Chapter 4
The Pharmacology of Extrasynaptic $GABA_A$ Receptors

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Abstract The different subunits that make up the family of $GABA_A$ receptor subtypes have unique distributions within the brain and nervous system. Their localization at the neuronal level is in many cases not necessarily associated with synaptic densities, and this has led to the hypothesis that extrasynaptic receptors perform a unique function in controlling excitability. In most cases, the subunits that make up extrasynaptic receptors are different to those on synaptic membranes and hence have their own unique pharmacological profile, both in respect to agonists and allosteric modulators. Here I will review the different receptor subtypes that have been classified as extrasynaptic, as well as those that may serve both roles depending on their location, with a view to illustrating their pharmacological properties, and their impact on neuronal function. The identification of functional differences and allosteric sites for specific modulation of these receptors offers an opportunity to gain more knowledge of the role of receptor subtypes and the potential to develop novel therapeutic agents that should impact a number of psychiatric and neurological disorders where these receptors are implicated.

Keywords δ subunit · α5 subunit · Gaboxadol · Anaesthetic · Allosteric modulator · Benzodiazepine

4.1 Introduction

Healthy and efficient activity of the mammalian brain relies on a careful balance between neuronal excitation and inhibition within the networks that control its activity. A major component of that inhibitory control is contributed via the neurotransmitter γ-aminobutyric acid (GABA) acting on a large family of ligand-gated ion channel (LGIC) receptors and G protein-coupled receptors. The ligand-gated $GABA_A$ receptors have a widespread distribution throughout the brain and until recently were believed to exert their prominent effects, inhibiting neuronal excitation, entirely through their clearly demonstrated presence on postsynaptic membranes of GABAergic synapses. More detailed localization experiments and electrophysiological

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studies have now shown that in addition to their postsynaptic location, $GABA_A$ receptors are also present outside the synapse both on cell bodies and peripheral to synapses. Thought at first to be non-functional or perhaps receptors in transit, recent evidence has suggested that these receptors play a major role in neuronal excitability and are involved in a number of important functions including sleep, cognition and epilepsy. Moreover, these receptors have physiological properties that differentiate them from their synaptic partners and make them ideally suited for their extrasynaptic role. The pharmacological properties of these receptors also differ in many respects, and offer an opportunity to specifically target them as a route to potential novel therapeutic drugs. This chapter will review the pharmacological properties of those $GABA_A$ receptors which are primarily extrasynaptic and compare their properties to postsynaptic $GABA_A$ receptor subtypes.

4.2 $GABA_A$ Receptor Subtypes

All $GABA_A$ receptors are made up of a pentameric arrangement of heterogeneous subunits which combine to form a pore in the plasma membrane that is selectively permeable to chloride (Cl^-) ions. Activation of the receptor allows chloride to pass through the channel in either direction depending on the driving force on the cell. This change in chloride levels will directly influence the excitability of the neuron and will be inhibitory in most cases but in some instances can elicit an excitatory response. The $GABA_A$ receptor family consists of a number of related subunits including α1–α6, β1–β3, γ1–γ3, δ, ε, θ, ρ1–3. The majority of $GABA_A$ receptors comprise two α subunits, two β subunits and one other, predominantly γ2. ρ–containing subunits are members of the of $GABA_A$ receptor family but form an independent receptor sometimes referred to as $GABA_C$ that has its own unique pharmacology and can assemble as a homomeric receptor. Each GABA receptor subunit has an individual distinctive pattern of expression throughout the central nervous system (CNS) and this determines the nature of receptors expressed in particular brain structures. For example, the most abundant receptor in the brain is the α1β2γ2 subtype which is expressed widely, particularly in the cortex, thalamus, olfactory bulb and some parts of the hippocampus (Pirker et al. 2000). Even in the same brain region, expression of subtypes can be cell specific; the major $GABA_A$ receptor subtype in cerebellar Purkinje neurons is α1β3γ2 but in cerebellar granule cells α6βδ predominates (Pirker et al. 2000). Another clear example is the nucleus reticularis of the thalamus which exclusively expresses the α3-containing subtype (Studer et al. 2006). Differential distribution of subunits has also been shown to be present within an individual cell with different receptors being targeted to different parts of the plasma membrane (Mangan et al. 2005). Different promoters control the regional expression of individual receptor subunits in different brain regions, and there are molecular chaperones which target and traffic the subunits on a cellular basis. There have been a number of these identified for $GABA_A$ receptors, including gephyrin, GABARAP, PLIC, GRIF-1, protocadherin γC5, radixin and GRIP (reviewed in Luscher et al. 2011; Tretter et al. 2012).

Although immunoprecipitation techniques have estimated that there are likely to be 10–12 major receptor subtypes, it is impossible not to preclude any minor combinations that might occur at very low levels and with highly specific regional expression (McKernan and Whiting 1996). The question remains however, which of these receptor subtypes are located postsynaptically, and which are extrasynaptic? Until recently it was thought that the γ2 subunit played a major part in determining postsynaptic clustering since receptors in γ2 subunit-deleted mice did not appear to form proper postsynaptic clusters (Günther et al. 1995), a feature which could be restored by transfecting neurons with γ2 cDNA (Baer et al. 2000). More recently, the role played by the protein gephyrin in determining postsynaptic clustering has been better understood and gephyrin binding sites identified on α1, α2 and α3 receptors, which seem to be more important for localizing receptors at postsynaptic membranes (Tretter et al. 2012; Wu et al. 2012). Co-immunolabelling with the GABAergic synaptic marker neuroligin-2 demonstrated that α1, α2 and β3 in CA1 hippocampal membranes are primarily expressed in synaptic membranes compared to extrasynaptic membranes (Kasugai et al. 2010). In contrast, similar experiments studying the δ subunit showed that receptors containing this subunit were excluded from synaptic locations revealing an extrasynaptic location (Nusser et al. 1998). α5 subunit-containing receptors are interesting in this regard since they are primarily thought to be extrasynaptic (Brunig et al. 2002). There is some evidence however, for α5 contributing to postsynaptic responses in the hippocampus (Zarnowska et al. 2009; Serwanski et al. 2006). Recent work has revealed that α5-containing receptors are able to form clusters on the neuronal cell-body membrane associated with the actin-binding protein radixin (Loebrich et al. 2006), and these authors speculate that α5-containing receptors might form specialised neuron–glia contacts. It is likely then that the majority of α1, α2 and α3 plus γ2-containing receptors are primarily postsynaptic in nature, while α4, α5 and α6 appear to be predominantly extrasynaptically located. Alpha 4 and α6 are preferentially co-expressed with the δsubunit, forming receptors with unique biophysical characteristics that enable them to perform quite different functions to postsynaptic receptors, however there is also a population of α4 and α6βγ2 receptors. The α5 subunit co-assembles with a β and γ2 subunit with similar properties to α1, α2 and α3. This receptor is highly sensitive to GABA, and with both extrasynaptic and synaptic locations appears to mediate both tonic extrasynaptic conductance and phasic inhibitory synaptic activity.

