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ORIGINAL PAPER

Neurochemicals for the Investigation of $GABA_C$ Receptors

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Abstract $GABA_C$ receptors are being investigated for their role in many aspects of nervous system function including memory, myopia, pain and sleep. There is evidence for functional $GABA_C$ receptors in many tissues such as retina, hippocampus, spinal cord, superior colliculus, pituitary and the gut. This review describes a variety of neurochemicals that have been shown to be useful in distinguishing $GABA_C$ receptors from other receptors for the major inhibitory neurotransmitter GABA. Some selective agonists (including (+)-CAMP and 5-methyl-IAA), competitive antagonists (such as TPMPA, (±)-cis-3-ACPBPA and aza-THIP), positive (allopregnanolone) and negative modulators (epipregnanolone, loreclezole) are described. Neurochemicals that may assist in distinguishing between homomeric ρ1 and ρ2 $GABA_C$ receptors (2-methyl-TACA and cyclothiazide) are also covered. Given their less widespread distribution, lower abundance and relative structural simplicity compared to $GABA_A$ and $GABA_B$ receptors, $GABA_C$ receptors are attractive drug targets.

Keywords GABA · $GABA_C$ · TPMPA · Allopregnanolone · Loreclezole · Cyclothiazide

Abbreviations

Aza-THIP	4,5,6,7-tetrahydropyrazolo[3,4-c] pyridin-3-ol
CACA	cis-4-aminocrotonic acid
(+)-CAMP	(1S,2R)-2-aminomethylcyclopropanecarboxylic acid
(±)-*cis*-3-ACPBPA	(±)-*cis*-(3-aminocyclopentanyl)butylphosphinic acid
5-Me-IAA	5-methyl-1H-imidazole-4-acetic acid
2-Methyl-TACA	*trans*-4-amino-2-methylbut-2-enoic acid
P4MPA	(piperidin-4-yl)methylphosphinic acid
SGS742	(3-aminopropyl)-*n*-butylphosphinic acid
THIP	4,5,6,7-tetrahydroisolazolo[5,4-c] pyridin-3-ol
TPMPA	(1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid

Introduction

$GABA_C$ receptors are being investigated for their role in many aspects of nervous system function including memory [1, 2], myopia [2, 3], pain [4] and sleep [5]. This review describes some representative neurochemicals that have been shown to be useful in distinguishing $GABA_C$ receptors from other receptors for the major inhibitory neurotransmitter GABA, thus aiding in the investigation of

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The authors are pleased to honour Abel Lajtha for his expert stewardship of this journal since its inception and his generous assistance to many Australian neurochemists.

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$GABA_C$ receptors. It is concerned mainly with studies on human recombinant receptors as a model system for the study of ionotropic GABA receptors of known subunit composition. For a comprehensive listing of GABA analogs that have been examined on human recombinant $GABA_C$ receptors, see the molecular modelling study by Abdel-Halim et al. [6].

The origin of the concept of $GABA_C$ receptors was the observation that CACA, cis-4-aminocrotonic acid (Fig. 1), inhibited the firing of cat spinal neurones in a manner insensitive to the $GABA_A$ antagonist bicuculline [7]. As CACA did not influence the binding of the $GABA_B$ agonist (−)-baclofen to rat brain membranes, this led to the suggestion that it was interacting with 'a class of binding site ($GABA_C$?) insensitive to (−)-baclofen and bicuculline [8]. The cloning of ρ1 [9, 10] and ρ2 receptors [11] together with the commercial availability of CACA led to a surge in studies on $GABA_C$ receptors. A third $GABA_C$ subtype ρ3 was subsequently cloned from rat retina [12]. $GABA_C$ receptors differ from $GABA_A$ and $GABA_B$ receptors on the basis of their significant physiological, pharmacological and molecular biological differences [13].

