GABA_B Receptors: From Monogamy to Promiscuity

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Key Words

GABA_B · GABA receptor · GPCR · Dimer · Review

Abstract

The aim of this review is firstly to describe the current understanding of the diverse physiology and pharmacology of GABA_B receptors in vivo. We will then focus on recent advances made, since the identification of the GABA_B receptor subunit genes, in our knowledge of the molecular nature of the receptor, and the recently discovered molecular determinants of functions such as ligand binding, trafficking and signalling. We will conclude with a summary of the GABA_B receptor-interacting proteins that have been described thus far, and discuss how these may, at least in part, account for the paradox of varied receptor pharmacology in the potential context of a single heterodimeric GABA_B receptor.

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Introduction

The GABA_B receptor was first described in 1981 as a bicuculline-insensitive, baclofen-sensitive GABA receptor widely expressed in the mammalian central nervous system [1]. However despite much effort in the years since

its discovery, it remained the last major neurotransmitter receptor to be cloned. This was resolved, at least partially, in 1997 with the expression cloning of the first putative GABA_B receptor subunit by Bettler and colleagues [2]. As predicted, this subunit, termed GABA_{B1}, turned out to be a member of the seven-transmembrane G protein-coupled receptor (GPCR) superfamily, with similarity to the metabotropic glutamate receptors (mGluRs). However, despite having been cloned by its ability to bind selective, high affinity GABA_B receptor antagonists, GABA_{B1} was shown to be non-functional when expressed alone and was indeed retained within the endoplasmic reticulum of transfected cells [3]. The solution to this problem came the following year with the cloning of a second subunit, GABA_{B2}. This molecule, a homologous family C GPCR, was shown to heterodimerize with GABA_{B1} and transport it to the plasma membrane. This in turn resulted in the formation of a functional GABA_B receptor with properties more similar to that of GABA_B receptors in vivo [4–7].

With respect to native GABA_B receptors in vivo, they can be separated into a postsynaptic population that controls postsynaptic excitability and a presynaptic population that controls neurotransmitter release (see fig. 1). Each population is capable of either increasing or decreasing the overall excitability in a neuronal network depending upon the magnitude and temporal/spatial pattern of

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1424–862X/02/0116–0299\$19.50/0

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activation relative to other synaptic inputs. This is exemplified by the effects of pre- and postsynaptic GABA_B receptor populations on a form of long-term synaptic plasticity known as long-term potentiation (LTP), where activation of postsynaptic GABA_B receptors on principal neurones is generally believed to restrict LTP [8, 9] whereas activation of presynaptic GABA_B receptors controlling the release of GABA from inhibitory interneurons is believed to promote the induction of LTP [10, 11]. Given these findings it is not surprising that GABA_B receptors have been implicated in numerous disease states (e.g. pain, spasticity, addiction, schizophrenia, epilepsy and dementia [12]) where synaptic reorganization occurs. Furthermore, it is widely believed that the development of pharmacological agents that selectively modify the function of specific pre- and postsynaptic GABA_B receptor populations may provide beneficial therapies with limited side effect profiles for these ailments.

Postsynaptic GABA_B Receptors

If we consider postsynaptic GABA_B receptors first, there is an extensive literature demonstrating that application of the GABA_B receptor agonist baclofen to nearly all principal and interneurone populations in the CNS causes a postsynaptic hyperpolarization that is mediated by activation of a potassium conductance (that depending upon the laboratory has been reported to exhibit linear, outwardly and inwardly rectifying IV characteristics) via pertussis toxin-sensitive Gi/o G proteins [e.g. 13, 14]. In many of these neuronal populations activation of GABAergic afferents produces a slow inhibitory postsynaptic potential (IPSP_B) (fig. 1A; time to peak 130–200 ms; duration 400–1,500 ms) that exhibits similar biophysical characteristics to the baclofen-induced hyperpolarization and is occluded or inhibited by GABA_B receptor agonists and antagonists, respectively [15–18]. The kinetics of this synaptic response are consistent with a membrane delimited signal transduction cascade that is independent of diffusible second messengers. Thus, IPSP_B exhibits a typical latency to onset of 12–50 ms [17, 19] reflecting G protein coupling and a rise time with fourth order activation kinetics possibly reflecting an interaction with the four subunits that constitute the potassium channel [19]. The slow decay time of IPSP_B, in turn, may represent the duration of GABA occupancy of GABA_B receptors or the rate of Gα subunit GTP hydrolysis.