4.3 Different Biophysical Properties of Synaptic and Extrasynaptic Receptors

The fact that $GABA_A$ receptors are found outside of the synapse has been known for many years. However, the precise nature of these receptors and identification of a functional role has only been uncovered fairly recently. Postsynaptic receptors clearly respond to activation by presynaptically released GABA, usually at relatively high localised concentrations. As the released GABA is cleared via reuptake mechanisms

and diffusion, the response diminishes rapidly. The majority of postsynaptic receptors will also rapidly desensitize following agonist exposure. Postsynaptic $GABA_A$ receptors usually have a relatively low sensitivity for GABA, typically in the 10–50 μM range. This may be related to the high concentrations of GABA usually found in the synaptic cleft. Having low sensitivity for GABA and rapid desensitization means that these types of receptors located on the neuronal membrane will not be activated following exposure to low concentrations of GABA. The delta subunit which co-assembles primarily with α4 and α6 is exclusively located extrasynaptically and confers several properties that differentiate these receptors from α1, α2 and α3 containing synaptic receptors. Firstly, these receptors possess a significantly higher sensitivity to agonists such as GABA; they are usually activated at concentrations of less than 1 μM GABA (Brown et al. 2002; Saxena and MacDonald 1996; Mortensen et al. 2011). Secondly, they exhibit a lower degree of receptor desensitization (Saxena and MacDonald 1996; Mortensen et al. 2010) to enable sustained or tonic levels of activation, and thirdly a lower open-channel probability, restricting the maximum level of receptor activation (Mortensen et al. 2010). These properties allow receptors to be activated by low levels of extracellular GABA in a continuous manner providing dynamic regulation of the resting membrane potential and a gain control on neuronal output (Semyanov et al. 2004; Cope et al. 2005; Duguid et al. 2012). The presence of a native tonic GABAergic current was first demonstrated in cerebellar granule neurons, which primarily express α6βδ receptors (Brickley et al. 1996). The same type of current was also later identified in various brain regions expressing the α4βδ subtype, such as dentate gyrus of the hippocampus (Stell and Mody 2002; Yeung et al. 2003) and thalamic relay neurons (Jia et al. 2005; Herd et al. 2009; Cope et al. 2005). In addition, δ-containing receptors are expressed at low levels in cerebral cortex and are localised on neurogliaform interneurons (Capogna 2011), which can release GABA to produce self-inhibition and inhibition of surrounding cells expressing δ-containing $GABA_A$ receptors (Oláh et al. 2009). Tonic currents have been demonstrated not only in brain slice preparations but also *in vivo*, for example in the cerebellum (Chadderton et al. 2004). The presence of a tonically activated GABA channel will directly influence the level of intracellular chloride and this in turn is dependent on the presence of chloride transporters such as KCC2 producing a dynamic balance. Interestingly, this has now come full circle, in that the level of intracellular chloride itself has recently been shown to regulate the expression of GABA receptor subunits (Succol et al. 2012). The source of GABA eliciting tonic currents is thought to result from either spillover at synapses or release from surrounding glia (Rossi et al. 2003)(see Chap. 6). Block of GABA uptake by drugs such as tiagabine or NO-711 can enhance tonic currents and in the cerebellum for example, one source of GABA is thought to be via release from astrocytes through bestrophin-1 anion channels (Lee et al. 2010). This group demonstrated that silencing bestrophin-1 could completely abolish tonic currents in granule cells (Lee et al. 2010) and that the levels of astrocytic GABA were generally higher in brain regions identified as expressing GABA-mediated tonic currents, suggesting that release from astrocytes may be a primary source of ambient GABA in these areas (Yoon et al. 2011).

4.4 δ Subunit-Containing Receptors

The δ subunit is highly expressed in cerebellar granule cells where it assembles with α6 and β2/3, and in hippocampal dentate gyrus and the ventrobasal thalamus where it assembles with α4 and β2/3 (Saxena and Macdonald 1996; Brickley et al. 1996; Jia et al. 2005; Stell and Mody 2002). Lower levels of δ subunit expression have been reported in cortex and striatum where it may co-assamble with other α subunits such as α1 (Drasbek and Jensen 2006; Santhakumar et al. 2010). The δ subunit substitutes for the γ subunit resulting in a number of effects on the pharmacology of these subtypes. Firstly, the γ2 subunit contributes a major part to the binding site for benzodiazepines (BZ), and substitution with the δ subunit ablates this binding site making these receptors insensitive to modulation by BZ (Quirk et al. 1995; Saxena and Macdonald 1996; Brown et al. 2002). This change to the binding site is not exclusively down to the δ subunit. The α4 and α6 subunits also co-assemble with βγ2 forming a modified binding site which can bind some BZ site ligands, particularly the imidazobenzodiazepines such as bretazenil and flumazenil, with reasonably high affinity. However, given the different nature of the binding site, the functional efficacy of these drugs is modified. For example, the BZ ligand Ro15-4513 behaves as a partial inverse agonist at α1β3γ2 but has full agonist efficacy at α4β3γ2 and α6β3γ2 (Brown et al. 2002). This change is due to a single histidine to arginine change between the α1 and α4/6 subunits (Wieland et al. 1992). The amino acids contributing to the BZ site are now well explored and pharmacophore modelling has revealed a number of residues important for the binding of BZs (Sigel and Luscher 2011). It is not just the binding site residues that make a difference in functional modulation however, and chimeric subunit studies using γ2 and δ have identified functionally relevant residues Tyr235, Phe236, Thr237 in transmembrane domain 1 (TM1) and Ser280, Thr281, Ile282 in TM2. The TM2–TM3 loop also forms a pocket responsible for transducing BZ signalling to potentiate channel opening in γ2 containing receptors, a region which is different in δ-containing receptors (Jones-Davis et al. 2005). It is currently unknown whether this binding pocket plays a role in the action of other allosteric modulators that can affect δ-containing receptors.