Genetic linkage and radiation hybrid mapping of the three human $GABA_C$ receptor subunit genes, GABRR1, GABRR2 and GABRR3, have shown that GABRR1 and GABRR2 are located close together, in a region of chromosome 6q that contains loci for inherited disorders of the eye, while GABRR3 maps to chromosome 3q11-q3.3 [14]. The mapping data suggests that the $GABA_C$ receptor subunit genes, which share a common ancestor with $GABA_A$ receptor subunit genes, diverged at an early stage in the evolution of this gene family with the $GABA_C$ and $GABA_A$ genes being located on different chromosomes [14]. Most neurochemical studies have been carried out on the ρ1 and to a lesser extent the ρ2 subtype of $GABA_C$ receptors, while only limited studies have been done on the ρ3 subtype. The bulk of these studies were on homomeric receptors although there is some evidence for heteromeric $GABA_C$ receptors made up of more than one ρ-subtype [15] and there is also evidence that all three ρ-subtypes occur in the same hippocampal neurones [16]. Polyclonal antibody studies suggest that $GABA_C$ receptors do not co-assemble with either $GABA_A$ or glycine receptor subunits in rat retina [17].

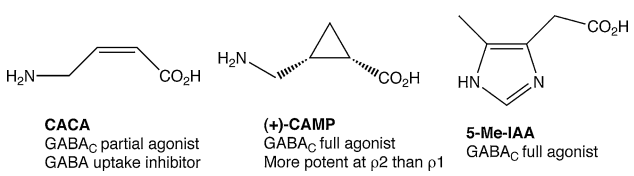

Fig. 1 Structures of some representative agonists at $GABA_C$ receptors

There is evidence for functional $GABA_C$ receptors in many tissues such as the retina, hippocampus, spinal cord, superior colliculus, pituitary and the gut [18]. The most extensive studies have been carried out in vertebrate retina [19]. Knockout studies show that elimination of the ρ1 subunit alters visual processing in mouse retina [20] and leads to changes in vascular permeability similar to the pathological changes induced by hypoxic conditions seen in diabetic retinopathy [21]. Knockout studies also show that ρ1 $GABA_C$ receptors mediate inhibitory modulation on the olfactory bulb [22] and that $GABA_C$ receptor mediated inhibition is altered, but not eliminated, in the superior colliculus suggesting that ρ2 or ρ3 $GABA_C$ receptors are functional in this tissue [23]. Knockout studies on the ρ2 $GABA_C$ receptor in mice suggest the involvement of these receptors in pain pathways [24]. Both ρ1 and ρ2 $GABA_C$ receptors are found in the hippocampus where there is evidence for their functional role as extrasynaptic receptors activated via spillover of synaptically released GABA [25] and in paired-pulse depression of inhibitory postsynaptic currents [26]. $GABA_C$ receptors may be involved in the regulation of thyrotropin release from the pituitary [27] and in synaptic transmission in the spinal cord [28]. $GABA_C$ receptors have also been described on neurones in the gastrointestinal system [29] where they may increase the release of nitric oxide from non-adrenergic, non-cholinergic inhibitory neurones [30].

$GABA_C$ Agonists

The prototype $GABA_C$ neurochemical, CACA, proved to be a partial agonist at human recombinant $GABA_C$ receptors expressed in *Xenopus* oocytes showing 70–80% of the efficacy of GABA and equally potent on ρ1 and ρ2 receptors (EC_{50} 70–74 μM) [31, 32]. Subsequently, CACA was found to have other actions including effects on GABA transport [33–35] and α6-containing $GABA_A$ receptors in the cerebellum [36]. Thus, CACA should be used with care as a selective $GABA_C$ agonist.