The accepted dogma is that, at the point of synaptic contact, IPSP_B represents an inhibitory synaptic input in most brain regions. Unlike GABA_A receptor-mediated IPSPs, which at rest provide inhibition primarily via membrane shunting because of a 90–140 nS conductance change, IPSP_B produces inhibition via a peak 10- to 20-mV membrane potential hyperpolarization that is accompanied by a macroscopic peak conductance change of only 13–19 nS [20] (single channel conductance of 5–6 pS) [21]. This hyperpolarization is substantial enough to significantly reduce the probability of action potential firing and restrict NMDA receptor-mediated glutamatergic synaptic transmission by maintaining the voltage-dependent magnesium block of the NMDA receptor channel [9, 22]. It should be noted, however, that the effectiveness of IPSP_B to inhibit strong synaptic inputs is far less than its ability to restrict weak synaptic inputs. Thus, in effect IPSP_B can act to enhance the neuronal signal to noise ratio by filtering out background noise. In this context it is interesting to note that GABA_B receptor sites are located extra- or perisynaptically; a suggestion consistent with the observation that inhibitors of GABA uptake, which will promote diffusion of GABA out of the synaptic cleft, greatly enhance IPSP_Bs [23–25]. Thus, it is generally accepted that IPSP_B is most likely to be generated physiologically during periods of synchronized activation of GABAergic interneurons (e.g. during epileptic discharge or cognitive processing) [26–28]. Even then only certain interneurone populations that innervate the dendritic, and not the somatic, aspects of the target neurone are thought to be responsible for activating this response [28, 29].

Despite this inhibitory role it is also clear that the membrane hyperpolarization afforded by IPSP_B can be ‘excitatory’ in certain brain regions. Thus, in the thalamus, for example, IPSP_B provides a mechanism for de-inactivating (by 50–100%) T-type Ca²⁺ channels in GABAergic thalamocortical neurones. Following activation of an IPSP_B, the inactivation of T-type Ca²⁺ channels that exists at resting membrane potentials is removed such that when the IPSP_B terminates and the membrane potential returns to resting levels or just depolarized to these levels, a T-type Ca²⁺ current is generated that causes a burst of action potentials. This in turn generates another IPSP_B, thereby self-perpetuating this excitatory cycle [18]. It is this form of neuronal activity that is thought to contribute to the aberrant synaptic activity responsible for absence epilepsy [30, 31]. It should be noted, however, that at the same time as promoting activation of T type Ca²⁺ channels GABA_B receptor activation may actually

inhibit this low threshold voltage-activated Ca^{2+} channel, an effect that can be clearly demonstrated using the application of baclofen [32]. Likewise, GABA_B receptors inhibit N-, L-, P- and Q-type high voltage-activated Ca^{2+} currents [33–35] through a direct membrane-delimited mechanism thought to involve the interaction of Go α subunits with the Ca^{2+} channel itself [36]. The role of $\beta\gamma$ subunits, if any, in this inhibitory effect is unclear.

Whilst this effect on high voltage-activated Ca^{2+} channel function may be perceived as inhibitory it is possible that it may actually represent a method for increasing postsynaptic excitability by reducing Ca^{2+} -activated K^+ channel-mediated afterhyperpolarizations that limit burst firing activity. Such an effect is further complicated if other synaptic inputs are activated that reduce the AHP through activation of Gs G proteins (e.g., β -adrenergic receptors). Under these circumstances prior activation of GABA_B receptors can release $\beta\gamma$ subunits from Gi/o G proteins which in turn prime adenylyl cyclase II in such a way that when the Gs α -subunit stimulates cAMP production there is enhanced productivity of cAMP and potentiated inhibition of the Ca^{2+} -activated K^+ channel mediating the AHP [37]. On their own GABA_B receptors have no direct effect on the Ca^{2+} -activated K^+ channel itself. Saying this, GABA_B receptors do interact with other K^+ channels distinct from those mediating the IPSP_B . Thus, Wagner and Dekin [38] have reported that GABA_B receptors open a Ba^{2+} -insensitive outward-rectifying K^+ channel in premotor respiratory neurones and Saint et al. [39] have demonstrated that baclofen shifts the voltage dependence of activation and inactivation of the A current.