$GABA_A$ receptors are also known to be modulated by some types of steroid, many of which are endogenous, for example, 5α-pregnan-3α-ol-20-one which is a metabolite of progesterone, and 5α-pregnan-3α,21-diol-20-one a metabolite of deoxycorticosterone (Belelli and Lambert 2005)(see Chap. 5). These neurosteroid type modulators are produced at levels which may influence inhibitory synaptic transmission, but given that they also affect tonically active, extrasynaptic receptors, they may actually exert a much more profound effect by modulating these types of receptors (Stell et al. 2003). The potentiating effect of neurosteroids was reported to be much greater at δ-containing receptors than other γ2 containing subtypes (Wohlfarth et al. 2002; Brown et al. 2002). Subsequent exploration of this mechanism however suggests that the apparent increase in neurosteroid efficacy is more likely due to the low efficacy of GABA itself at δ-containing receptors,

resulting in a greater potential for modulation (Bianchi and MacDonald 2003; Shu et al. 2012). The $\delta^{-/-}$ mouse displays a reduced sensitivity to neurosteroids *in vivo*, suggesting that δ-containing receptors are an important target for these modulators (Mihalek et al. 1999). δ subunit expression can also be regulated by the levels of endogenous steroid in females (Maguire and Mody 2009) and these receptors may play a role in the pathology of some types of menstrual disorder such as anxiety associated with premenstrual dysphoria, catamenial epilepsy and postpartum depression (Maguire and Mody 2008; Brickley and Mody 2012)(see Chap. 12). Several residues on the α-subunit have been identified to play a role in conferring the allosteric modulation by neurosteroids (Hosie et al. 2006) and indeed the binding site is conserved in α4β3δ receptors suggesting that the δ subunit does not form part of this site (Hosie et al. 2009). An additional complicating factor with neurosteroid modulation of $GABA_A$ receptors is that it appears to be somewhat dependent on the phosphorylation state of the receptor and reduction of protein kinase C- (PKC) or protein kinase A- (PKA) dependent phosphorylation reduces the degree of potentiation by neurosteroids (Harney et al. 2003). This factor is also likely to influence steroid effects on extrasynaptic δ–containing receptors, since the α4 and β3 subunits also contain sites for phosphorylation and these receptors are regulated by protein kinases (Abramian et al. 2010; Saliba et al. 2012).

Another important pharmacological property of $GABA_A$ receptors is their sensitivity to anaesthetics and it is believed that this mechanism underlies the *in vivo* activity of many commonly used anaesthetics such as propofol and etomidate, as well as the volatile anaesthetics such as halothane and isoflurane (Franks 2008). Like neurosteroids, the majority of $GABA_A$ receptor subtypes have binding sites for anaesthetics and robust enhancement of GABA activity is observed with these agents (Forman and Miller 2011). Site-directed mutagenesis studies and photolabelling using an analogue of etomidate have identified a potential pocket in the transmembrane domain incorporating residues from TM1 and TM3 where anaesthetics can bind (Jenkins et al. 2001; Li et al. 2006; Chiara et al. 2012). GABA responses at both α4βδ and α6βδ subtypes are enhanced by anaesthetic agents, for example propofol (Brown et al. 2002), etomidate and isoflurane (Lees and Edwards 1998; Jia et al. 2008a). In $\delta^{-/-}$ mice, the sleep time produced by propofol, pentobarbital, etomidate and ketamine were not different to wild-type mice and loss of righting reflex to halothane was unchanged suggesting that δ-containing receptors do not play a major role in mediating anaesthetic activity of these agents (Mihalek et al. 1999). While loss of righting reflex and degree of immobility with isoflurane were unaffected in the α4−/− mouse, amnesic effects were reduced, suggesting a potential role for α4-containing receptors in anaesthetic mediated cognitive deficits (Rau et al. 2009). Additional studies have suggested agent-specific differential effects on synaptic and extrasynaptic receptors when comparing thiopental and isoflurane on hippocampal brain slice activity (Bieda et al. 2009) and propofol effects on tonic and phasic GABA currents in supraoptic magnocellular neurons (Jeong et al. 2011). While the gene knockout data would suggest that with the exception of neurosteroids, anaesthetics exert their effects primarily through synaptic receptors, the α4βδ receptors in thalamus have been demonstrated to play a role in hypnotic

activity, and more specifically in the generation of slow-wave sleep with GABA agonists (see later section). Recent work using α6βδ receptors and cerebellar granule cells has also demonstrated that the level of enhancement of tonic GABA-mediated currents by anaesthetic agents is also dependent on the ambient GABA concentrations which will differ from one brain region to the other (Houston et al. 2012). These authors demonstrate that at higher ambient GABA concentrations, propofol enhancement was reduced, whereas other types of modulator such as neurosteroids maintained their efficacy, perhaps accounting for the differences observed in the $\delta^{-/-}$ mouse.