(+)-CAMP, (1S,2R)-2-aminomethylcyclopropanecarboxylic acid (Fig. 1), is a more selective $GABA_C$ agonist than CACA [37]. It is a full agonist at recombinant $GABA_C$ receptors, more potent at ρ2 (K_d 27 μM) than at ρ1 (K_d 40 μM) and does not influence GABA transport. It shows little activity at $\alpha1\beta2\gamma2L$ $GABA_A$ receptors. While both CACA and (+)-CAMP are conformationally restricted analogs of GABA in a folded conformation, conformational restriction in (+)-CAMP is achieved via a cyclopropane ring, rather than a *cis* double bond as in CACA. This has less influence on the ionisation of the carboxyl group, which is thus more acidic in CACA than in (+)-CAMP and GABA. (−)-CAMP, the enantiomer of

(+)-CAMP, surprisingly shows the opposite pharmacology at $GABA_C$ receptors being a weak antagonist (IC_{50} 900 and 400 μM at $\rho1$ and $\rho2$ receptors respectively) [37]. Rarely do enantiomers exert opposite pharmacological effects as in this case. The difficulty of separating the CAMP enantiomers means that supplies of enantiomerically pure (+)-CAMP are limited.

5-Me-IAA, 5-methyl-1H-imidazole-4-acetic acid (Fig. 1), is a full agonist that is more potent than (+)-CAMP at human $\rho1$ $GABA_C$ receptors expressed in HEK293 cells (EC_{50} 22 and 40 μM for 5-Me-IAA and (+)-CAMP respectively) [38]. It shows little activity at $\alpha1\beta2\gamma2S$ $GABA_A$ receptors. Extensive structure–activity studies on substituted imidazole acetic acid derivatives together with mutagenesis and molecular modelling studies indicated that a major difference in the orthosteric sites of $\alpha1\beta2\gamma2S$ $GABA_A$ and $\rho1$ $GABA_C$ receptors is a threonine residue (Thr129) in the $\alpha1$ subunit and a serine (Ser168) residue in the equivalent position in $\rho1$. The larger size of the threonine may hinder access of 5-Me-IAA to the orthosteric sites of $\alpha1\beta2\gamma2S$ $GABA_A$ receptors [38]. 5-Me-IAA, or a related 5-substituted imidazole-4-acetic acid derivative, may well be the agonist of choice with which to study $GABA_C$ receptors but more studies need to be carried out.

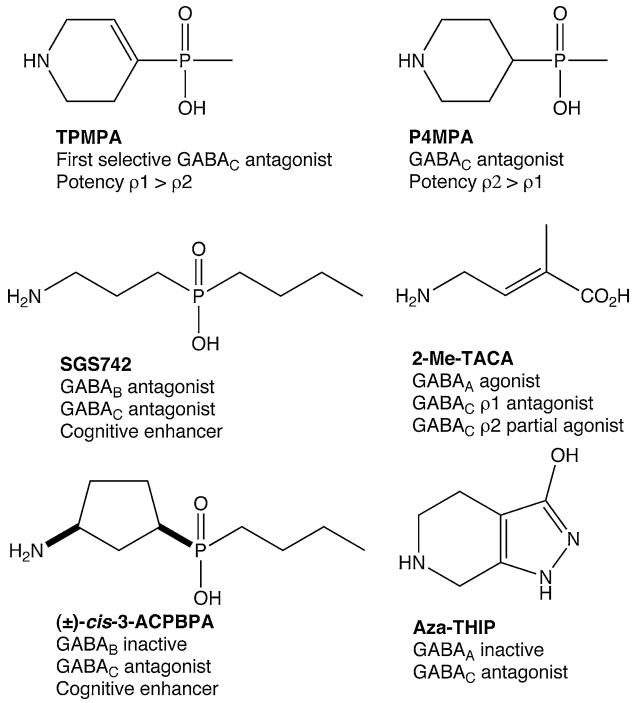

Fig. 2 Structures of some $GABA_C$ receptor antagonists

$GABA_C$ Competitive Antagonists

Agents that are structurally related to GABA that competitively inhibit the activation of GABA receptors by GABA are considered to be competitive antagonists acting at the GABA orthosteric site, as distinct from negative modulators that act at allosteric sites [39].