Presynaptic GABA_B Receptors

Turning to presynaptic GABA_B receptors, it is possible to subdivide these on the basis of those that control GABA release (autoreceptors) and those that inhibit all other neurotransmitter release (heteroreceptors).

GABA_B Autoreceptors

GABA_B autoreceptors are not universally present on all GABAergic interneurone terminals, and they are not activated following GABA release from individual interneurones but are activated after synchronized activation, possibly indicating a low affinity for GABA or remote localization from the synaptic cleft [40–42]. Once activated, however, by synaptically released GABA, there is a delay of 20–50 ms prior to initiation of inhibition. Maximal inhibition then occurs after 100–200 ms (fig. 1B) and

lasts up to 5 s [17, 27, 43–47] closely mirroring, albeit with an extended duration, the characteristic time course of IPSP_B . It is generally agreed that GABA_B autoreceptors are activated and inhibited by agonists and antagonists that also modulate the activity of postsynaptic GABA_B receptors [43, 48] although one or two exceptions to this rule have been reported [49, 50]. This aside, the concentration of antagonist required to inhibit physiological activation of GABA_B autoreceptors is consistently 5- to 10-fold higher than that required to inhibit the IPSP_B [48]. Conversely, activation of GABA_B autoreceptors by the selective GABA_B receptor agonist baclofen requires much lower concentrations than are necessary to activate postsynaptic GABA_B receptors [17, 51]. However, this does not mean that molecularly distinct GABA_B receptor subtypes per se explain the apparent pharmacological differences between physiologically activated pre- and postsynaptic GABA_B receptors. Indeed, it is possible that these differences occur because pre- and postsynaptic GABA_B receptor populations (1) exist with different receptor reserves, (2) are differentially exposed to endogenously released GABA, and/or (3) couple to distinct signal transduction cascades. In this respect, there are numerous conflicting reports regarding the precise signal transduction mechanism involved in regulating GABA release. However, the most consistent finding is that GABA_B autoreceptors inhibit release by inhibiting presynaptic Ca^{2+} entry into terminal release sites [27] through a pertussis toxin-sensitive signal transduction cascade [34, 52, 53]. Whether this occurs by activation of a potassium channel-restricting terminal depolarization or inhibition of high voltage-activated Ca^{2+} channels in the presynaptic terminal is still a hotly disputed topic and may differ from synapse to synapse [39, 41, 45, 54, 55].

GABA_B Heteroreceptors

As with GABA_B autoreceptors, GABA_B heteroreceptors are widely distributed throughout the CNS, but are more diverse in that they control the release of numerous neurotransmitters including glutamate, monoamines and many neuropeptides [56–60]. Neurochemical analysis has suggested that pharmacological differences occur between heteroreceptors controlling the release of different neurotransmitters. However, the pharmacological fingerprints that have been reported [59] have not generally been replicated using electrophysiological approaches, although this analysis has focussed almost exclusively on GABA_B heteroreceptors controlling glutamate release. Thus as a general rule agonists and antagonists that affect postsynaptic GABA_B receptors and GABA_B autoreceptors also affect

