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REVIEW ARTICLE

GABA-ρ receptors: distinctive functions and molecular pharmacology

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The homomeric GABA-ρ ligand-gated ion channels (also known as $GABA_C$ or $GABA_A$-ρ receptors) are similar to heteromeric $GABA_A$ receptors in structure, function and mechanism of action. However, their distinctive pharmacological properties and distribution make them of special interest. This review focuses on GABA-ρ ion channel structure, ligand selectivity toward ρ receptors over heteromeric $GABA_A$ receptor sub-types and selectivity between different homomeric ρ sub-type receptors. Several GABA analogues show selectivity at homomeric GABA-ρ receptors over heteromeric $GABA_A$ receptors. More recently, some synthetic ligands have been found to show selectivity at receptors formed from one ρ subtype over others. The unique pharmacological profiles of these agents are discussed in this review. The classical binding site of GABA within the orthosteric site of GABA-ρ homomeric receptors is discussed in detail regarding the loops and residues that constitute the binding site. The ligand-residue interactions in this classical binding and those of mutant receptors are discussed. The structure and conformations of GABA are discussed in regard to its flexibility and molecular properties. Although the binding mode of GABA is difficult to predict, several interactions between GABA and the receptor assist in predicting its potential conformation and mode of action. The structure–activity relationships of GABA and structurally key ligands at ρ receptors are described and discussed.

Abbreviations

2-MeGABA; 4-amino-2-methylbutanoic acid; 2-MeTACA; *trans*-2-methyl-4-aminocrotonic acid; 3-AMOHP, 3-(aminomethyl)-1-oxo-1-hydroxy-phospholane; 3-AOHP, 3-(amino)-1-oxo-1-hydroxy-phospholane; 3-GOHP, 3-(guanidino)-1-oxo-1-hydroxy-phospholane; 4-ACPAM, 4-aminocyclopent-1-enecarboxamide; 4-ACPHA, 4-amino-N-hydroxycyclopent-1-enecarboxamide; ACPBPA; 3-aminocyclopentenyl-butylphosphinic acid; (±)-ACPECA; (±)-4-aminocyclopent-2-ene-1-carboxylic acid; CACA; *cis*-4-aminocrotonic acid; CAMP; *cis*-2-(aminomethyl)cyclopropane carboxylic acid; I4AA; imidazole-4-acetic acid; LGIC; ligand-gated ion channel; TACA; *trans*-4-aminocrotonic acid; TAMP; *trans*-2-(aminomethyl) cyclopropane carboxylic acid; THIP; 4,5,6,7-tetrahydroisoxazolo[5,4-c] pyridin-3-ol; TM; transmembrane; TPMPA; (1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid

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Cys-loop pentameric ligand-gated ion channels

The **$GABA_A$-ρ receptors** as designated by the International Union of Pharmacology (IUPHAR) (Alexander *et al.,* 2015) and also known as GABA-ρ or $GABA_C$ receptors, are homopentameric ligand-gated ion channels (LGIC) composed of ρ subunits. They are members of the pentameric or Cys-loop LGIC superfamily comprising excitatory cation selective receptors such as **nicotinic acetylcholine receptors**, **5-HT_3 receptors** and **zinc-activated channels**, and inhibitory anion-selective receptors such as $GABA_A$ receptors, strychnine-sensitive **glycine receptors** and invertebrate **glutamate-gated chloride channels** (Thompson *et al.,* 2010; Baenziger and Corringer, 2011). Receptors of this superfamily require five subunits to assemble a single ion channel. The ion channel may be homomeric formed by five identical subunits as is the case of GABA-ρ receptors or heteromeric, consisting of a combination of at least two different subunits, such as $GABA_A$ receptors (Olsen and Sieghart, 2009).

The Cys-loop receptors are analogous to each other in their structure, and they consist of three domains. The N-terminal extracellular domain is generally formed by 10 β-strands in two sheets that form a sandwich and two α-helices (Figure 1). This domain contains the orthosteric binding site and also the Cys–Cys disulfide bond forming the characteristic Cys-loop of 13 residues (also called β6–β7 loop). This loop is conserved across subunits belonging to this superfamily and is the basis of the name "Cys-loop receptors" (Miller and Smart, 2010). This structure is believed to be important for both cell surface expression and cooperative interaction between the agonist binding sites and the channel gate (Wong *et al.,* 2014). The second domain consists of the four transmembrane α-helices (TM1–TM4). TM2 forms the pore of the ion channel, whereas the remaining three TM helices form a hydrophobic environment to incorporate the pore into the plasma membrane (Figure 1) (Corringer *et al.,* 2000). The third domain is an intracellular loop between TM3 and TM4 that is variable and of unknown structure. This loop has little residue conservation between different subunits or subunits of different subtypes. There is evidence that the intracellular domain is involved in modulating the receptor by phosphorylation and binding to other intracellular molecules. There is a short extracellular C terminus after M4 (Filippova *et al.,* 1999).

Ionotropic GABA receptor subunit composition

In humans, there are 19 isoforms of $GABA_A$ subunits, that is, six α, three β, three γ and one of δ, ε, π, θ, known to form heteromeric $GABA_A$ receptors, and three ρ subunits that were reclassified by the Nomenclature Committee of IUPHAR (Olsen and Sieghart, 2009) to $GABA_A$ from a distinct class of receptors known as $GABA_C$. This reclassification is controversial due to the differing pharmacology, physiology and molecular biology of GABA receptors containing ρ-subunits from those containing non ρ-subunits. Most $GABA_A$ receptors are heteromeric and require at least two different $GABA_A$ subunits (Olsen and Sieghart, 2009). The predominant $GABA_A$ receptors in brain tissues are formed from two α, two β and one γ subunit, for example, $GABA_A$ α1β2γ. The formation of active GABA-gated ion channels requires the presence of at least α and β subunits. However, GABA-ρ receptors express as homomeric ion channels, and some studies suggest pseudoheteromeric channels consisting of different ρ subunits (Connolly *et al.,* 1996). There is some limited *in vitro* evidence to support co-assembly of ρ subunits with other $GABA_A$ subunits, particularly with α1 and γ2 (Milligan *et al.,* 2004; Pétriz *et al.,* 2014), and also with glycine receptor α1 and α2 subunits (Pan *et al.,* 2000).