TPMPA, (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (Fig. 2), was the first selective $GABA_C$ antagonist to be developed and widely used [40, 41]. It shows more than 100 fold selectivity in blocking $\rho1$ $GABA_C$ than $\alpha1\beta2\gamma2$ $GABA_A$ receptors. It is some 8 times less potent at $\rho2$ than at $\rho1$ $GABA_C$ receptors [42]. The piperidine analog of TPMPA, P4MPA ((piperidin-4-yl) methylphosphinic acid, Fig. 2), shows the reverse selectivity being more potent at $\rho2$ than at $\rho1$ $GABA_C$ receptors [43].

TPMPA and related $GABA_C$ antagonists have been patented for the treatment of myopia [3]. TPMPA has been used to study $GABA_C$ receptor function in the retina [44], cerebral cortex zone [45], cerebellum [46], hippocampus [26], lateral geniculate nucleus [47], superior colliculus [48], spinal cord [28], anterior pituitary [49] and duodenum [30]. It has been used to study the involvement of $GABA_C$ receptors in sleep-waking behavior [5] and in antinociception in the periphery [4]. Intracranial injections of TPMPA and P4MPA have been used to study the role of $GABA_C$ receptors in short term memory formation in young chicks [1], however it is not clear whether or not TPMPA crosses the blood brain barrier on systemic administration.

Several phosphinic, phosphonic and seleninic acid bioisosteres of isonipecotic acid act as novel and selective $GABA_C$ antagonists, with piperidin-4-ylseleninic acid (SEPI) being more potent than TPMPA [50].

SGS742, (3-aminopropyl)-*n*-butylphosphinic acid (Fig. 2), also known as CGP36742), is one of a range of phosphinic acid analogs of GABA that act as $GABA_C$ antagonists [51]. It was developed as an orally active $GABA_B$ receptor antagonist [52] and showed therapeutic potential for the treatment of cognitive deficits, petit mal epilepsy and depression [53]. The discovery that it was also a $GABA_C$ receptor antagonist with potency about half that at $GABA_B$ receptors [51] led to the development of cyclopentane analogs in which the conformational flexibility of the 3-aminopropyl moiety was constrained. These cyclopentane analogs were inactive at $GABA_B$ receptors but retained the $GABA_C$ receptor antagonist activity of SGS742. Of particular interest was (±)-*cis*-3-ACPBPA, (±)-*cis*-(3-aminocyclopentanyl)butylphosphinic acid (Fig. 2), which was shown to be a selective $GABA_C$ antagonist that enhanced learning and memory following intraperitoneal injection in rats and inhibited the development of myopia on intravitreal injection in chicks [2]. (±)-*cis*-3-ACPBPA and related cyclopentane and cyclopentane analogs have been patented

for use in enhancing cognitive activity [54, 55]. They are lead compounds for further drug development.

2-Methyl-TACA, *trans*-4-amino-2-methylbut-2-enoic acid (Fig. 2), a known $GABA_A$ agonist, is of interest as it has contrasting effects on $\rho1$ and $\rho2$ $GABA_C$ receptors [42]. It is a competitive antagonist at $\rho1$ $GABA_C$ receptors (Kb 45 μM) and a partial agonist at human $\rho2$ $GABA_C$ receptors (K_d 101 μM, Imax 34%). It may be useful in distinguishing between native homomeric $\rho1$ and $\rho2$ $GABA_C$ receptors in vitro.