Fig. 1. Physiological activation of GABA_B receptors. The schematic diagram represents synaptic inputs onto a target principal neurone (or interneurone) illustrating the location of postsynaptic GABA_B receptors and presynaptic GABA_B autoreceptor and heteroreceptor populations. AMPA, GABA_A and GABA_B receptors are represented for simplicity as single molecular subunits in red, blue and green, respectively. The right hand traces are electrophysiological recordings illustrating physiological activation of postsynaptic GABA_B receptors (A), GABA_B autoreceptors (B) and GABA_B heteroreceptors (C). Thus, A is an intracellular single electrode voltage clamp recording of a pharmacologically isolated monosynaptic GABA_B receptor-mediated inhibitory postsynaptic current (IPSC). B is also an intracellular single electrode voltage clamp recording but of pharmacolog-

ically isolated monosynaptic GABA_A receptor-mediated IPSCs evoked by two identical stimuli delivered 200 ms apart to illustrate the depression of the second IPSC that is produced by activation of GABA_B autoreceptors. C is an extracellular recording of glutamatergic AMPA receptor-mediated field excitatory postsynaptic potentials (fEPSPs) illustrating the depression afforded by activation of GABA_B heteroreceptors. Thus, the red trace represents an fEPSP evoked by a single stimulus where little or no GABA_B heteroreceptor activation occurs whereas the black trace represents fEPSPs evoked by 5 stimuli delivered at 100 Hz followed by a single test stimulus 200 ms later where substantial GABA_B heteroreceptor activation has occurred causing a significant reduction in the fEPSP evoked by this stimulus.

Fig. 2. Agonist binding causes a conformational change in the GABA_B receptor heterodimer resulting in receptor coupling to effector systems. According to family C-based structural information, lobes 1 (LB1) and 2 (LB2) make up a single protomer in GABA_{B1} (orange) and GABA_{B2} subunits (green). In the inactivated 'open' state, the ligand-binding pocket situated in the GABA_{B1} extracellular binding domain is open and the extracellular and TM domains of GABA_{B1} and GABA_{B2} are apart. Agonist binding to GABA_{B1} induces 'closing' of the ligand-binding pocket and an 'activated' receptor state. This conformational change results in the extracellular and TM domains of GABA_{B1} and GABA_{B2} coming closer together (yellow arrows) and is key in the inception of downstream signalling cascades.

GABA_B heteroreceptors [25, 61]. That said, it is often the case that higher concentrations of baclofen are required to inhibit release via heteroreceptors than via autoreceptors (e.g. IC₅₀ for inhibition of a glutamatergic EPSP is typically 3–4 μ M vs. IC₅₀ for inhibition of a GABAergic IPSP of 0.5 μ M) [62, 63]. Once again, the most likely explanation for this observation is differences in the signal transduction pathways coupling the receptor to the effector. Thus the balance of opinion suggests that, in stark contrast to GABA_B autoreceptors that inhibit release by inhibiting presynaptic Ca²⁺ entry into the synaptic terminal, GABA_B heteroreceptors inhibiting glutamate release do so via direct inhibition of the release machinery at a step beyond Ca²⁺ entry into the presynaptic terminal [64]. As with GABA_B autoreceptors, however, there are many conflicting reports on this subject because of the operational difficulties encountered when trying to accurately study mechanisms operating within presynaptic terminals. Indeed it has even been suggested that GABA_B receptors may regulate spontaneous and evoked release of glutamate through different cellular mechanisms [65]. This aside, GABA_B heteroreceptors on glutamatergic terminals, like GABA_B autoreceptors, are thought to be located at some distance from GABA release sites and thus require a spillover of GABA from highly active GABAergic afferents for activation [25, 66]. Once activated, however, the time course of GABA_B heteroreceptor-induced inhibition of glutamate release is very similar to that of the IPSP_B and autoreceptor-mediated inhibition of GABA release (fig. 1C).

Clearly then in vivo there exists a complex and diverse GABA_B receptor physiology. Attempts to pharmacologically dissect this using selective antagonists have generally failed to identify compounds that specifically target individual GABA_B receptor populations. That said, however, quantitative pharmacological analyses of native GABA_B receptors in experimental systems that do not rely on endogenously released GABA to activate the receptor have suggested clear differences in GABA_B receptor responses to both agonists and antagonists. In particular, neurotransmitter release studies have identified pharmacological differences not only between auto- and heteroreceptors but also between heteroreceptor populations [59, 67–72]. Differences have also been observed in agonist coupling to adenylate cyclase [73], as well as between autoreceptors in tissues such as brain and spinal cord [74–76]. To date, however, no reports have defined the precise molecular nature of the GABA_B receptor mediating any physiological response in native tissue, although recently there have been reports claiming that synthetic agonists and antagonists that target specific GABA_B receptor sub-