$GABA_A$ receptors are known to be sensitive to alcohols and these molecules are believed to bind in the same region as anaesthetics (McCracken et al. 2010). There have been several papers published demonstrating that δ-containing receptors are particularly sensitive to low concentrations of ethanol (Wallner et al. 2003; Sundstrom-Poromaa et al. 2002) and that there is a binding site conferred by the δ subunit that is also sensitive to the BZ inverse agonist Ro15-4513 (Wallner et al. 2006). In addition, tonic currents in hippocampal recordings are modulated at low concentrations of ethanol (Wei et al. 2004). These findings are however still controversial, as other publications have failed to replicate these effects with low concentrations of ethanol (Borghese et al. 2006; Borghese and Harris 2007; Baur et al. 2009; Yamashita et al. 2006; Shu et al. 2012) or binding of Ro15-4513 (Korpi et al. 2007; Mehta et al. 2007). In addition, for the most part α4−/− and δ−/− mice have normal responses to ethanol (Chandra et al. 2008; Mihalek et al. 2001; Shannon et al. 2004). It is quite possible that similar to the case for neurosteroids, there are other factors that can influence receptor sensitivity to ethanol, for example, PKC isoforms (Werner et al. 2011; Choi et al. 2008; Qi et al. 2007) which are not controlled for in these studies. A recent report has identified an interesting sex difference with regard to the α4 dependence of Ro15-1453 and ethanol induced ataxia, where only male α4−/− were resistant to the effects of Ro15-4513 (Iyer et al. 2011). However, this effect is likely to be mediated through α4βγ2 receptors rather than α4βδ (Linden et al. 2011).

While the agonist binding site is thought to be located at the α/β subunit interface, there are very clear effects on GABA agonist pharmacology when the γ2 subunit is replaced by the δ subunit, indicating that the nature of the third subunit can influence agonist-induced gating. δ containing receptors are generally much more sensitive to agonists (Brown et al. 2002; Mortensen et al. 2011; Stórustovu and Ebert 2006) with GABA EC_{50} values usually less than 1 μM. In addition, the amplitude of maximum currents produced by GABA is reduced relative to other subtypes (Mortensen et al. 2010). Despite the presence of the GABA binding site at the α/β interface, there have been several recent reports showing that the δ-subunit can also contribute to the GABA binding site. Using concatenated subunits, Baur et al. showed that the δ-subunit can assume various positions in the pentameric structure replacing the position of an α-subunit and can form part of a GABA-binding site (Baur et al. 2009; Kaur et al. 2009). In addition, mutating the residue Arg218 of the δ-subunit, equivalent to the GABA-binding residue Arg207 of the β2-subunit, reduced the potency of GABA by 670-fold, again suggesting a potential role for GABA binding to the δ-subunit (Karim et al. 2012a).

Receptor desensitization is reduced although not completely abolished, and the steady-state tonic current that remains when activated by ambient GABA is rendered insensitive to transient increases in GABA, for example from spillover at synaptic junctions (Bright et al. 2011). The agonist pharmacology is also somewhat different on δ–containing receptors compared to γ2-containing receptors. For example GABA behaves as a partial agonist relative to other agonists such as the constrained analogue 4,5,6,7-tetrahydoisoxazolo[5,4-c]pyridin-3(2H)-one (THIP or Gaboxadol), as well as other amino acids such as β-alanine and taurine which, like GABA, have increased potency at δ-containing receptors (Brown et al. 2002; Mortensen et al. 2010; Jia et al. 2008b, Bianchi and Macdonald 2003). The potency and efficacy of THIP at δ-containing receptors led to this being classified as a selective extrasynaptic agonist, and when applied to thalamic slices which express α4βδ, THIP can selectively activate tonic currents with no effect on synaptic transients (Belelli et al. 2005; Cope et al. 2005). These properties combined with its favourable brain distribution following oral dosing, supported the discovery that THIP could induce slow-wave activity and increase sleep in both rodents and humans (Lancel and Langebartels 2000; Lankford et al. 2008; Wafford and Ebert 2006). Despite not being approved as a sleep-enhancing agent, THIP has proven to be a useful tool in exploring the role played by δ-subunit containing, extrasynaptic $GABA_A$ receptors, since its sedating effects are lost in both $\delta^{-/-}$ and $\alpha4^{-/-}$ mice (Winsky-Sommerer et al. 2007; Chandra et al. 2006), implicating α4βδ $GABA_A$ receptors in sleep–wake control mechanisms.

Like other $GABA_A$ receptors, δ-containing receptors are also allosterically potentiated by barbiturates such as pentobarbital (Brown et al. 2002). As with other allosteric modulators, the degree of potentiation observed when using GABA as the agonist appears much greater than with other receptor subtypes, however the potency is not affected. This difference can be accounted for by the partial agonist nature of GABA at this subtype (Feng et al. 2004). Chimeric δ/γ2 subunits have been used to demonstrate a key region from the amino-terminus to proline 241 in the TM1 transmembrane domain that is critical for pentobarbital potentiation, as well as being a part of the subunit that confers reduced desensitization (Feng and Macdonald 2010).

Given the very different properties and role of extrasynaptic δ-containing receptors, there has been recent interest in developing allosteric modulators that would be selective for this subtype. The first δ-selective modulator identified by Wafford et al. was 4-chloro-N-[2-(2-thienyl)imidazo[1,2-a]pyridine-3-yl benzamide or DS2. This drug-induced marked potentiation of α4βδ receptor mediated currents with sub-micromolar potency and robust efficacy comparable to that of neurosteroids and was devoid of effects at other subtypes (Wafford et al. 2009). A close analogue, DS1 was also very potent, but had a strong GABA-mimetic effect, directly activating the receptor, while maintaining subtype selectivity. Further work has shown a dependence on the δ-subunit for potent activity of DS2 and suggests a novel binding site exists for these types of allosteric modulators, since mutations known to affect steroids and anaesthetics do not alter modulation by DS2 (Jensen et al. 2013). The compound also enhances tonic currents in thalamic and cerebellar brain slices

