to be a primary requirement for functional inhibition (Maksay *et al.*, 2009; Yang *et al.*, 2007).

Recently, several studies addressed the location of the tropeine binding sites on GlyRs. In agreement with studies performed in 5-HT_3 receptors (Joshi *et al.*, 2006; Yan and White, 2005), tropeines appear to bind to cavities within the extracellular domain located close to the ligand binding sites. Using recombinant GlyRs, Yang *et al.* (2007) showed that mutations to N102 in the α1, but not in the β subunit (N125), abolished tropisetron inhibition without affecting the potentiation. Subsequent work performed with a structurally related tropeine (3α-(3'-methoxy-benzoyloxy) nortropane, MBN) determined that other amino acid substitutions close to the agonist-binding domain of α1-GlyRs also alter the MBN inhibition or potentiation of GlyRs (see Fig. 2, Maksay *et al.*, 2009). In addition, homology models and

molecular docking simulations also suggest that the bi-phasic modulation elicited by tropeines on GlyRs is likely to involve different docking modes in adjacent binding sites within the agonist-binding region (Maksay *et al.*, 2009).

The high affinity binding and the remarkable sensitivity of GlyRs to tropeines makes this group of compounds one of the most promising candidates for the development of specific drugs targeting GlyRs. Despite the existence of some interesting differences between the chemical determinants required for tropeine binding to GlyRs and 5-HT_3 receptors, most tropeines still bind and modulate 5-HT_3 receptors with high affinity (Maksay *et al.*, 2004). In addition, the bi-phasic nature of tropeine-GlyR modulation and the significant overlap between the requirements for potentiation and inhibition is also an important impediment to their use as enhances of GlyR function. A better understanding of the mechanisms underlying the potentiation of GlyR subtypes by tropeines will hopefully lead to new tropeine agents lacking glycinergic inhibition and 5-HT_3 receptor binding.

Zinc

The interaction of GlyRs with the cation zinc is probably at present the best characterized form of allosteric modulation of GlyR. Previous research has not only consistently established the molecular sites involved, but work in point mutated mice has also firmly established a physiological role of this modulation. Zinc modulates GlyRs in a bi-phasic manner. Potentiation dominates at low (< 10 µM) concentrations while inhibition occurs at higher concentrations (>10 µM) (Bloomenthal *et al*., 1994; Doi *et al*., 1999; Laube *et al*., 2000). This bi-directional modulation involves different molecular sites. Potentiation is due to an increase in the affinity of GlyRs to glycine, while inhibition occurs through reduced efficacy. Amino acids involved in the potentiation by zinc are D80, E192, E194 (Laube *et al.*, 2000; Lynch *et al.*, 1998), while inhibition involves H107, H109, T112, and T133 (all positions refer to α1-GlyR) (Harvey *et al*., 1999; Laube *et al*., 2000; Miller *et al*., 2005). The different glycine receptor isoforms differ in their susceptibility to modulation by glycine. Zinc inhibits α2-GlyR and α3-GlyR to a lesser degree than α1-GlyR. This difference is apparently due to the substitution of amino acid H107 in α1-GlyR by an asparagine residue in the corresponding positions in α2 and α3-GlyR. Generation of a point mutated mouse carrying a D80A substitution, which largely ablates the potentiating effects of zinc without changing glycine sensitivity, expression level, or receptor trafficking to the synapse, revealed a physiological function of this modulatory site (and of zinc itself) in spinal cord neuronal circuits (Hirzel *et al*., 2006). Homozygous D80A point mutated mice exhibit a progressive hyperekplexia-like phenotype starting about at day P12, when α1-GlyRs replace embryonic α2-GlyRs. Whether these zinc modulatory sites are suitable for therapeutic targeting is however at present not known.