units selectively activate or inhibit specific GABA_B receptor populations [77, 78]. Currently, there is an ongoing debate as to whether this reflects (1) a differential distribution pattern of GABA_B receptor subunit splice variants pre- and postsynaptically [79, 80] (see later) or (2) differences in GABA_B receptor-activated signal transduction cascades operating pre- and postsynaptically [67]. As such, the second part of this review will focus on our current understanding of the molecular nature of the GABA_B receptor, and whether the progress made since the initial cloning of GABA_{B1} and GABA_{B2} can begin to explain the ‘receptor subtypes’ observed in vivo.

GABA_B Splice Variants and Pharmacological Diversity

The cloning of GABA_{B1} uncovered the existence of two alternatively spliced forms of this receptor subunit in human and rodent, GABA_{B1a} and GABA_{B1b} [2]. These two isoforms differed by the insertion of a pair of tandem ‘Sushi’ domains in the N-terminus of GABA_{B1a}, which are not present in GABA_{B1b}. These domains, previously shown in other proteins to be involved in protein-protein interactions [81], have yet to exhibit any function in the context of the GABA_B receptor, although preliminary evidence has demonstrated an interaction of the N-terminal domain of GABA_{B1} with the extracellular matrix laminin-binding protein fibulin [82]. However the functional relevance of this interaction is still unknown. Despite this differential localization patterns of these splice variants by in situ hybridization have suggested a specific pre- and postsynaptic location of GABA_{B1a} and GABA_{B1b}, respectively [83], although this has not been confirmed at the protein level [80].

The human GABA_{B1} gene encodes a third isoform, GABA_{B1c} (unpubl., Genbank accession No. AJ012187), in which the second of the aforementioned Sushi domains is deleted, although again a functional role for this subunit has yet to be determined. However, the combined circumstantial evidence that other proteins containing Sushi domains are important in development, and that the GABA_{B1} isoforms containing Sushi domains (GABA_{B1a} and GABA_{B1c}) are relatively more highly expressed in fetal brain than GABA_{B1b} [84, 85] suggests a possible role for these variants in the developing human brain. In the rat there are two further splice variants, GABA_{B1c} and GABA_{B1d}. The former contains an insertion at the start of the fifth transmembrane domain and the latter has a divergent C-terminus that deletes approximately half the

coiled coil region including the ER retention motif [86]. Although they have both been shown to be widely expressed in rat tissues, activity in a functional GABA_B receptor has only been reported for GABA_{B1c} [87]. Finally for GABA_{B1}, a truncated form of the protein has been described that only contains the extracellular ligand binding domain (GABA_{B1c}). Although this protein is non-functional when expressed with GABA_{B2}, it appears to be the primary isoform expressed in the periphery and interestingly is capable of forming heterodimers with GABA_{B2}, supporting other findings that the coiled coil domain is not the only region of association between GABA_{B1} and GABA_{B2} [88, 89]. Thus despite its non-functionality this isoform may have an important role in the modulation of GABA_B receptor function in a dominant negative manner [90].

The first apparent and significant breakthrough in GABA_B receptor subunit-specific pharmacology was proposed in two recent studies by Ng and colleagues [77, 91]. In recombinant experiments, the novel anticonvulsant gabapentin was reported to act as an agonist at GABA_{B1a}, but not GABA_{B1b}, containing heterodimers. Furthermore, gabapentin was shown to selectively activate postsynaptic GABA_{B1a}-containing receptors, in CA1 pyramidal neurons, and to be either positively coupled to GIRK channels and/or negatively coupled to voltage-gated calcium channels, with no effect observed on presynaptic GABA_B receptor-mediated inhibitory currents. However, several groups, working independently of one another, were unable to replicate any of the above findings using similar experimental protocols and systems. In these latter studies, no evidence was found to suggest that gabapentin had any agonist activity through GABA_B receptors, either in recombinant systems [92, 93], in brain slice preparations [92] or in vivo [93, 94], despite the use of high concentrations of gabapentin. Given that the only difference between the GABA_{B1a} and GABA_{B1b} receptor subunits is the additional Sushi domains at the N-terminus of the GABA_{B1a} protein, it is hard to conceive structurally how this difference can result in the proposed pharmacological differentiation. The putative gabapentin-binding site would have to lie within the Sushi domains of GABA_{B1a}, and be competitively insensitive to known agonists and antagonists which are known to bind within a binding pocket common to all GABA_{B1} subunits [95] (see later). Although results from these later studies are disappointing, they are difficult to reconcile with the original observations made by Ng and colleagues, and do cast doubt on these original findings.