Significant differences have been identified between various ionotropic GABA receptors based on physiological,

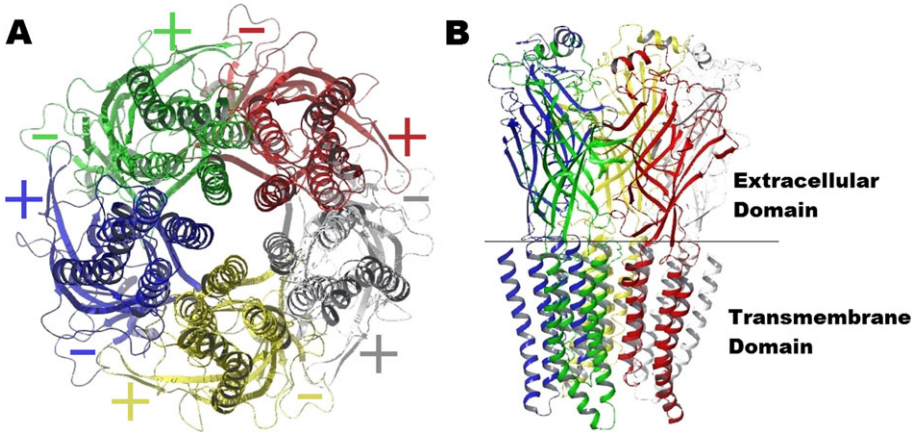

Figure 1

Pentameric ligand-gated ion channel receptor of GABA-ρ homology model based on GluCl structure prepared has been previously described (Naffaa *et al.,* 2015). A single ion channel showing the main domains with five identical subunits, coloured differently to show the intersubunit interfaces located between the principal (+) and complementary (−) sides. (A) Bottom view and (B) side view. As the structure of the intracellular domain has not yet been determined by crystallography, it is not included.

pharmacological and biochemical properties (Johnston, 2002). The amino acid sequence identity between various $GABA_A$ subunits and the ρ subunits ranges between 35 and 45% but is as high as 75% in the TM region (Le Novere and Changeux, 1999). GABA-ρ ion channels expressed in *Xenopus* oocytes have different properties to other $GABA_A$ receptors in terms of potency, channel opening time and receptor desensitization. In general, **GABA** is between 10- and 100-fold more potent at GABA-ρ receptors than heteromeric $GABA_A$ receptors, with slow activation and deactivation and less readily desensitized (Feigenspan *et al.*, 1993; Amin and Weiss, 1994; Feigenspan and Bormann, 1994; Bormann, 2000). Heteromeric $GABA_A$ receptors are also well known to be modulated by agents such as benzodiazepines, barbiturates and neurosteroids (Johnston, 1996; Bormann, 2000). GABA-ρ receptors are insensitive to these $GABA_A$ receptor modulators. However, some $GABA_A$ modulators can also modulate GABA-ρ receptors, such as zinc, lanthanides (Wu *et al.*, 1993; Calvo *et al.*, 1994; Chang *et al.*, 1995) and some synthetic neurosteroids (Morris *et al.*, 1999).

GABA-ρ receptors

The concept of a third sub-type of GABA receptor arose in 1984 from the studies demonstrating the lack of inhibition by ***cis*-4-aminocrotonic acid (CACA)** (Figure 2), a GABA analogue that has a bicuculline-insensitive depressant action on the firing of cat spinal neurones, on the binding of [^{3}H]-baclofen to the rat cerebellar membranes and thus unlikely to act as an agonist on bicuculline-sensitive $GABA_A$ or baclofen-sensitive **$GABA_B$** receptors (Drew *et al.*, 1984). Later, bovine retinal mRNA expressed in *Xenopus* oocytes was found to result in receptors sensitive to GABA but insensitive to both bicuculline and baclofen (Polenzani *et al.*, 1991), and the novel **ρ1 subunit** from a human retina cDNA library was cloned in the early 1990s (Cutting *et al.*, 1991). Originally known as (and often still referred to as) $GABA_C$ receptors, the second member of this subfamily, **ρ2 subunits** were cloned from human retina a year later (Cutting *et al.*, 1992) and is also found in brain tissue such as hippocampus, cerebellum and pituitary, with significant abundance (Lopez-Chavez *et al.*, 2005). A third member of this subfamily, **ρ3**, has been detected in retina and at lower expression levels in higher brain regions (Boue-Grabot *et al.*, 1998; Bailey *et al.*, 1999). In the retina, ρ3 subunits are expressed in ganglion neurons, while ρ1 and ρ2 subunits are specifically expressed in bipolar and horizontal cells (Qian and Dowling, 1993; Fletcher *et al.*, 1998; Lopez-Chavez *et al.*, 2005). ρ1 knockout mice studies have indicated that ρ receptors are present in the superior colliculus (Schlicker *et al.*, 2009). The presence of GABA ρ1 and ρ2 subunits, either homomerically or combined with other $GABA_A$ (α1 and γ2) subunits, has also been identified in cultured cerebellar astrocytes. It is proposed that these GABA-ρ subunits may contribute to the regulation of glial development in the cerebellum (Pétriz *et al.*, 2014). Although GABA-ρ receptors are mainly expressed in the CNS, they are also found in the peripheral nervous system, for example, in the gastrointestinal tract (Jansen *et al.*, 2000), and sperm cells (Li *et al.*, 2008). Members of this ρ subfamily share more than 70 and 95% amino acid identity and similarity respectively (Figure 3) (Zhang *et al.*, 2001).

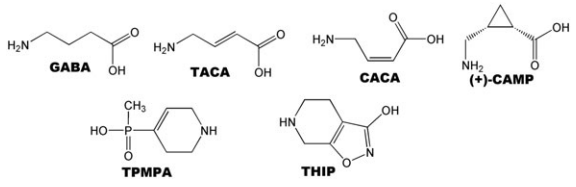

Figure 2
Chemical structures of ligands that selectively distinguish GABA-ρ1 receptors from $GABA_A$ receptors.

The involvement of GABA-ρ receptors in a range of physiological processes has been suggested, including the inhibition of ammonia-induced apoptosis in hippocampal neurons (Yang *et al.*, 2003) and hormone release in the pituitary (Boue-Grabot *et al.*, 2000). The GABA-ρ antagonist (1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid; **TPMPA**) (Figure 2) was shown to improve the symptoms of retinitis pigmentosa in rats (Jensen, 2012) and inhibit the development of myopia in chicks (Chebib *et al.*, 2009b). Both GABA-ρ1 and -ρ2 receptors are found in the hippocampus as extrasynaptic receptors activated by GABA through spillover (Alakuijala *et al.*, 2006) and are believed to be involved in paired-pulse depression of inhibitory postsynaptic currents (Xu *et al.*, 2009). More recently GABA-ρ2 receptors expressed pre-synaptically in the spinal dorsal horn have been implicated in pain perception and identified as a novel target for analgesia (Tadavartya *et al.*, 2015). Behavioural pharmacological studies have shown an important role for ρ1 receptors in the sleep-waking behaviour of rats (Arnaud *et al.*, 2001), learning and memory in chicks and rats (Chebib *et al.*, 2009b; Gibbs and Johnston, 2005), the inhibitory modulation of the olfactory bulb (Chen *et al.*, 2007) and evidence that ρ1 and ρ2 receptors may be important for some specific, *in vivo*, effects of ethanol (Blednov *et al.*, 2014).

Structure and function of GABA-ρ1 receptors

Homology models of GABA-ρ receptors (Harrison and Lummis, 2006b; Abdel-Halim *et al.*, 2008; Osolodkin *et al.*, 2009; Tai *et al.*, 2009; Naffaa *et al.*, 2015) have been based on the five different LGIC crystal structures, AChBP, ELIC, GLIC, $GABA_A$ β_3 and GluCl (Brejc *et al.*, 2001; Hilf and Dutzler, 2008; Chen *et al.*, 2010; Hibbs and Gouaux, 2011; Miller and Aricescu, 2014). With the exception of AChBP, these crystal structures are of full ion channels, with varying percentages of residues conserved with GABA-ρ. AChBP, ELIC and GLIC have limited amino acid conservation with ρ1 (~20% amino acid identity), while for $GABA_A$ β_3 and GluCl receptors, this is ~40% (Figure 4) (Naffaa *et al.*, 2015).