Conclusions

Pharmacological modulation of glycinergic inhibition could represent a novel therapeutic strategy against a variety of diseases involving altered synaptic inhibition primarily in the spinal cord and brain stem but possibly also at supraspinal sites. Several endogenous molecules including neurotransmitters and neuromodulators, and exogenous substances such as anaesthetics and alcohols have been identified that modulate GlyR function. As most pathologies linked to GlyR dysfunction involve diminished GlyR activity, positive allosteric modulation appears desirable in the majority of cases. Most currently available GlyR modulators are rather promiscuous and by no means specific for GlyRs (compare Tab. 2). These compounds are therefore not suitable for a therapeutic approach targeted specifically towards GlyRs. However, for several of them, direct modulation through allosteric sites is either firmly established or very likely. Yet in most cases (with the exception of zinc) the sites responsible are not yet firmly established. The existence of putative distinct sites for allosteric modulation on GlyRs however indicates future possibilities for a specific modulation of GlyR subtypes by novel synthetic ligands. This perhaps optimistic view is supported by the recent report which showed that an unbiased high throughput screening approach led to the identification of several highly specific GlyR modulator peptides (Tipps *et al.*, 2010). A comprehensive mapping of the molecular sites and mechanism involved will certainly facilitate the identification and development of small molecules specifically targeting GlyRs. In the absence of high-resolution structures for GlyRs (and hence also of structural data of these receptors with bound allosteric modulators or agonists), our knowledge is restricted to what can be inferred from functional studies using recombinant mutant receptors. Alternatively, advances might also come from the analysis of structurally related channels. High-resolution X-ray structures have recently been obtained from bacterial pentameric ligand-gated ion channel (reviewed recently by Baenziger and Corringer, 2011). For the bacterial proton-activated ion channel GLIC crystal structures have even been obtained with a general anaesthetics bound (Nury *et al.*, 2011). Such data will hopefully foster future structure-function studies on GlyRs, for example through improved homology modelling and molecular dynamic simulations. Through these and other new approaches, the discovery and development of new synthetic drugs targeting GlyRs with improved specificity and efficacy appears to be not too far fetched.

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Statement of conflicts of interest

The authors state no conflict of interests.

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Compound	***Native GlyRs*** (EC_{50} or concentration range examined)				***Comments***	***Reference***
AEA	↓ 0.1 – 1 μM neonatal rat hippocampal pyramidal neurons[1]				100 μM glycine (≈ EC_{50})	Lozovaya *et al.*, 2005
2-AG	↓ 1 μM neonatal rat hippocampal pyramidal neurons[1]					
WIN 55,212-2	Little effect on amplitude, but τ_{des} and τ_{on} decreased (0.1 – 10 μM)					
AEA	↑ (230 nM) acutely dissociated VTA neurons				5 μM glycine	Hejazi *et al.*, 2006
Δ^9- THC	↑ (115 nM) acutely dissociated VTA neurons					
Δ^9- THC	↑ (0.03-1 μM) cultured spinal neurons				10 μM glycine ≈ EC_2)	Xiong *et al*., 2011
meth-AEA	No effect on the amplitude or kinetics of glycinergic mIPSCs from spinal cord dorsal horn slices				5 μM meth-AEA	Anderson *et al.*, 2009
	Recombinant GlyRs (EC_{50} or concentration range examined)					
	α1	α1/β	α2	α3		
Δ^9-THC	↑ (86nM)	↑(73nM)	-	-	3-5 μM glycine ≈ EC_2	Hejazi *et al.*, 2006
Δ^9-THC	(0.03-50 μM)[2]	-	↑(0.03-50 μM)[2]	↑(0.03-50 μM)[2]	(subunit-dependent)	Xiong *et al*., 2011
AEA	↑ (320nM)	↑ (320nM)	-	-	3-5 μM glycine	Hejazi *et al.*, 2006
AEA	↑ (38 nM)	↑	no effect	no effect	≈ EC_{10} (subunit-dependent)	Yang *et al.*, 2008
HU-210	↑ (270nM)	↑	↑ (90 nM)	↑ (50 nM)		
WIN 55,212-2	no effect	no effect	↓ (0.22μM)	↓ (86 nM)		
N-arachidonyl-glycine	complex action[2]	complex action[2]	↑(3.03μM)	↑(1.32μM)		
Ajulemic acid	↑(9.7 μM)[3]	↑(12.4μM)[3]	-	-	10 μM glycine	Ahrens *et al.*, 2009b
Cannabidiol	↑(12.3 μM)[3]	↑(18.1 μM)[3]	-	-	10 μM glycine	Ahrens *et al.*, 2009a