With regard to GABA_{B2}, initial cloning reports demonstrated the existence of three alternatively spliced forms of this subunit in human tissues [96]. These were termed GABA_{B2a}, GABA_{B2b} and GABA_{B2c}, and differed in the C-terminal region within and downstream of the coiled coil domain. However, a more recent report has conclusively demonstrated that these variants do not in fact occur in vivo, and were probably the result of artefacts arising during cDNA synthesis [97]. This is in agreement with unpublished observations from our laboratory, in which using the polymerase chain reaction we were unable to amplify GABA_{B2b} or GABA_{B2c} sequences from any human tissue examined, despite the robust and reproducible amplification of GABA_{B2a}. Thus, it seems likely now that there are no alternative splice forms of GABA_{B2}, only the sequence originally described as GABA_{B2a}.

Therefore, despite the near complete sequencing of the human genome and extensive molecular biology efforts, a lack of functional GABA_B receptor subtypes still remains with the currently identified receptor subtypes and splice variants not easily accounting for differences in receptor pharmacology. Indeed to date no convincing pharmacological or physiological differences have been ascribed to any of the functional GABA_B splice variants. A more detailed study of the in vivo function and anatomical distribution of the various isoforms needs to be carried out in order to resolve these and other issues with respect to receptor diversity, although it seems unlikely at this point that alternative splicing of the GABA_B receptor subunits will completely answer these questions. Certainly, other factors (e.g. additional receptor subunits; see later) that have yet to be identified may modify GABA_B receptor pharmacology as suggested by the report of Wood et al. [98] where recombinant GABA_B receptors are insensitive to certain antagonists that are active at GABA_B receptors endogenously expressed in the brain.

GABA_B Receptor Knockout Mice

The development of transgenic mice deficient in GABA_B receptor subunits is one approach that is currently being used to address the question of whether true GABA_B receptor subtypes fulfill distinct physiological roles in vivo. To date, knockout of the GABA_{B1} receptor subunit suggests that this subunit is critical for all GABA_B receptor function irrespective of its pre- or postsynaptic location, a finding consistent with the existing dogma that this subunit and not the GABA_{B2} receptor subunit is responsible for binding GABA. Indeed, no evidence for

functional homomeric GABA_{B2} (or novel GABA_B receptor subunits) forming receptors either in the CNS or the periphery of GABA_{B1}-deficient mice was presented in the three studies so far published [99–101].

Irrespective of this, the fact that these animals exhibit generalized (absence and tonic-clonic) epilepsy by postnatal week 3, cognitive impairment, hyperalgesia and sensorimotor gating abnormalities independent of genetic background strain suggests that GABA_B receptors are critically involved in normal development. Certainly, acute and chronic administration of GABA_B receptor antagonists to adult animals has not generally produced such overt behavioural changes [102–104] presumably because of (1) the differences in adult versus infantile synaptic circuitry and (2) dosing of these compounds producing partial rather than complete GABA_B receptor blockade. This aside, pharmacological or genetic intervention of the function of specific GABA_B receptor subunit splice variants together with the development of a GABA_{B2} receptor subunit knockout mouse will provide further valuable insights into whether molecularly distinct GABA_B receptor subtypes exist *in vivo*.