The human homopentameric $GABA_A$ β3 receptor was the first of the $GABA_A$ receptor structures to be resolved (Miller and Aricescu, 2014). The β3 subunit has a relatively high sequence identity to the ρ1 subunit, and it is unlikely to form functional homomeric receptors *in vivo* (Tretter and Moss, 2008). In $GABA_A$ heteromeric receptors, the orthosteric

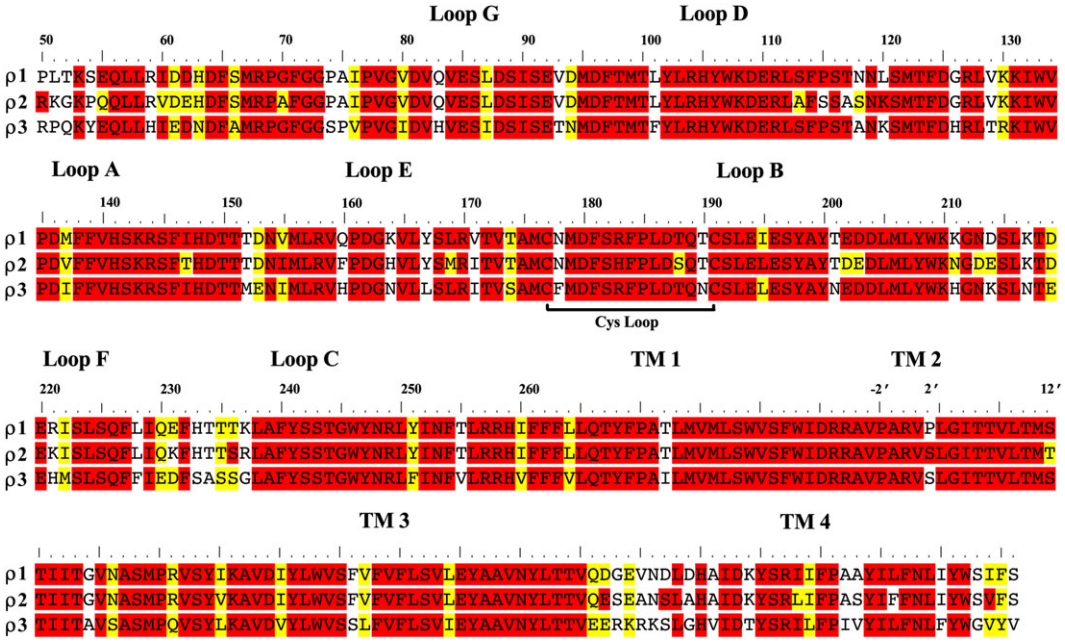

Figure 3

The amino acid sequence alignment of GABA-ρ1, GABA-ρ2 and GABA-ρ3 subunits. Alignments were prepared as previously described (Naffaa *et al.,* 2015). The amino acid sequence of various human GABA-ρ receptors was obtained from the Universal Protein Resources (http://www.uniprot.org/) (UniProt Consortium, 2013). The UniProt IDs for GABA-ρ1, GABA-ρ2 and GABA-ρ3 subunits are P24046, P28476 and A8MPY1 respectively.

binding site is between the β subunit on the principal (+) side (comprising loops A–C) and an α subunit on the complementary (−) side (comprising loops D–G), as defined in the same manner as shown for GABA-ρ receptors (Figure 1). The (−) side of the GABA β3 homomeric receptor lacks the key arginine residue that forms a critical salt-bridge between all α and ρ subunits and the carboxylate of GABA (Naffaa *et al.,* 2015). Therefore, the (+) side of the interface may provide accurate predictions, but the (−) side is lacking residues that form essential interactions with GABA. The homology model based on the $GABA_A$ β_3 template is not able to fully predict the critical interactions of GABA and its binding mode (Figure 4) (Baumann *et al.,* 2002; Naffaa *et al.,* 2015). In contrast, the ρ1 homology model based on GluCl is in excellent agreement with most previous ρ1 receptor experimental findings. Additionally, GluCl has been co-crystalized with its endogenous ligand, L-glutamate, which is similar to GABA in structure and flexibility that also helps to predict GABA interactions (Naffaa *et al.,* 2015).

GABA-ρ1 agonist binding site

Mutational studies on a number of ρ1 residues equivalent to some previously studied in the $GABA_A$ β2 subunit (Amin and Weiss, 1993) led to significant decreases in GABA sensitivity, revealing the importance of residues such as Tyr^{198}, Tyr^{200}, Tyr^{241}, Tyr^{247} and Thr^{244} (Figure 5) (Amin and Weiss, 1994; Bormann and Feigenspan, 1995). Mutation of Tyr^{102} to serine was also found to produce spontaneously active receptors (Torres and Weiss, 2002), and structurally diverse antagonists were found to exhibit different effects at these spontaneously active receptors suggesting differing affinities for the open and closed state of the receptor (Yamamoto *et al.,* 2012b). Other mutational studies have identified Tyr^{102}, Tyr^{106}, Phe^{138} and Phe^{240} in GABA-ρ1 receptors to be major determinants for antagonist selectivity at ρ1 receptors when compared with the $GABA_A$ α1β2γ receptors (Figure 4) (Zhang *et al.,* 2008). Mutation of the Tyr^{102} residue leads to significant changes in GABA activity but does not appear to be involved in stabilizing GABA in the binding site (Figure 5B). Therefore, Tyr^{102} may play a role in the conformational changes that lead to gating, as has been proposed for the equivalent residue (Phe^{64}) in the $GABA_A$ α1 subunit (Szczot *et al.,* 2014), or it may also have a critical role in stabilizing the protein by forming inter-subunit interactions (Figure 5B) (Miller and Aricescu, 2014; Naffaa *et al.,* 2015).

In a mapping study on loops A, E and F (Figure 5A) (Sedelnikova *et al.,* 2005), only residues Asp^{136} in loop A, Leu^{166}, Ser^{168} and Arg^{170} in loop E and Gln^{226} in loop F were noted to significantly decrease GABA sensitivity when cysteine was introduced at these sites. Interestingly, cysteine mutation of $Leu^{169,}$ located in loop E but orientated away from the binding site and not thought to be involved in GABA binding, resulted in a several-fold increase in the potency of GABA. Residues at the 169 position may be involved in the conformational changes of channel gating or form interactions with residues in the same subunit, with the interactions formed by the cysteine leading to greater stability. Trp^{133}, Pro^{135} and Phe^{139} in loop A and Gln^{160} in loop E, when individually mutated to cysteine resulted in non-functional receptors. As these residues are also some distance away from the GABA binding site, these residues