where α4βδ and α6βδ generate the extrasynaptic tonic current (Wafford et al. 2009), and these effects are absent in thalamic slices from the $\delta^{-/-}$ mouse (Jensen et al. 2013). Unfortunately, DS2 has very limited brain penetration so is not a useful tool for *in vivo* experiments, however other compounds have been proposed that have δ-subunit activity with some *in vivo* effects. AA29504 is a retigabine analogue with modest activity at KCNQ ion channels that has been shown to potentiate α4βδ receptor responses activated by GABA and gaboxadol. Like other modulators, AA29504 produced a greater maximal effect on α4βδ than α1βγ2, but was not selective for δ-containing receptors since α1β2γ2 receptors were also potentiated at the same concentrations, and synaptic inhibitory postsynaptic currents (IPSCs) were prolonged in cerebellar granule neurons (Hoestgaard-Jensen et al. 2010; Vardya et al. 2012). When administered subcutaneously, the drug produced anxioytic activity and motor impairment, but the mechanism underlying these effects is not yet determined. A set of dihydropyrimidinones related to barbiturates have also been shown to potentiate α1β2δ receptors with some selectivity over other subtypes (Lewis et al. 2010). These compounds are relatively weak with EC_{50} values from between 190 μM for monastrol, and 410 μM for JM-II-43A. Further investigation revealed that potentiation by these drugs was not completely dependent on the δ subunit since GABA currents at α4β2 receptors were also potentiated. No effects were observed on γ2-containing receptors however, suggesting at least some level of receptor selectivity with this class of molecule (Lewis et al. 2010). Interestingly there appeared to be some differences in potentiation between α1β2δ, α4β2δ and α6β2δ receptors, supporting the hypothesis that the effects of these compounds are modulated by δ, but their binding site may be linked more to the α-subunit.

The NMDA antagonist ketamine has recently been demonstrated to be a weak potentiator of $GABA_A$ receptors with some selectivity for the α6β2/3δ receptor subtype. In addition, at concentrations above 100 μM, ketamine could directly activate these receptors (Hevers et al. 2008). This property was not shared by other NMDA channel blockers such as MK-801 or phencyclidine. Ketamine could potentiate tonic currents in cerebellar granule neurons at 30 μM or above suggesting this effect may contribute to the CNS depressant features of this drug *in vivo*.

Another well-characterised GABA-related drug, gamma-hydroxybutyric acid (GHB), has previously been shown not to affect $GABA_A$ receptors, but exert its effects primarily through $GABA_B$ receptors and potentially bind to a specific site which remains to be identified. More recently, the GABA α4 subunit has been proposed as a potential binding protein for [^{3}H]NCS-382, a potent 'GHB-receptor' ligand, and photo-affinity labelling using [^{125}I]BnOPh-GHB which labels high-affinity GHB sites is inhibited by the $GABA_A$ antagonist gabazine (Absalom et al. 2012). These authors also showed that GHB can behave as a partial agonist at α4β1δ receptors with a potency of 140 nM and can be blocked by gabazine. Interestingly, GHB had remarkable β1 selectivity over β2 and β3 containing receptors and had no effect on γ2-containing receptors. This suggests that α4β1δ may represent a high-affinity binding site for GHB, a hypothesis which is supported by reduced [^{3}H] NCS-382 binding in $\alpha4^{-/-}$ mice. This interesting finding needs to be followed up, and several questions remain, particularly around the pharmacological profiling of

other GHB ligands such as NCS-382. In addition α4 and δ primarily associate with β2 and β3, whereas β1 has low abundance in regions expressing α4 (Pirker et al. 2000; Herd et al. 2008; Belelli et al. 2005).

A number of other agents have claimed to have some activity at extrasynaptic δ-containing receptors, however it is as yet unclear what contribution these receptors may be making to the *in vivo* activity of any of these drugs. For example, 3-hydroxy-20-methoxy-6-methylflavone has been reported to enhance GABA-mediated responses at α1, α2, α4 and α6 but not α3 and α5 containing $GABA_A$ receptors, in addition to acting as a direct agonist at α4βδ and α6βδ receptor subtypes (Karim et al. 2012b). The compound is anxiolytic *in vivo*, but the role played by extrasynaptic receptors in this response remains to be elucidated. Biphenolic extracts from magnolia tree bark, magnolol and honokiol which have been shown to be anxiolytic and sleep inducing in humans also potentiated GABA-mediated currents in recombinant receptors, with no particular subtype selectivity, but more marked effects on δ-containing receptors (Alexeev et al. 2012). Some endocannabinoids such as 2-arachidonoyl glycerol (2-AG) have been shown to potentiate $GABA_A$ receptors between 1 and 10 μM. These compounds seem to show a degree of β2 selectivity and some synergistic effects with neurosteroids. It is possible that endogenous 2-AG can modulate $GABA_A$ responses to neurosteroids (Sigel et al. 2011) and these authors suggest that extrasynaptic receptors would be preferentially affected given a larger effect of 2-AG at low GABA concentrations.

An indirect modulator of $GABA_A$ receptors is insulin, which is able to rapidly recruit new receptors to the plasma membrane and consequently increase the amplitude of induced pluripotent stem cells (IPSCs) (Wan et al. 1997). This is thought to be mediated via tyrosine phosphorylation of residues on the β-subunit and is dependent on the $GABA_A$ receptor-associated protein, phospholipase C-related catalytically inactive protein (PRIP) (Fujii et al. 2010). This process also affects extrasynaptic receptors and insulin enhances tonic currents in hippocampal slices, subsequently reducing neuronal excitability (Jin et al. 2011). Given the relatively larger charge transfer produced by extrasynaptic receptors relative to synaptic receptors, this effect may predominate where extrasynaptic receptors are present and functional. While the δ-subunit itself does not appear to be a substrate for PKC, both the β2 and β3 subunits do contain consensus sites for phosphorylation and can modulate the function of extrasynaptic $GABA_A$ receptors (Saliba et al. 2012). In addition, α4 is also phosphorylated by PKC at Ser^{443} (Abramian et al. 2010). Like insulin PKC phosphorylation can increase the number of receptors in the plasma membrane and stabilizes the receptor, preventing run-down of the current over time when α4βδ receptors are expressed in human embryonic kidney (HEK) cells (Abramian et al. 2010). 8-bromo-cAMP has also been shown to enhance currents produced by expression of α4β3δ, implicating the potential for PKA phosphorylation as another modulatory mechanism for these extrasynaptic receptors (Tang et al. 2010).