Structural Determinants of GABA_B Ligand Binding and Allosteric Modulation

The development of pharmacological agents that target specific GABA_B receptor subunits may require the synthesis of compounds that target molecular aspects of specific subunits other than the GABA-binding site itself. To develop such compounds it is important to identify the structural determinants required for GABA_B receptor activation. In this respect, GPCRs exhibit a highly diverse range of strategies for both agonist binding and receptor activation [105]. Ligands can interact through residues embedded within the membrane spanning domains, through residues on the external surface of the seven-transmembrane bundle or through proteolytic cleavage of the extracellular amino terminal domain. The Venus Flytrap hypothesis for agonist binding has been the accepted model for activation of family C receptors. This mechanism of activation involves ligand binding through a cleft in the large N-terminal extracellular domain, made up of two lobes separated by a hinge region [95]. The recent solving of the crystal structure of the dimeric N-terminal extracellular domains of mGluR1 in the presence and absence of glutamate has further validated this model and helped clarify our understanding of ligand-mediated receptor activation [106, 107]. In mGluR1, each extracellu-

lar N-terminal domain consists of a protomer made of two distinct globular lobes, termed LB1 and LB2. The two protomers associate to form a homodimer via a disulphide bridge in LB1. In the inactive, agonist-free, state, LB1 and LB2 in each protomer are in an open conformation. However, in the presence of glutamate, one of the protomers in the dimer (with glutamate now bound to it) shifts its conformation, such that LB1 and LB2 come together to form a closed and activated conformational state [106, 107]. This change from the open to closed state, in a single protomer of mGluR1, is sufficient to bring together the lower lobes (LB2) of each protomer and presumably changes the conformation of the adjacent transmembrane domains such that signal transduction can occur (a schematic model similar to this is depicted for GABA_B receptors in fig. 2).

Following resolution of the crystal structure of the mGluR1 ligand-binding domain, previously published observations for the activation of GABA_B receptors via a Venus Flytrap model remain true (fig. 2). Detailed analysis of the GABA_{B1} receptor subunit using truncated and chimeric receptors clearly demonstrate that all the determinants for agonist and antagonist binding are present in the extracellular amino terminal domain of this subunit [108]. Pin and colleagues [108] have generated *in silico* three-dimensional models based on structural information for bacterial periplasmic binding proteins and other members of the group C GPCR family. Using such techniques, they have demonstrated that the extracellular domain of GABA_{B1} and GABA_{B2} can also adopt globular two-lobed structures similar to those described for mGluR1. Extensive mutagenesis studies on the extracellular domain of the GABA_{B1} receptor subunit have demonstrated that residues in LB1 (Ser246, Ser269, Asp471 and Glu465) and LB2 (Tyr366) are key for both agonist and antagonist binding [95, 109–111]. Based on these studies it is believed that the orientation of the hydroxyl groups on Ser246 and Tyr366 allows them to form salt bridges with the carboxylic group of GABA (and baclofen), whereas Glu465 interacts with the amino terminal end of GABA [109, 111]. Asp471, which was originally also thought to form a salt bridge with GABA, now appears more likely to form part of a network of hydrogen bonds important for the correct folding of LB1 [111]. Ser247 and Gln312 also appear to play a role in ligand binding, by modulating the closure of the ligand-binding pocket. Mutation of these two residues was found to increase the affinity for agonists and decrease the affinity for antagonists [112].

In contrast to the clear role played by the GABA_{B1} subunit in ligand binding, there has been some debate in the literature as to the properties of the GABA_{B2} subunit in this regard. Two groups originally suggested that expression of GABA_{B2} in isolation was sufficient to enable functional GABA_B responses in recombinant systems [6, 113]. However, these observations were not observed by other groups [4, 5, 7]. Since that time, numerous independent publications have reported GABA_{B2} to be non-functional in the absence of the GABA_{B1} subunit [see 12]. More recently, mutagenesis of the GABA_{B2}-binding pocket failed to alter ligand-binding affinity at the heterodimeric receptor, and sequence analysis further demonstrated a complete lack of evolutionary constraint placed on the ligand-binding pocket of GABA_{B2}. This is in stark contrast to the amino acid sequence of GABA_{B1} which is highly conserved in species ranging from *Caenorhabditis elegans* to human [111]. Overall, these data suggest that agonists, such as GABA, are unable to bind directly to the ligand-binding pocket of GABA_{B2}. Nevertheless, this does not preclude a pivotal role for the GABA_{B2} subunit both in terms of modulating ligand binding and mediating downstream signalling events (see later). For example, although the rank order of agonist potency is conserved between recombinant GABA_{B1} and native GABA_B receptors, the binding affinity of agonists is a hundred fold lower for recombinant GABA_{B1} when compared to native GABA_B receptors [2, 5, 7]. In contrast, agonist potency more closely approximates that of native receptors, when the GABA_{B2} subunit is co-expressed with GABA_{B1}.