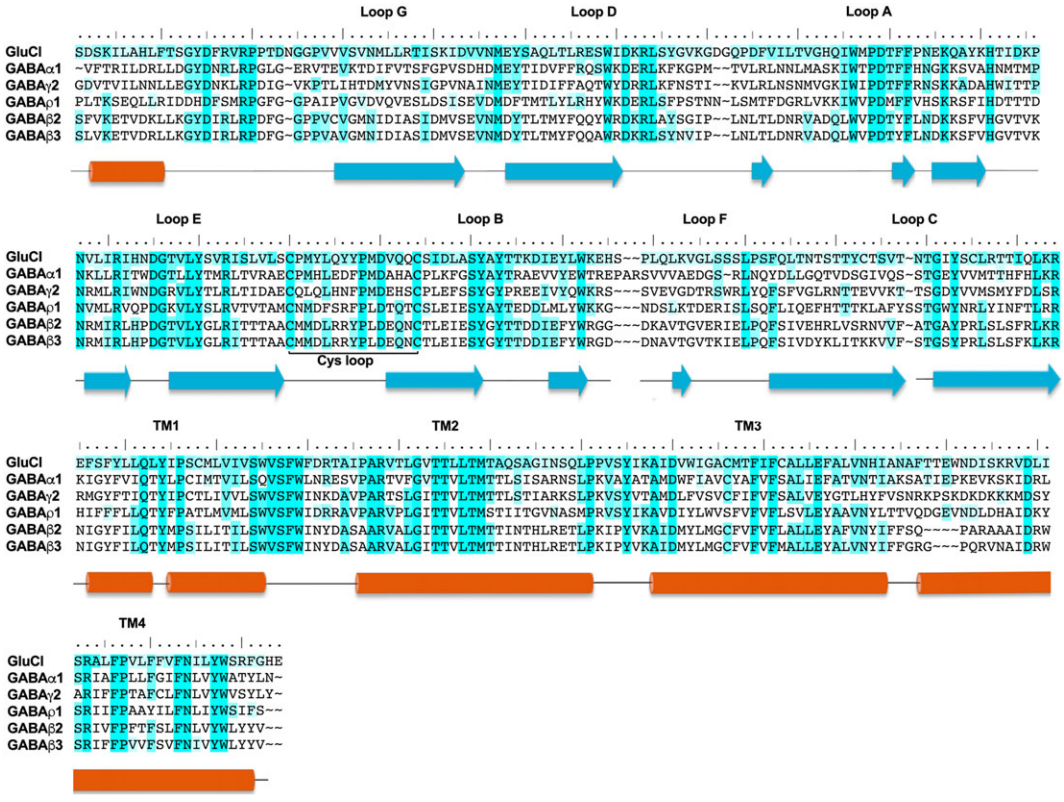

Figure 4

The amino acids sequence alignment of GluCl, GABA α1, GABAγ2, GABA ρ1, GABA β3 and GABA β2 subunits. The different shades of blue highlights show the conserved amino acids between the template 'GluCl' and the studied GABA subunits in this review. Dark blue indicates that the residue is conserved between all or most of the subunits, while light blue indicates that residue is conserved or related to another residue in two or three subunits only. Alignments were prepared as previously described (Naffaa *et al.,* 2015). The amino acid sequence of human GABA and GluCl subunits was obtained from the Universal Protein Resources (http://www.uniprot.org/) (UniProt Consortium, 2013) and Protein Data Bank (PDB). UniProtKT ID for GABA α1, GABAγ2, GABAρ1 and GABA β2 subunits are P14867, P18507, P24046 and P47870–1 respectively. PDB ID for GluCl and GABA β3 are 3RIF and 4COF respectively.

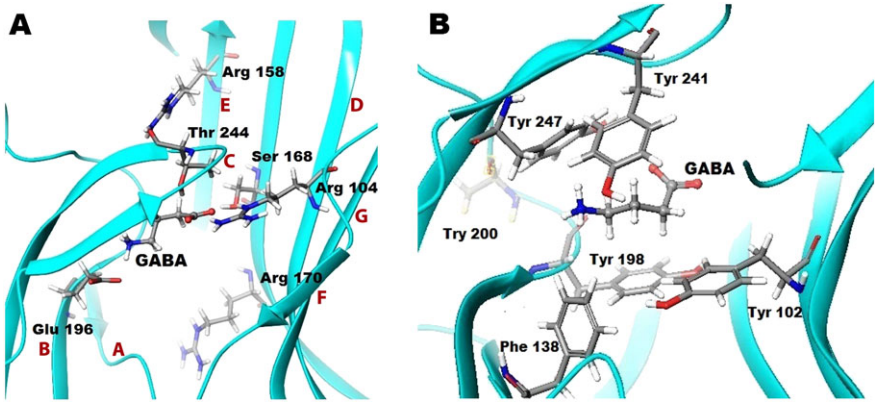

Figure 5

Molecular basis of GABA bound in the orthosteric binding site of GABA ρ1 homology model based on GluCl. (A) GABA bound GABA-ρ1 homology model based on GluCl, showing loops A–G (labelled in red), and H-bond interactions formed between GABA and Thr^{244} and Ser^{168}, and salt bridges with Arg^{104} and Glu^{196}. Arg^{158} and Arg^{170} are two important residues for protein stability either by forming interaction within the same subunit or between neighbouring subunits. (B) GABA and aromatic residues (Tyr^{102}, Tyr^{198}, Tyr^{241} and Tyr^{247}) forming the aromatic box that stabilizes GABA in the binding site during the channel gating. The Phe^{138} residue may form important interactions as it has a benzene ring system that partially oriented to both the GABA binding site and the adjacent subunit.

may have critical roles in protein stability through intra-subunit interactions rather than a direct effect on GABA binding. The same study showed that when cysteine replaces Gln^{160} in loop E, the receptors become spontaneously active, suggesting that this residue is involved in the conformational changes that lead to gating (Sedelnikova *et al.*, 2005).

Mutations of residues in the binding site within a distance of 7 Å of the carboxylate group of GABA identified Arg^{104} and Ser^{168} (Figure 5A) (Harrison and Lummis, 2006a) to be essential for GABA activity and have been predicted by modelling studies to form a salt bridge and a H-bond interaction, respectively, with GABA (Harrison and Lummis, 2006b; Abdel-Halim *et al.*, 2008; Melis *et al.*, 2008; Osolodkin *et al.*, 2009; Naffaa *et al.*, 2015). However, Arg^{158} and Arg^{170} residues (Figure 5A), which are also in close proximity to the carboxylate group of GABA in the binding site and have been found to be critical for receptor activation by GABA, are predicted by modelling not to be involved in GABA binding but important for protein stability (Harrison and Lummis, 2006b; Abdel-Halim *et al.*, 2008; Osolodkin *et al.*, 2009; Naffaa *et al.*, 2015).

Similar to other LGIC receptors, the orthosteric binding site of GABA-ρ1 receptors contains many aromatic residues. Mutational studies have demonstrated the significance of the aromatic residues Tyr^{198}, Tyr^{241} and Tyr^{247} (Figure 5B). Mutation of these tyrosine residues to the aromatic amino acid phenylalanine leads to only a minor effect on GABA response whereas mutation to the aliphatic serine leads to a significant decrease (Lummis *et al.*, 2012). These residues are predicted by modelling studies to have their aromatic group oriented toward the binding site and to form an aromatic box surrounding the ammonium group of GABA (Harrison and Lummis, 2006b; Abdel-Halim *et al.*, 2008; Osolodkin *et al.*, 2009; Naffaa *et al.*, 2015). Tyr^{200} (Figure 5B) was also found to be important for GABA activity. However, according to modelling studies, this residue has its functional group oriented away from the orthosteric binding site, indicating that it is unlikely to interact directly with GABA (Lummis *et al.*, 2012; Naffaa *et al.*, 2015).