As mentioned with regard to anaesthetic sensitivity, the contextual nature of extrasynaptic receptors compared to synaptic receptors can also contribute to their pharmacology. For example, the open channel blocker penicillin was reported to selectively block phasic currents in hippocampal neurons, but be relatively inef-

fective at blocking tonic extrasynaptic currents in the same neurons (Yeung et al. 2003). Using directly applied GABA to recombinant receptors, γ2- and δ-containing receptors were both found to be sensitive to block with penicillin, but in both cases only peak currents were reduced, leaving steady-state currents unaffected (Feng et al. 2009). Likewise δ-containing receptors stimulated in a physiological manner, for instance by continuous application of low concentrations of GABA, produced a steady-state current that was insensitive to penicillin, whereas γ2-containing receptors stimulated with brief, high concentrations of GABA were almost completely inhibited. Penicillin may be one example of this type of contextual-dependent selectivity, and potentiators may also give apparent differences when applied in context. For example propofol, gaboxadol and neurosteroids behave differently depending on the ambient concentration of GABA, and this is likely to influence their *in vivo* properties (Houston et al. 2012).

Another potential contributor to the pharmacology of both types of receptors is the possibility of constitutive activity in the absence of agonist. There is still some debate as to whether there are endogenous constitutively active $GABA_A$ channels; however, in recombinant expression systems there are some instances where channel activity can be measured in the absence of GABA (Maksay et al. 2003). There are a few reports of spontaneous activity with δ-containing receptors (Tang et al. 2010), and one recent study has demonstrated a small amount of activity with α4β3δ expressed in *Xenopus* oocytes. This activity, which appeared to be dependent on the β-subunit isoform as it was not seen in α4β2δ expressing cells, could be potentiated by DS2 (Jensen et al. 2013). In other expression systems, however, the spontaneous activity of this combination is not present (Brown et al. 2002), so it is currently unclear if this represents the native situation or just an artefact of the expression system.

4.5 α5 Subunit-Containing Receptors

In adult brain, the α5 subunit has a very limited distribution. Early on in development however there is more widespread expression, hence this receptor may play an important role during brain maturation (Laurie et al. 1992; Sebe et al. 2010). In adults, the most abundant form of this receptor is $\alpha5\beta_x\gamma2$ and its highest level of expression is in the CA1 and CA3 regions of the hippocampus (Pirker et al. 2000), and lower levels of expression is in the cortex.

The α5βγ2 subunit was one of the first $GABA_A$ receptors to be proposed to function in a tonic fashion (Caraiscos et al. 2004; Glykys and Mody 2006) despite containing a γ2 subunit and being sensitive to BZs. More recent studies have revealed an actin-binding protein radixin, co-associated uniquely with α5, which may be responsible for trafficking and clustering α5-containing receptors within the membrane (Loebrich et al. 2006). There is still some debate about the presence of α5-containing receptors at hippocampal synapses, but accumulating evidence now suggests this receptor may contribute to synaptic responses (Collinson et al. 2002;

Prenosil et al. 2006; Zarnowska et al. 2009) and is located at synaptic terminals in addition to existing in a larger extrasynaptic pool (Serwanski et al. 2006). More specifically, $GABA_A$ α5-containing receptors have been shown to contribute to a slow IPSC termed '$GABA_{A\ slow}$' in CA1 dendrites which is large in amplitude, prolonged by diazepam and activated by stimulation of the Schaffer-collateral pathway (Zarnowski et al. 2009; Vargas-Caballero et al. 2010; Capogna and Pearce 2011).

Given the high level of expression of α5βγ2 receptors in the hippocampus, this receptor has been strongly implicated in cognitive processing, and evidence suggests that inhibition or deletion of α5-containing receptors promotes cognitive performance in both rodents and humans (Collinson et al. 2002; Dawson et al. 2006; Nutt et al. 2007). The potential for the treatment of cognitive disorders such as Alzheimer's disease and schizophrenia has led to the development of selective BZ-site targeted molecules with either binding or functional selectivity for α5βγ2 over other receptor subtypes. Several different molecules have been identified and used as tools in this context. L-655,708 was demonstrated to be 50–100-fold more selective for α5βγ2 over other γ2-containing subtypes using [^{3}H]-flumazenil binding, and behaved as a partial inverse agonist at the receptor (Atack et al. 2006). The compound-enhanced long-term potentiation in hippocampal brain slices and improved cognitive performance in the Morris water maze (Atack et al. 2006). Several other molecules were developed with greater efficacy at inhibiting α5βγ2 receptors and it was discovered that compounds could be designed to exhibit functional selective inhibition at this subtypes versus other receptor subtypes. α5IA has potent affinity at all BZ-sensitive receptors but inhibits only α5βγ2, behaving as a neutral antagonist at the other subtypes with no functional consequences, for example no convulsant liability. α5IA improved cognitive performance in rodent hippocampal-dependent tests of learning and memory (Dawson et al. 2006) as well as reversing alcohol induced memory impairment in humans (Nutt et al. 2007). Other examples of selective α5-negative modulators have also been developed (Ballard et al. 2009; Atack 2011), but none have yet been evaluated in cognitively impaired patients. There have been several recent reports that inhibiting $GABA_A$ α5-containing receptors can improve cognitive deficits in the mouse Ts65Dn model of Down's Syndrome (Braudeau et al. 2011; Möhler 2012) and an α5 inverse agonist (RG1662) is now being evaluated in Down's Syndrome patients.

Recent publications have suggested that there may be an age-related component to modulation of α5-containing receptors and cognitive improvement (Koh et al. 2013). Koh et al. report an improvement in cognitive performance in young rats with an α5−selective allosteric inhibitor, but not in older rats. In contrast, in their older cohort they demonstrate an improvement when the animals are injected with positive α5-selective modulators, such as Compound 44 [6,6-dimethyl-3-(3-hydroxypropyl) thio-1-(thiazol-2-yl)-6,7-dihydro-2-benzothiophen-4(5H)-one] and Compound 6 [methyl 3,5-diphenylpyridazine-4-carboxylate], which are partial positive allosteric modulators at α5βγ2 receptors. They suggest that the hippocampus is overactive in older animals and in conditions such as Alzheimer's disease, and by potentiating α5-containing $GABA_A$ receptors this activity can be normalised (Koh et al. 2013).