Table 1. Cannabinoid ligand effects on native and recombinant GlyRs

[1] Qualitatively similar effects were obtained in cerebellar Purkinje neurons.
[2] EC_{50} values were not reported. The sensitivity to Δ^9-THC was α1=α3>α2
[3] Complex actions with initial potentiation and subsequent inhibition
[4] Direct activation was observed at 10-20 fold higher concentrations.

Group	*Representative ligands*	*GlyR* (relative potency[1])	*Additional targets*	*References*
Volatile anaesthetics	Isoflurane Enflurane	↑↑	$GABA_A$-R Voltage-gated Ca^{2+} channels NMDA-R 2P-domain K^+ channels	Yamakura *et al.*, 2001 Franks, 2008
Intravenous anaesthetics	Propofol	↑↑	$GABA_A$-R L-type Ca^{2+} channels 11β-hydroxylase	Yamakura *et al.*, 2001 Rudolph and Antkowiak, 2004 Franks, 2008
n-alcohols	Ethanol	↑	$GABA_A$-R NMDA-R GIRK channels	Aguayo *et al.*, 2002 Harris *et al.*, 2008
Avermectins	Ivermectin	↑↑**	nAch-Rs $GABA_A$-R $P2X_4$-R	Krusek and Zemkova, 1994 Krause *et al*., 1998 Silberberg *et al.*, 2007 Jelinkova *et al.*, 2008 Adelsberger *et al.*, 2000
Tropeines	Tropisetron	↑↑↑ / ↓↓[3]	$5HT_3$-R	Thompson and Lummis, 2007
Cannabinoid ligands	Anandamide THC	↑↑↑ / ↓↓	CB-R TRPV1 nAch-Rs / $5HT_3$-R	Piomelli, 2003 Oz, 2006
Bivalent cations	Zn^{2+}	↑↑ / ↓↓[3]	$GABA_A$-R NMDA-R TrkB-R	Smart *et al.*, 2004 Mony *et al.*, 2009 Sensi *et al.*, 2009
Glutamatergic ligands	AP5 NMDA	↑↑	NMDA-R	Dingledine *et al.*, 1999

Table 2. GlyR positive allosteric modulators and their additional targets

Potentiation or inhibition ranges: ↑↑↑ or ↓↓↓, nM; ↑↑ or ↓↓, μM; ↑ or ↓, mM
[1] Mainly from functional studies using electrophysiology (see text)
[2] Direct activator
[3] Bi-phasic modulation, inhibition at >10-50 μM