Mutation of the ρ1 Thr^{244} residue in loop C, which has its hydroxyl group oriented toward the GABA binding site (Figure 5A), found that only the T244S mutation resulted in functional receptors, which were 35-fold less sensitive to GABA (Amin and Weiss, 1994). A range of agonists studied at the T244S mutant receptor demonstrated many-fold decreases in potency, while antagonist activity remained unaffected by the mutation (Yamamoto *et al.*, 2012a). Thr^{244} is proposed to be essential for the formation of an H-bond with agonists initiating conformational changes through movement of Loop C to open the channel (Naffaa *et al.*, 2016).

GABA-ρ1 channel

The structure of the TM2 domains of most LGIC receptors is highly conserved across species and subunits, with variation at only a few sites in the pore. The structures of the ρ1 and ρ2 subunits differ at two sites within TM2 regions only, Pro^{294} (−2′) and Ser^{304} (12′) in ρ1 are Ser^{290} (2′) and Thr^{300} (12′), respectively, in ρ2 subunits (Figure 6).

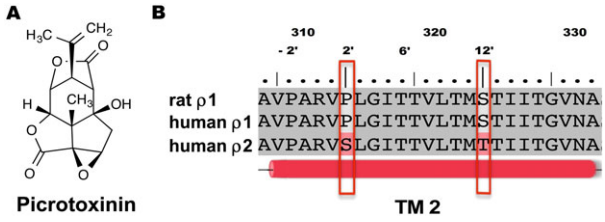

Figure 6

(A) Chemical structure of picrotoxinin. (B) The amino acids sequence alignment of the second TM domain of human GABA ρ1 and ρ2 subunits against the GABA rat ρ1 subunit.

Picrotoxinin is a pore-blocker at many ligand-gated ion channels (Akaike *et al.*, 1985; Jarboe *et al.*, 1968; Pribilla *et al.*, 1992). It binds to a unique site in TM2 (Gurley *et al.*, 1995; Wang *et al.*, 1995), inhibiting the chloride flux through the anionic ligand-gated channels (Inoue and Akaike, 1988; Etter *et al.*, 1999).

Picrotoxinin has different effects at ρ1 and ρ2 homomeric receptors and also at native GABA-ρ receptors from different species (Qian and Dowling, 1993; Lukasiewicz and Werblin, 1994; Zhang *et al.*, 1995). In GABA and glycine channels, the residues located at the 2′ positions play significant roles in the picrotoxinin activity (Lynch *et al.*, 1995; Shan *et al.*, 2001; Sedelnikova *et al.*, 2006). Introduction of mutations at Pro^{294} (−2′) and Pro^{298} (2′) (Figure 6) of human ρ1 homomeric receptors expressed in *Xenopus* oocytes changed not only picrotoxinin sensitivity but also the response of the mutant receptors to the agonists and partial agonists (Carland *et al.*, 2004). Rat GABA-ρ2 receptors are relatively insensitive to picrotoxinin (Feigenspan *et al.*, 1993). Mutation of Thr^{314} (6′) in the TM2 of the rat ρ1 subunit to methionine (Met^{299}), the equivalent residue in the rat ρ2 subunit, resulted in mutant receptors with smaller chloride currents (Figure 6). Co-expression of rat WT ρ1 and rat T314 M ρ1 mutant subunits resulted in receptors with picrotoxinin sensitivity that is similar to co-expressed rat WT ρ1 and ρ2 subunits, which led to the suggestion of native pseudo-heteromeric $GABA_A$ ρ receptors composed of ρ1 and ρ2 subunits in rat (Zhang *et al.*, 1995). In the ρ2 channel, the serine residue at the 2′ position is predicted by homology models to form multiple hydrogen bonds and hydrophobic interactions with picrotoxinin, whereas the homologous proline residues of ρ1 channels are predicted to form only hydrophobic contacts with picrotoxinin (Naffaa and Samad, 2016).

The Trp^{328} in TM3 of ρ1 receptors is important for sensitivity to **pentobarbital**. Although ρ1 wild-type receptors are generally insensitive to pentobarbital, when Trp^{328} is mutated to various hydrophobic residues, these ρ1 mutant receptors become sensitive to pentobarbital. This is possibly due to a change in structure that results from substitution of Trp^{328} by smaller, hydrophobic residues exposing the gate of the channels to the extracellular membrane components or affording a binding cavity for pentobarbital (Amin, 1999). Moreover, mutation of Ile^{323} in TM2 to serine also renders human homomeric ρ1 mutant receptors sensitive to barbiturates, which is believed to be due to allosteric rather than direct effects (Belelli *et al.*, 1999). Wild-type GABA-ρ receptors are also insensitive to

modulation by benzodiazepines. However, the double mutation of I307S/W328 M in ρ1 subunits renders homomeric mutant receptors sensitive to micromolar concentrations of diazepam (Walters *et al.,* 2000). These ρ1 I307S/W328 M mutant GABA receptors still resemble ρ1 WT receptors in terms of the pharmacological properties of agonists and antagonists, demonstrating that the receptors retain GABA-ρ character (Hall *et al.,* 2014).

Many divalent cations such as **Zn^{2+}**, Ni^{2+} and Cu^{2+} have modulatory actions at ρ1 receptors (Calvo *et al.,* 1994; Dong and Werblin, 1995). The zinc cation, which is present in the synaptic terminal of photoreceptors (Wu *et al.,* 1993), is able to reduce GABA responses at ρ1 receptors with an effect that depends on the extracellular pH. Because of this pH effect, the His^{156} residue in the orthosteric binding site was suggested to have a direct role in zinc modulation. Introduction of a tyrosine at this site leads to receptors insensitive to divalent cations such as Zn^{2+}. However, other pharmacological and electrophysiological properties were the consistent with those of wild-type receptors, supporting the involvement of this residue in the modulation by Zn^{2+} (Calvo *et al.,* 1994; Wang *et al.,* 1995).

Ligand selectivity for GABA-ρ receptors and structure activity relationships

Structure and conformations of GABA

GABA is a low molecular weight, zwitterionic ligand that has three rotatable carbon–carbon bonds, which afford flexibility and the ability to adopt many different conformations. The flexible rotation about C2–C3 and C3–C4 bonds allows GABA to exist in a range of low-energy conformations. Because of the variation in binding residues and sensitivities of specific subunit combinations to GABA, the conformational flexibility is critical for its biological activity at different receptors sub-types (Figure 2, Table 1). It is believed that GABA adopts different conformations at the orthosteric binding sites of different GABA receptor sub-types (Crittenden *et al.,* 2005a,c; Majumdar and Guha, 1988; Ottosson *et al.,* 2014). However, the question of the conformation(s) of GABA in the binding site has not yet been fully answered, and there is currently no evidence to support whether GABA is able to activate a receptor subtype in only one or more than one conformation. Indeed, this conformational flexibility may be an important factor in the ability of GABA to activate receptors.