Interestingly, another condition that exhibits abnormal hippocampal overactivity is the methylazoxymethanol acetate (MAM) model of schizophrenia (Lodge and Grace 2007) and application of another positive allosteric modulator at α5βγ2 receptors SH-053-2′F-R-CH3 appears to normalise this activity when administered either systemically or directly injected into hippocampus (Gill et al. 2011). This drug also reduced the enhanced locomotor response to amphetamine seen in this model; however, it has not yet been evaluated in cognitively based tests.

As described above, because general anaesthetics interact with most $GABA_A$ receptors, it is likely that $\alpha5\beta_x\gamma2$ receptors are also involved in their *in vivo* effects. It has recently been shown by using the $\alpha5^{-/-}$ mice that this receptor contributes to the memory impairing effects of both etomidate and the volatile anaesthetic isoflurane on anaesthetic recovery (Martin et al. 2009; Zurek et al. 2012). In addition, by blocking α5-containing receptors with L-655,708, the deficits in short-term memory induced by isoflurane could be prevented (Zurek et al. 2012).

It is not currently clear which population of α5-containing receptors are contributing to the *in vivo* effects observed with α5-selective inhibitors. Given the function and significant effects extrasynaptic receptors have on controlling baseline neuronal excitability, it is quite likely that this population are playing a major role in the *in vivo* effects of allosteric modulators, both positive and negative. While $\alpha5^{-/-}$ mice exhibit enhancements in tests of learning and memory (Collinson et al. 2002), further experiments need to be done to understand the role of the tonic current in the hippocampus and its contribution to spatial learning.

While the majority of effort has focused on α5-containing receptors in the hippocampus and their role in cognition, there are some reports of α5-mediated tonic current in other regions, for example a recent report of tonic GABAergic currents in spinal ventral cord interneurons which are sensitive to α5-selective pharmacological agents (Castro et al. 2011). This opens the possibility that α5-selective agents may have some analgesic properties, although this has yet to be reported.

4.6 Other Potential Extrasynaptic Receptor Subtypes

While a major receptor subtype in the ventrobasal thalamus is α4βδ, there is also a high level of expression of γ2 in this region, which by immunoprecipitation co-assembles with α4 and β (Sur et al. 1999). The α4βγ2 subtype can be expressed in recombinant systems and has some unique properties with its modified BZ binding site being sensitive to certain BZ-site molecules such as the imidazobenzodiazepines, Ro15-4513, bretazenil and flumazenil, all of which behave as positive allosteric modulators (Whittemore et al. 1996; Brown et al. 2002). Like $\alpha6\beta_x\gamma2$, the $\alpha4\beta_x\gamma2$ receptor is also more sensitive to blockade by furosemide than other subtypes (Wafford et al. 1996). Despite the immunoprecipitation data, native receptor pharmacology in the ventrobasal thalamus has not actually revealed a receptor with this pharmacology, and

individual subunit deletion experiments suggest just two populations in this region, α4β2δ and α1β2γ2 (Herd et al. 2009; Peden et al. 2008). In the hippocampus, there is some evidence for receptors which exhibit this unique pharmacology appearing transiently following progesterone withdrawal (Gulinello et al. 2002), which results in up-regulation of α4 in this region. In addition, lorazepam becomes inactive, and flumazenil which is normally silent exhibits an anxiolytic-like effect under these conditions, suggesting α4βγ2 may underlie the response in these animals (Gulinello et al. 2002). It is not clear whether this receptor is located at synapses or extrasynaptically, however the miniature IPSC decay time constant is decreased in many neurons, and these become insensitive to the prolongation normally observed with lorazepam, suggesting that at least a proportion are located synaptically (Hsu et al. 2003).

A recent publication studying $GABA_A$ receptors in the amygdala has identified a tonic current present in principal cells of the basolateral nucleus which expresses predominantly α3-containing receptors (Marowsky et al. 2012). The tonic current is sensitive to BZ and is potentiated by the α3-selective potentiator TP003 and hence contains a γ2 subunit. The current is also significantly reduced in the $\alpha3^{-/-}$ mouse suggesting this receptor may play a role in controlling excitability of the basolateral amygdala. The presence of α3 in extrasynaptic receptors is surprising given it has the lowest sensitivity to GABA, but the local concentration of ambient GABA in this region is unknown.

The majority of $GABA_A$ receptors in the CNS appear to be pentamers comprising a preferred arrangement of two α, two β and one other subunit. Evidence does exist however for native receptors containing only α and β subunits, an arrangement which does function in recombinant systems, and will form in the $\gamma2^{-/-}$ mouse (Günther et al. 1995). In a recent report, a low conductance component of the tonic current in hippocampal pyramidal neurons appeared to be highly sensitive to block by zinc, a feature of receptors composed of α and β subunits alone (Mortensen and Smart 2006). Further investigation revealed this to be insensitive to BZs and have a conductance state similar to αβ but not αβγ or αβδ receptors, making up approximately 10 % of the tonic current in these neurons (Mortensen and Smart 2006). This suggests that at least in some parts of the CNS, there may be extrasynaptic receptors composed of just α and β subunits with their own distinct pharmacology, including high sensitivity to zinc and insensitivity to BZs.