THIP (Gaboxadol) is widely used as a $GABA_A$ receptor partial agonist of high efficacy [56]. It is of particular interest as a non-opioid analgesic and a novel hypnotic agent [57]. It has been recently patented for the treatment of stress-related depression [58]. The action of THIP on $GABA_A$ receptors varies widely dependent upon the subunit composition [59]. While it is a partial agonist at the most ubiquitous subunit composition of $\alpha1\beta2\gamma2$ (ED_{50} 143 μM, Imax 76%), it is a full agonist at α5 containing $GABA_A$ receptors (ED_{50} 28–129 μM, Imax 93–99%) and a 'super agonist' at $\alpha4\beta3\delta$ $GABA_A$ receptors (ED_{50} 6 μM, Imax 163%) [60, 61]. THIP is, however, an antagonist at $\rho1$ $GABA_C$ receptors (IC_{50} 25 μM) [62]. Clinical studies with THIP have indicated that sleep quality improving effects are obtained at plasma concentrations of the order of 1 μM [63]. Classical benzodiazepine-sensitive, bicuculline-sensitive $GABA_A$ receptors do not appear to be involved in the effects of THIP on pain perception and sleep. THIP-induced analgesia is not sensitive to the selective $GABA_A$ antagonist bicuculline [64], and benzodiazepine-sensitive $GABA_A$ receptors are not involved in the effects of THIP on sleep patterns [63]. Thus, bicuculline-insensitive, benzodiazepine-insensitive ρ-containing $GABA_C$ receptors are possible candidates contributing to the effects of THIP on pain and/or sleep.

Aza-THIP, 4,5,6,7-tetrahydropyrazolo[3,4-c]pyridin-3-ol (Fig. 2), is an analog of THIP that is inactive at $GABA_A$ receptors but shows similar potency (K_i 31 μM) to THIP as a $GABA_C$ receptor antagonist [62]. Aza-THIP may be a useful tool for molecular and behavioural pharmacological studies, particularly in distinguishing the $GABA_C$ antagonist effects of THIP from its $GABA_A$ agonist/partial agonist action.

Insights into the molecular basis of the distinct antagonist properties of $GABA_A$ and $GABA_C$ receptors have been made via mutation and modelling studies [65]. Single mutations at each of nine residues in $\rho1$ $GABA_C$ receptors were unable to impart dramatic sensitivity to the classic $GABA_A$ receptor antagonist bicuculline, which is inactive at wild type $\rho1$ $GABA_C$ receptors. A $GABA_C$ triple mutant Y106S F138Y FYS240VF however exhibited relative high bicuculline sensitivity (K_i 29 μM), compared to the bicuculline sensitivity of the wild-type $GABA_A$ receptor (K_i 5 μM) [65]. Similar studies with gabazine, a potent antagonist at wild type $GABA_A$ receptors (K_i 0.12 μM) with much lower activity at wild type $GABA_C$ receptors (K_i 58 μM), showed that $GABA_C$ sensitivity could be imparted by the mutations Y106S, F138Y, and Y102F. For the $GABA_C$ receptor antagonist 3-aminopropylphosphonic acid (3-APA), its sensitivity was mainly dependent on residues Tyr102, Val140, FYS240–242, and Phe138. Thus, the residues Tyr102, Tyr106, Phe138, and FYS240–242 in the $\rho1$ $GABA_C$ receptors are major determinants for $GABA_C$ antagonist properties distinct from those in the $GABA_A$ receptors [65]. Unfortunately these studies were not extended to TPMPA, the most widely used $GABA_C$ receptor antagonist due to the then commercial unavailability of TPMPA in the USA [65].

A model of the molecular basis for agonist and antagonist actions at $GABA_C$ receptors predicts distinctive conformations of loop C, with agonists eliciting loop C closure, while antagonists interacted with a more open loop C [6]. This and other models of the $GABA_C$ receptors [38, 66–68] seek to integrate computational and experimental data to aid the discovery of important interactions with existing neurochemicals known to influence $GABA_C$ receptor function and to design new, more selective agents. Of particular interest are enantiomers that show contrasting effects including *cis*-constrained and flexible 2-substituted GABA analogs [69] and 3-hydroxy-substituted GABA analogs [70] with competing interactions at the GABA binding pocket where steric bulk on one side favours agonist action while steric bulk on the other side favours antagonist action.