Structure activity relationships of GABA-ρ receptors

The synthesis of conformationally restricted GABA analogues led to the initial identification of GABA-ρ receptors using ligands that are selective at this subfamily, relative to heteromeric $GABA_A$ receptors (Johnston *et al.,* 1975). The conformationally restricted GABA analogues, **CACA** and *trans*-3-aminocrotonic acid (TACA) possess an unsaturated bond at C2–C3 and are therefore only freely rotatable at the C3-C4 bond. The *cis*-isomer (CACA, Figure 2, Table 1) offers a folded conformation and is a potent and selective GABA receptor partial agonist. CACA depressed firing of cat spinal neurons with **bicuculline**-insensitive properties, and this depressant effect, not reproduced by **baclofen**, suggested pharmacologically distinct GABA receptors in the mammalian CNS, which were not $GABA_A$ or $GABA_B$ (Johnston, 1996). CACA is inactive at heteromeric $GABA_A$ receptors but is a partial agonist (70% efficacy) at GABA-ρ1 recombinant homomeric receptors. The *trans*-isomer (TACA, Figure 2, Table 1) exhibits an extended conformation and is a potent agonist. TACA has greater potency than GABA at ρ receptors but is non-selective, also acting as an agonist at heteromeric $GABA_A$ receptors (Woodward *et al.,* 1993; Kusama *et al.,* 1993a; Kerr and Ong, 1995).

The restricted cyclic analogue, **(+)-*cis*-2-(aminomethyl)cyclopropane carboxylic acid (CAMP)** (Figure 2), demonstrates greater selectivity for GABA-ρ receptors than CACA, which also inhibits GABA reuptake transporters. Interestingly, (+)- and (−)-CAMP show opposite pharmacological actions, with (+)-CAMP being a full agonist and (−)-CAMP being a weak antagonist on both ρ1 and ρ2 receptors (Duke *et al.,* 2000). Both (±)-CAMP and (±)-*trans*-2-(aminomethyl) cyclopropane carboxylic acid (TAMP) (Figure 7, Table 1) have been shown to differentiate between subtypes of GABA-ρ receptors (Table 1). (±)-CAMP is a partial agonist at ρ3 receptors while (±)-TAMP is an antagonist (Vien *et al.,* 2002). The area of steric interaction encountered by the antagonists (−)-TACP (Figure 8, Table 1) and (−)-CAMP (Figure 2, Table 1) is thought to be the same area of the binding site (Chebib *et al.,* 2001) and may be responsible for the antagonist action of these ligands.

(*S*)-2MeGABA (Figure 7) and (+)-4-aminocyclopent-2-ene-1-carboxylic acid [(+)-ACPECA] (Figure 8, Table 1) are full agonists at ρ1 and ρ2 receptors with preferred orientations that place the bulk of the molecule behind the plane. Their enantiomeric pairs, (*R*)-2MeGABA and (−)-ACPECA, are antagonists and orientate such that the bulk of the molecule is in front of the plane (Figure 8) (Crittenden *et al.,* 2006). This difference in orientation is thought to contribute to their opposing pharmacological activities. This finding is in accordance with the pharmacological profile of GABA analogues at the ρ1 receptor where substitution behind the double bond leads to agonist activity while substitution in front of the double bond results in antagonist activity (Crittenden *et al.,* 2005b). These results are also consistent with the observed antagonist activity of (−)-CAMP. Substitution in the front of the plane may lead to steric interactions that prevent the movement of loop C required for receptor activation, thus resulting in antagonist activity (Abdel-Halim *et al.,* 2008; Naffaa and Samad, 2016).

4,5,6,7-Tetrahydroisoxazolo[5,4-c] pyridin-3-ol (THIP) (**Gaboxadol**, Figure 2, Table 1), a relatively rigid analogue of GABA, is a potent antagonist at all GABA-ρ receptor subtypes (Johnston *et al.,* 2003) but acts as a partial agonist at synaptic $GABA_A$ receptors and as a potent super-agonist at extra-synaptic α4β3δ receptors (Krogsgaard-Larsen *et al.,* 2004). THIP has demonstrated therapeutic effects on the area of sleep and analgesia (Krogsgaard-Larsen *et al.,* 2004). The THIP derivative, **aza-THIP** (Figure 8, Table 1), is approximately equipotent to THIP as an antagonist at GABA-ρ1 receptors but is more selective for GABA-ρ receptors than THIP, with negligible activity at heteromeric $GABA_A$ receptors. Thio-THIP (Figure 8, Table 1) in which the

Table 1

The activities of the ligands at various GABA-ρ, $GABA_A$ and $GABA_B$ receptors.

	$GABA_A$-ρ1	$GABA_A$-ρ2	$GABA_A$-ρ3	$GABA_A$ ($\alpha_1\beta_2\gamma_{2L}$)	$GABA_B$
GABA	EC_{50} = 2.5μM[a]	EC_{50} = 2.5μM[b]	EC_{50} = 2.5μM[b]	EC_{50} = 21.1μM[c]	EC_{50} = 1.7 μM[c]
TACA	EC_{50} = 0.44μM[d]	EC_{50} = 0.3μM[a]	EC_{50} = 3.8 μM[e]	EC_{50} = 133 μM[f]	Inactive
CACA	EC_{50} = 74 μM[g] (70% efficacy)	EC_{50} = 70 μM[g]	EC_{50} = 139 μM[e]	Inactive	Inactive
(+)-CAMP	EC_{50} = 40 μM[g]	EC_{50} = 17 μM[g]	EC_{50} = 28 μM[g]	Weak antagonist ≥ 1mM[g]	Inactive
TPMPA	K_B = 2.1 μM[h]	K_B = 15.6 μM[i]	K_B = 10.2 μM[e]	Weak Antagonist K_B = 320 μM[h]	Weak agonist EC_{50} ~ 500 μM[h]
THIP	K_i = 25 μM[k]	-	IC_{50} = 10.2 μM[e]	EC_{50} = 355 μM[l]	Inactive
Picrotoxinin	IC_{50} = 48μM[m]	IC_{50} = 4.8μM[m]	-	IC_{50} = 5 μM[n]	Inactive
I4AA	IC_{50} = 1.45 μM[i]	IC_{50} = 3.18 μM[j]	IC_{50} = 12.6 μM[i]	EC_{50} = 138 μM[a]	-
2-MeTACA	K_b = 45 μM[i]	Partial agonist K_B = 101 μM, I_{MAX} = 34%)[i]	Inactive[e]		
(-)-TAMP	EC_{50} = 9 μM, I_{MAX} = 39%[g]	EC_{50} = 55 μM, I_{MAX} = 55%[g]	Antagonist[g]	EC_{50} = 50 μM, I_{MAX} = 51%[g]	Inactive
(R)-ACPBPA	K_B = 60 μM[c]	K_B = 6 μM[o]	-	Inactive[c]	Inactive[c]
(S)-ACPBPA	K_B = 5 μM[c]	K_B = 11 μM[o]	-	Inactive[c]	Inactive[c]
(S)-2MeGABA	EC_{50} = 65μM[p]	EC_{50} = 20μM[p]	EC_{50} = 25μM[q]	-	-
(R)-2MeGABA	IC_{50} = 16μM[p]	IC_{50} = 36μM[p]	-	-	-
(+)-ACPECA	EC_{50} = 135μM[p]	EC_{50} = 60μM[p]	EC_{50} = 110μM[p]	-	-
(−)-ACPECA	IC_{50} = 300 μM Inhibit 25% of GABA EC_{50}[p]	-	IC_{50} = 300 μM Inhibit 25% of GABA (3μM)[p]	-	-
(+)-TACP	EC_{50} = 2.7 μM, I_{MAX} = 83%[q]	EC_{50} = 1.5 μM, I_{MAX} = 85%[q]	EC_{50} = 2.7 μM, I_{MAX} = 27%[q]	-	-
(−)-TACP(100 μM)	2% activation (56% blockade of 1 μM GABA)[q]	4.5% activation (62% blockade of 1 μM GABA)[q]	-	-	-
Aza-THIP	K_i = 31 μM[k]	-	-	K_i > 100 μM[k]	-
Thio-THIP	K_i = 91 μM[k]	-	-	K_i = 52 μM[k]	-
3-AMOHP	IC_{50} = 20 μM[r]	IC_{50} = 60 μM[r]	-	Inactive[q]	Weak agonist[r]
3-GOHP	IC_{50} = 30 μM[r]	IC_{50} = 50 μM[q]	-	Inactive[r]	Inactive[r]
3-AOHP	Inactive[r]	-	-	Inactive[r]	Inactive[r]
4-ACPAM	IC_{50} = 10 μM[s]	-	-	IC_{50} = 23 μM[s]	Inactive[s]
4-ACPHA	IC_{50} = 13 μM[s]	-	-	Inactive[s]	Inactive[s]