There are two additional $GABA_A$ subunits (θ and ε) which are of very low abundance and in at least the case of ε can dramatically alter the pharmacology of receptors when expressed with α and β subunits. θ remains relatively unexplored (Bonnert et al. 1999) but it has been proposed that α3, θ and ε are expressed together in postnatal spinal cord (Pape et al. 2009), as well as in other brain regions, including the locus coeruleus, the amygdala and various nuclei of the hypothalamus (Moragues et al. 2002; Sinkkonen et al. 2000). The native pharmacology of such a receptor has yet to be defined and the contribution of θ to these receptors is still unknown. The ε-subunit is unusual in that when co-expressed with α and β subunits, it can reduce the sensitivity to anaesthetics (Davis et al. 1997; Thompson et al. 2002) and is insensitive to

BZs. More importantly, it can produce constitutively open receptors which can also be activated by GABA and have a level of spontaneous channel activity (Maksay et al. 2003). Clearly this is likely to be an important feature for an extrasynaptic receptor where a level of constitutive or tonic activity can be maintained even in the absence of GABA. Unfortunately, it has been difficult to demonstrate the presence of such a receptor in native neuronal preparations, even those with the highest levels of expression of these subunits. One study reports a BZ-insensitive GABA synaptic current in locus coeruleus, however this did not exhibit spontaneous activity (Belujon et al. 2009). So at the present time, it is still unclear whether these represent a novel subtype of extrasynaptic receptor, or what the pharmacology of these receptors might be.

In addition to the identification of tonic currents elicited by $GABA_A$ receptor subtypes, it has recently been shown that $GABA_C$ or ρ-containing receptors can also be localised extrasynaptically on retinal bipolar neurons (Jones and Palmer 2009). These are thought to be predominantly ρ1 containing due to their insensitivity to the ρ2-selective antagonist cyclothiazide (Jones and Palmer 2011).

4.7 Beta Subunits

Unlike the α, δ and γ2 subunits, the identity of the β subunits in different populations of extrasynaptic and synaptic receptors has had little investigation. One study has utilized $\beta2^{-/-}$ and the point mutant β2N265S transgenic mice to differentiate populations of receptors in the dentate gyrus of the hippocampus. They discovered that the extrasynaptic α4βδ population contained primarily β2 subunits while the synaptic αβγ2 population contained primarily β3 subunits. The delineation of β3 and β2 to synaptic and extrasynaptic receptors is not universal however, since the diazepam sensitive tonic current present in these neurons, made up of α5βγ2, contained primarily β3 subunits and was unaffected by β2 deletion (Herd et al. 2008). In addition, in thalamic relay neurons, both extrasynaptic and synaptic $GABA_A$ receptors contained primarily β2 subunits (Belelli et al. 2005). Another study using cultured hippocampal pyramidal cells identified α1/2β2/3γ2 in clusters in both synaptic membranes and extrasynaptic membranes, and diffusely distributed α4β1δ receptors in extrasynaptic membranes (Mangan et al. 2005). There are few pharmacological tools that exhibit β-subunit selectivity. Loreclezole and etomidate both show a degree of β2/3 selectivity over β1, and PI 24513, a dioxane analogue has been identified which exhibits preferential allosteric potentiation of β1-containing receptors (Sergeeva et al. 2010). These have recently been used to demonstrate the presence of synaptic β1-containing receptors in the tuberomammilary nucleus and their potential involvement in the sedating effects of PI 24513 (Yanovsky et al. 2012). This tool may be useful to identify the potential presence of β1 in other extrasynaptic receptor populations.

Table 4.1 Direct and indirect modulators of extrasynaptic and synaptic $GABA_A$ receptor subtypes

	α1βxγ2	α2βxγ2	α3βxγ2	α5βxγ2	α4βxγ2	α4βxδ	α6βxδ	α1βxδ	αβ	ρ1
Classical BZ	✓✓✓	✓✓✓	✓✓✓	✓✓✓	—	—	—	—	—	—
imidazoBZ	✓✓	✓✓	✓✓	✓✓✓	✓✓✓	—	—	—	—	—
Zolpidem	✓✓✓	✓✓	✓✓	—	—	—	—	—	—	—
THIP	✓	✓	✓	✓	✓	✓✓✓	✓✓✓		✓✓✓	antag.
DS2	—	—	—	—	—	✓✓✓	✓✓✓		—	
TP003	BZ antag.	BZ antag.	✓✓	BZ antag.	—	—	—	—	—	—
steroids	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓✓	✓✓✓	✓✓✓		—
IAα5	BZ antag.	BZ antag.	BZ antag.	✓✓✓	—	—	—	—	—	—
EtOH	✓	✓	✓	✓✓	✓	✓✓?	✓✓?	✓✓?	✓	—
AA29504	✓	✓	✓	✓	✓	✓✓	✓✓		✓	
Dihydrop.	—	—	—	—	✓	✓✓	✓✓		✓	
Propofol/barbs	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓✓	✓✓✓	✓✓✓	✓✓	—
furosemide	✓	✓	✓	✓	✓✓	✓✓	✓✓		✓	
GABA uptake blockers	✓	✓	✓	✓	✓	✓✓	✓✓	✓✓	✓	✓✓

Illustrates predominantly synaptic receptors

Illustrates predominantly extrasynaptic receptors

Illustrates both synaptic and extrasynaptic receptors

Conclusions

The rich and diverse pharmacology of the $GABA_A$ receptor family can now be extended to that of those receptors that are located extrasynaptically and serve a different function to synaptic receptors. Many of the existing allosteric modulators of $GABA_A$ receptors show overlapping pharmacology on these different populations, while others are more specific (see Table 4.1), and while the understanding of the functional contribution of non-synaptic receptors is growing, it is still to be fully appreciated. It is clear now that some populations are insensitive to BZs such as those containing δ, while others such as those containing α5 can still be modulated via this site. It also appears that the level of receptor activation by GABA can affect some allosteric modulators but not others, which will have major consequences to the *in vivo* activity of these types of drugs and will be an important consideration when developing novel pharmacologically selective molecules. Extrasynaptic receptors also appear to be more plastic, and can respond quickly to changes in their environment, for example, in response to steroid exposure, or an epileptic seizure. The therapeutic potential for modulation of extrasynaptic receptors is extremely diverse, with associated changes in many neurological disorders such as epilepsy, depression, menstrual disorders, anxiety, sleep and Alzheimer's disease (Brickley and Mody 2012). Development of pharmacologically selective agents is still in the early stages and it is hoped that novel agents will be forthcoming in the near future that will prove beneficial in one or more of these disorders.

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