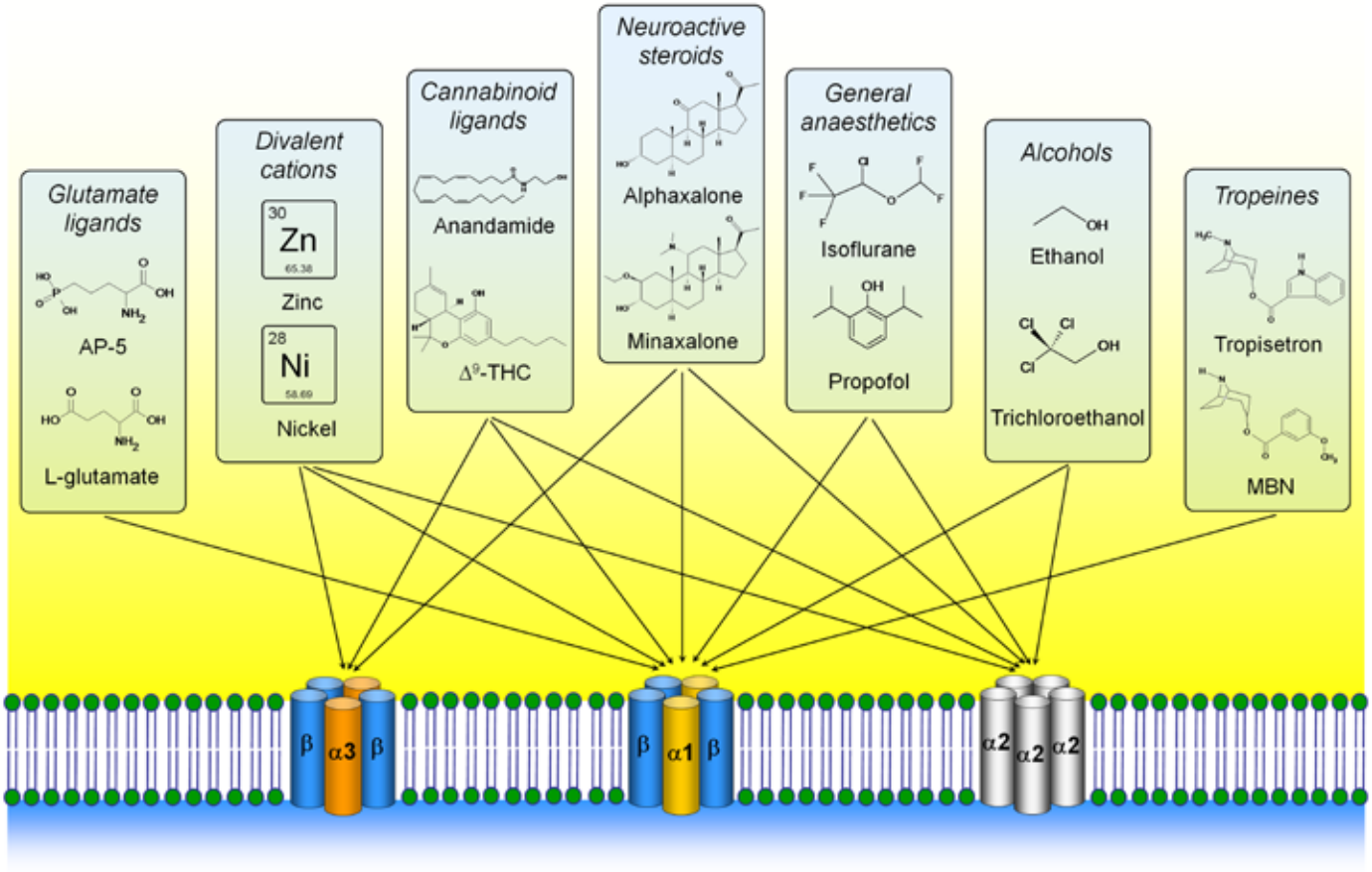
Figure legends

Figure 1. Allosteric modulation of GlyR subtypes

The scheme summarizes the interactions between several groups of allosteric modulators with different GlyR subtypes. Examples of some representative chemical structures for each group of compounds are shown.

Figure 2. Molecular sites for positive allosteric modulators of GlyR function

Critical residues are shown for several allosteric modulators that exert positive effects on GlyR function. The amino acid positions described were identified in functional experiments performed on the α1-GlyR mutants. The molecular sites involved in the effects elicited by neuroactive steroids, some cannabinoid ligands (i.e. endocannabinoids and synthetic cannabinoids) and glutamate receptor ligands remain to be defined. Data are from Mascia *et al.* (1996b), Michic *et al.* (1997), Laube *et al.* (2000), Lynch *et al.* (1998), Maksay *et al.* (2009), Lynagh and Lynch (2010), Xinog *et al.* (2011) and Yevenes *et al.* (2008).