Note: K_B = the molar concentration of a competitive antagonist that would occupy 50% of the receptors at equilibrium; K_i = the molar concentration of a competing ligand that would occupy 50% of the receptors if no radioligand was present; IC_{50} = the molar concentration of an agonist or antagonist which produces 50% of its maximum possible inhibition in a functional assay. 2-MeTACA, *trans*-2-methyl-4-aminocrotonic acid; 3-AMOHP, 3-(aminomethyl)-1-oxo-1-hydroxy-phospholane; 3-AOHP, 3-(amino)-1-oxo-1-hydroxy-phospholane; 3-GOHP, 3-(guanidino)-1-oxo-1-hydroxy-phospholane; 4-ACPAM, 4-aminocyclopent-1-enecarboxamide; 4-ACPHA, 4-amino-N-hydroxycyclopent-1-enecarboxamide.

[a]Data from Kusama *et al.* (1993a).
[b]Data from Kusama *et al.* (1993b).
[c]Data from Kumar *et al.* (2008).
[d]Data from Chebib *et al.* (1997).
[e]Data from Vien *et al.* (2002).
[f]Data from Ragozzino (1996).
[g]Data from Duke *et al.* (2000).
[h]Data from Murata (1996).
[i]Data from Chebib *et al.* (1998).
[j]Unpublished data Nafaa et al
[k]Data from Krehan *et al.* (2003a).
[l]Data from Ebert (1997).
[m]Data from Wang *et al.* (1995).
[n]Data from Xu (1995).
[o]Data from Kim *et al.* (2008).
[p]Data from Crittenden *et al.* (2006).
[q]Data from Chebib *et al.* (2001).
[r]Data from Gavande *et al.* (2011).
[s]Data from Locock *et al.* (2013).

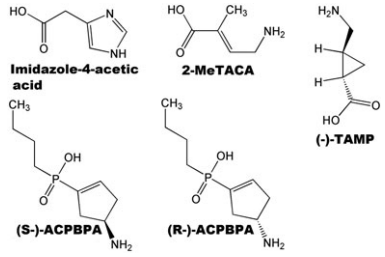

Figure 7
Chemical structures of ligands demonstrate subunit selectivity between homomeric receptors composed of different GABA ρ subunits.

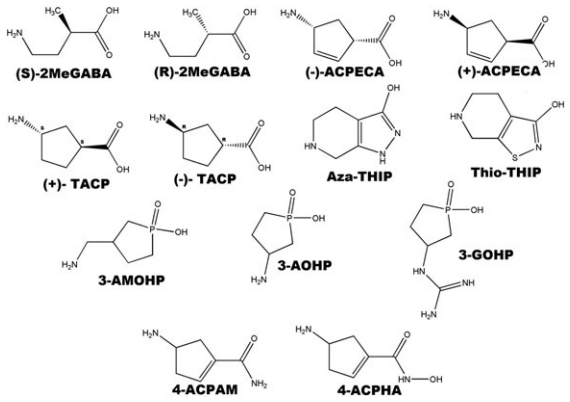

Figure 8
Chemical structures of ligands used to study structure activity relationships at GABA-ρ receptors.

isoxazole oxygen is replaced by a bulkier sulfur atom has threefold lower potency than THIP at GABA-ρ receptors, which is likely due to increased steric bulk of the sulfur atom (Krehan *et al.,* 2003a).

The interaction between the acidic moiety of ligands and the GABA-ρ receptor proteins is a common interaction among many GABA ligands. Ligands containing different acidic groups including carboxylic, phosphinic, methylphosphinic, phosphonic and seleninic acids have been investigated for their activity at GABA-ρ receptor (Chebib *et al.,* 1997; Krehan *et al.,* 2003b; Kumar *et al.,* 2008; Chebib *et al.,* 2009a,b; Gavande *et al.,* 2011). Additionally, replacement of the carboxylic acid moiety with either a hydroxamic acid (4-amino-N-hydroxycyclopent-1-enecarboxamide) or amide group (4-aminocyclopent-1-enecarboxamide) (Figure 8, Table 1) results in antagonists of moderate potency with increased selectivity for GABA-ρ receptors. This indicates that a zwitterionic structure is not essential for strong ligand–receptor interactions (Locock *et al.,* 2013) but is required for activation of the receptor. The proposed antagonist binding site as determined by homology modelling has been predicted to be larger in the *apo* conformation of the GABA-ρ receptor than in the open conformation of the receptor, allowing the antagonists to be substituted with larger acidic moieties (Abdel-Halim *et al.,* 2008). This has been confirmed through a number of studies where changing the carboxylic acid to a larger acid such as alkyl phosphinic, phosphonic or seleninic acid leads to the conversion of agonist to antagonist activity (Chebib *et al.,* 1997; Krehan *et al.,* 2003b; Kumar *et al.,* 2008).