Positive Modulators

While positive modulation by a variety of neurochemicals including barbiturates, benzodiazepines, flavonoids and steroids is a hallmark of $GABA_A$ receptors, it is relatively rare concerning $GABA_C$ receptors [18].

Certain steroids, such as allopregnanolone, 3α-hydroxy-5α-pregnan-20-one (Fig. 3), do act as positive modulators of wild type $GABA_C$ receptors but only at μM concentrations, whereas steroids act on $GABA_A$ receptors at nM concentrations [71, 72]. Structure–activity studies have shown that the interactions of steroids with $GABA_C$ receptors are complex. In general, in a pair of steroids active at $GABA_C$ receptors with identical structures except for the stereochemistry at the C5-position, the 5α-isomer acted as a positive modulator while the 5β-isomer was a negative modulator [72]. This highlights a major difference between steroid actions on $GABA_C$ and $GABA_A$ receptors in addition to differences in potency and efficacy: 5β-isomers negatively modulate $GABA_C$ receptors and positively modulate $GABA_A$ receptors [72].

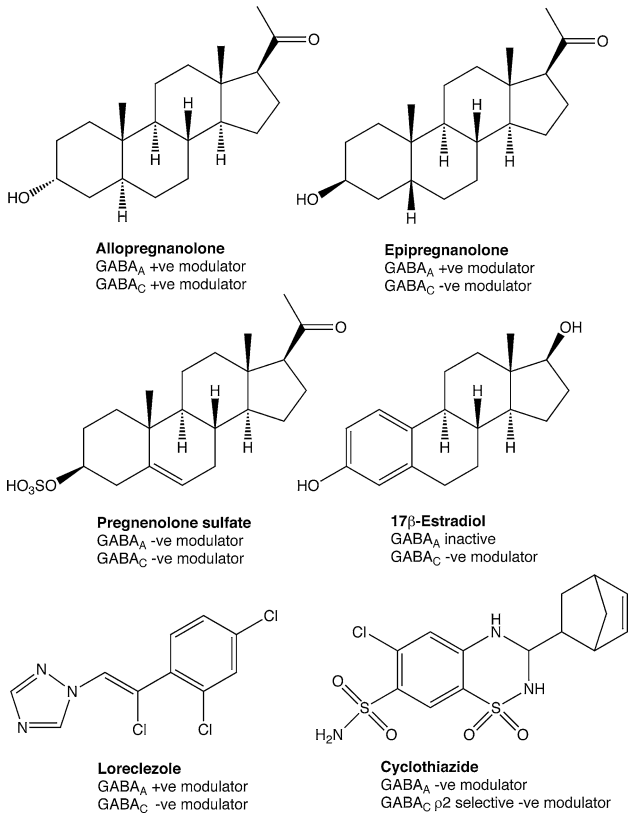

Fig. 3 Structures of some positive and negative modulators of the activation of $GABA_C$ receptors

It is possible to confer a degree of positive modulation by barbiturates by introducing mutations into $GABA_C$ receptors but such modulation is a pale imitation of that observed in wild type $GABA_A$ receptors. A single mutation of an isoleucine to serine (I307S) in TM2 of wild type $GABA_C$ receptors enables pentobarbitone to act as a relatively weak positive modulator (EC_{50} 226 μM) [73]. Similarly, a mutation of tryptophan 328 in TM3 to a spectrum of amino acids confers weak sensitivity to positive modulation by pentobarbitone [74]. Thus, serine 307 and tryptophan 328 contribute to the lack of positive modulation by barbiturates of $GABA_C$ receptors. A double mutation of these amino acids, ρ1 I307S/W328 M, is necessary to confer positive modulation by diazepam and such modulation is low affinity and flumazenil-insensitive [75]. Neither of the single mutants I307S nor W328 M is sensitive to modulation by diazepam.