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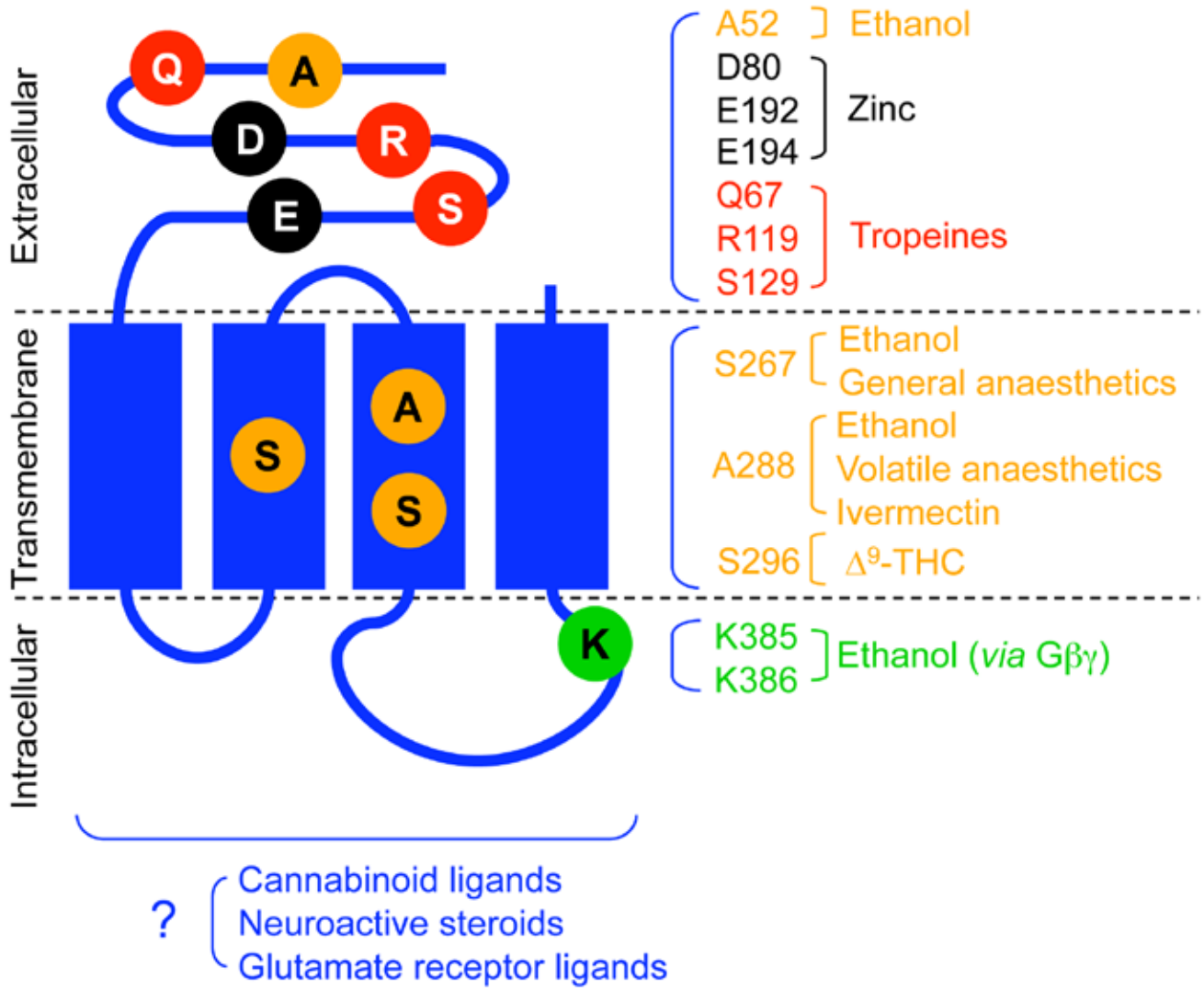

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