Modification of the amine moiety of amino-substituted phospholanes has been used to investigate the role of the basic functional group ($-NH_2$). 3-(aminomethyl)-1-oxo-1-hydroxy-phospholane (Figure 8, Table 1) is a potent antagonist at GABA-ρ1 receptors but inactive at $GABA_A$ (Gavande *et al.,* 2011). Shortening the carbon backbone by one carbon, 3-(amino)-1-oxo-1-hydroxy-phospholane (Table 1), led to loss of activity across all GABA receptors. This demonstrates the importance of the distance between the acid and amine groups in determining the ligand affinity, potency and selectivity (Chang *et al.,* 2000; Chebib *et al.,* 2009a; Gavande *et al.,* 2011). 3-(Guanidino)-1-oxo-1-hydroxy-phospholane (Figure 8 Table 1), with a guanidino group replacing the amine shows greater selectivity for GABA-ρ receptors with reduced inhibition at $GABA_A$ receptors (Gavande *et al.,* 2011). Replacement of the amine group by a carboxylic acid or hydroxyl group led to total loss of activity at mM concentrations at all GABA receptors. This indicates that a basic moiety such as an amine or guanidine group is essential for the affinity and potency of GABA (Gavande *et al.,* 2011).

Subunit-selectivity of ligands at GABA-ρ receptors

The GABA-ρ1 receptors are the most studied of the GABA-ρ subfamily, while ρ2 and ρ3 receptors have been much less studied. Although the three subunits are highly similar in their amino acid sequences across most regions, there are some important differences between them. However, a detailed study of these differences has not yet been undertaken.

Some ligands that bind in the orthosteric binding site can differentiate between different GABA-ρ subunits. Imidazole-4-acetic acid (I4AA) (Figure 7, Table 1) is a partial agonist at both ρ1 and ρ2 homomeric receptors expressed in oocytes. This ligand is a weak, low-efficacy partial agonist (EC_{50} = 60 μM, I_m = 8%) at ρ1 receptors, while it is potent and moderately efficacious (EC_{50} = 3 μM, I_m = 40%) at ρ2 receptors (Kusama *et al.,* 1993b; Chebib *et al.,* 1998; Madsen *et al.,* 2007). As a partial agonist at GABA-ρ receptors, I4AA also acts as an antagonist and shows a similar potency at both ρ subtypes (Table 1). Consistent with its weaker agonist potency and efficacy, I4AA is more efficacious as an antagonist at GABA-ρ1 receptors, inhibiting 97 and 20% of GABA responses at GABA-ρ1 and GABA-ρ2 homomeric receptors respectively (MN Nafaa *et al.*, unpublished data).

trans-2-Methyl-4-aminocrotonic acid (Figure 7, Table 1) is a moderate antagonist at ρ1 receptors, a partial agonist at ρ2 receptors (Chebib *et al.,* 1998), but inactive at ρ3 receptors (Vien *et al.,* 2002). However, *trans*-2-aminomethylcyclopropane carboxylic acid (−)-TAMP (Table 1) is a partial agonist at both human ρ1 and ρ2 receptors but an antagonist at ρ3 receptors (Vien *et al.,* 2002), although it should be noted that the only data available for (−)-TAMP at ρ3 receptors is from rat subunits.

(*S*)-4-Amino-2-methylbutanoic acid ((*S*)-2MeGABA) (Figure 8) is an agonist at ρ1 and ρ2 receptors and a partial agonist (90%) at ρ3 receptors. The stereoisomer, (*R*)-4-amino-2-methylbutanoic acid ((*R*)-2MeGABA) (Figure 8), is an antagonist at all three receptor subtypes. Furthermore, the stereoisomers showed selective potencies at ρ1 and ρ2 receptors as (*S*)-2MeGABA is a more potent agonist at ρ2 whereas (*R*)-2MeGABA is a more potent antagonist at ρ1 (Crittenden *et al.*, 2006).

The achiral antagonist, **TPMPA** (Table 1), is approximately equipotent at all GABA-ρ receptor subtypes. However, (*S*)- and (*R*)-aminocyclopentenyl-butylphosphinic acids ((*S*)-/(*R*)-ACPBPA) (Figure 8, Table 1) are potent stereoisomeric antagonists with differential selectivity at ρ1 and ρ2 receptors. (*R*)-ACPBPA, the only antagonist known at this time to be selective for ρ2 over ρ1 receptors, shows 10-fold selectivity at ρ2 receptors, while (*S*)-ACPBPA is a weakly selective for ρ1 over ρ2 receptors showing twofold selectivity (Kim *et al.*, 2008).

Conclusions

The testing of conformationally restricted GABA analogues led to the initial discovery of a distinct class of GABA receptors that did not fit the pharmacology of the sub-types known at that time. Following the naming strategy, these new receptors were termed $GABA_C$ and shown later to be composed of ρ subunits that were first cloned from the retina (ρ indicating retina) and later shown to form homopentameric LGICs. Although ρ subunits showed clear sequence homology to $GABA_A$ subunits, both the structure and pharmacology of these $GABA_C$ receptors were found to be much simpler than the complex heteromeric $GABA_A$ receptors with multiple subunit combinations. Many initial studies on these receptors focused on their physiological role in the retina, and many in the ophthalmic physiology field refer to the receptors simply as GABA-ρ, indicating that they are GABA-sensitive receptors composed of ρ subunits. The potential confusion through the identification of this class of receptor as either $GABA_C$ or GABA-ρ led the IUPHAR to review the nomenclature of GABA receptors (Olsen and Sieghart, 2008; Alexander *et al.*, 2015). It was 'especially recommended that the name $GABA_C$ receptor should not be used as the sole name for the ρ-receptors in an article including, especially, the title and abstract' (Olsen and Sieghart, 2008). Instead, $GABA_C$/GABA-ρ receptors should be referred to as a sub-class of $GABA_A$ receptor '$GABA_A$-ρ'. Rather than removing the confusion around the identity of these homomeric GABA LGICs, the classification of $GABA_A$-ρ has introduced a third term for these receptors, one that is in fact used by few researchers active in the field. This has further increased the possibility for confusion, complicating literature searches, and suggests that it may be time for a further review of GABA receptor nomenclature.

Despite confusion regarding the nomenclature, it is the lack of a GABA-ρ receptor crystal structure that has significantly hampered efforts to design selective ligands. However, the availability of X-ray crystal structures of related LGIC receptors has led to the development of GABA-ρ homology models that have been used to study interactions between the receptor and ligands. The development of ligands that are selective either for GABA-ρ over $GABA_{A/B}$ receptors or between different ρ subunit homomeric receptors has led to some understanding of the different structural features required for activity at the different receptors. It is particularly difficult to determine the binding conformation adopted in the receptor, as many ligands are able to exist in a number of low-energy conformations. The use of conformationally restricted GABA analogues and mutational studies combined with computational docking studies using homology models has facilitated understanding binding interactions and the conformational changes that may be required for receptors activation. However, there has not yet been any detailed study examining differences in interactions between conformationally restricted ligands at different ρ subunits.

Although the physiological function of GABA-ρ receptors is not fully understood, the selective distribution of ρ subunits indicates that GABA-ρ are specifically involved in visual image processing. The limited distribution and lower abundance of GABA-ρ receptors relative to heteromeric $GABA_A$ receptors suggests that GABA-ρ ligands are promising leads for developing agents with improved selectivity and therefore reduced unwanted effects. Further studies are encouraged to understand that the binding interactions and development of ligands with improved selectivity will facilitate greater knowledge of GABA-ρ receptors at both the molecular and physiological levels.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

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Conflict of interest

The authors declare no conflicts of interest.

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