Taurine is a weak agonist at ρ1 $GABA_C$ receptors, EC_{50} 5 mM [76]. Co-applied with GABA, however, taurine at 0.3–30 μM acts as a positive modulator of the activation by GABA. Given the abundance of taurine in the retina, these observations suggest that taurine may play an important role in modulating retinal transmission mediated via $GABA_C$ receptors [76]. Analogs of taurine may be worth exploring as positive modulators of $GABA_C$ receptors activation.

Negative Modulators

Epipregnanolone, 3β-hydroxy-5β-pregnan-20-one (Fig. 3), is an example of a number of steroids that are weak negative modulators of $GABA_C$ receptor activation [71, 72]. Sulfated steroids, such as pregnenolone sulfate, 3β-sulfoxypregn-5-en-20-one (Fig. 3), also negatively modulate $GABA_C$ receptor activation, less potently than they negatively modulate $GABA_A$ receptors [72]. Surprisingly, 17β-estradiol (Fig. 3) is a negative modulator of $GABA_C$ receptor activation, although it is relatively inactive at $GABA_A$ receptors and positively modulates human α4β2 nicotinic receptors [72]. From such studies it appears that steroids interact with multiple sites on $GABA_C$ receptors that differ from steroid modulatory sites on other members of the nicotinicoid receptor superfamily.

Loreclezole has been described as a 'simple functional marker for homomeric $GABA_C$ receptors' based on its ability to negatively modulate (IC_{50} 0.5 μM) recombinant ρ1 $GABA_C$ receptors [77]. It is known as a potent positive modulator of $GABA_A$ receptors containing β2 or β3 but not β1 subunits [78]. In addition to many neurosteroids, several neurochemicals are known to act as potent positive modulators of $GABA_A$ receptors but act as weaker negative modulators of $GABA_C$ receptors, including (+)-ROD188, pentobarbitone and propofol [73, 77].

Cyclothiazide is a negative modulator of perch ρ2 $GABA_C$ receptors (IC_{50} 12 μM) without effect on human ρ1 receptors [79]. In addition to its widespread use as a positive modulator of AMPA receptors, cyclothiazide is a potent negative modulator of $GABA_A$ receptors [80]. The differential effect of cyclothiazide on human ρ1 and perch ρ2 $GABA_C$ receptors may be attributed to a serine residue at the 2′ position in the second transmembrane domain. It would be interesting to know what effect cyclothiazide has on human as distinct from perch ρ2 $GABA_C$ receptors.

Some flavonoids such as apigenin act as negative modulators of ρ1 $GABA_C$ receptor activation [81] in contrast to their complex modulatory effects on $GABA_A$ receptors [82]. Many more flavonoids have been tested on $GABA_A$ receptors than on $GABA_C$ receptors.

Picrotoxinin and related cage compounds such as bilobalide negatively modulate $GABA_C$ receptors by acting in the ion channel in a manner similar but not identical to the manner in which such compounds negatively modulate $GABA_A$, 5-HT_{3A} and most glycine receptors [83, 84].

Ethanol and related alcohols act as negative modulators of $GABA_C$ receptors [85, 86], whereas they act as positive

modulators of $GABA_A$ receptors particularly those containing δ-subunits [87, 88].

Conclusions

Given their less widespread distribution, lower abundance and relative structural simplicity compared to $GABA_A$ and $GABA_B$ receptors, $GABA_C$ receptors are attractive drug targets. Considerable progress has been made in the discovery of neurochemicals with which to probe $GABA_C$ receptor function and as leads for drug development. It is already clear that $GABA_C$ receptors are very different from $GABA_A$ and $GABA_B$ receptors in terms of agonist, antagonist and modulator profiles. The development of subtype-selective agents for $\rho1$, $\rho2$, $\rho3$ and combinations thereof $GABA_C$ receptors is an urgent need.

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