Modulation of the GABA_B heterodimer can also occur via allosteric interactions and this could in some instances confer a degree of pharmacological diversity to the receptor. Calcium has been described to increase the affinity of agonists such as GABA and APPA, but not baclofen, at the heterodimer [114, 115]. This allosteric modulation by calcium is thought to be through interaction with Ser269, in the ligand-binding pocket of GABA_{B1}, and may stabilize the activated 'closed' conformational state of the receptor [114, 116]. Although the reported EC₅₀ value for calcium is low, at 37 μ M, it is conceivable that under certain physiological conditions, extracellular calcium levels could drop dramatically to levels where such allosteric modulation may have a pharmacological consequence on GABA_B receptor function [114]. More recently, a novel series of small molecule organic allosteric modulators have been described CGP7930 and CGP13501 [117]. These compounds have no intrinsic activity in the absence of agonists, but do potentiate the action of known GABA_B receptor agonists. The compounds bind to sites

distinct from known agonist and antagonist receptor-binding sites, and require the presence of the GABA_{B2} receptor subunit. At present it is unclear whether these compounds interact with one or both of the GABA_B receptor subunits, or whether they bind to extracellular or other domains of the subunits. However, it should be noted that allosteric modulators of another family C receptor, mGluR1, suggest that these types of compounds may bind within the transmembrane regions of the receptor complex [118].

Molecular Determinants of GABA_B Receptor Trafficking

Depending upon the level of cell penetrance of such allosteric modulator compounds, it is conceivable that they might act by interfering with the intracellular trafficking of one or other of the GABA_B receptor subunits. In this respect, it is now well established that an interaction between GABA_{B1} and GABA_{B2} receptor subunits is required to translocate GABA_{B1} from the ER to the cell surface as an active heterodimer with GABA_{B2} [2–7] and the molecular determinant for the retention of the GABA_{B1} receptor subunit within the ER resides in the intracellular C-terminus of this subunit [88, 89, 119, 120]. More specifically, the four-amino acid motif RXR(R) presents just downstream of the putative coiled coil domain of GABA_{B1}, and in addition a di-leucine motif at the N-terminal end of the coiled coil appears to be crucial for ER retention. Upon co-expression of GABA_{B2}, the interaction of the two homologous coiled coil domains is then sufficient to mask these signals from an as yet unknown ER anchoring mechanism, and thus the heterodimeric receptor traffics to the cell surface. Although it is clear that the RXR(R) motif is important in the mechanism of GABA_{B1} retention, it appears that the molecular context of this signal is also crucial. There are conflicting reports as to whether a truncated GABA_{B1} that ends at the amino acid just prior to this motif reaches the cell surface, although this may be as a result of differences in the detection level of the various techniques used [88, 120]. However, a GABA_{B1} construct with the coiled coil deleted but leaving the RXR(R) intact is surface expressed [89], whereas two separate GABA_{B2}/GABA_{B1} chimeric subunits, both containing the RXR(R), are either retained or not depending on the amount of GABA_{B1} sequence present upstream of the retention motif [88, 89]. Thus the sequence environment of the RXR(R) motif has profound implications for its function, and as a result of these stud-

ies Grünewald et al. [89] have proposed a functional retention motif in GABA_{B1} of QLQXRQQLRSRR.

The core RXR(R) within this sequence is reminiscent of a similar sequence (RKR) present in certain ATP-sensitive potassium channels [121]. This ER retention signal serves to prevent incorrectly assembled multimeric channels from reaching the cell surface, which would possibly result in incorrect channel functionality, and therefore a similar 'checkpoint' control function has been proposed for the GABA_{B1}-derived RXR(R) motif [119]. As described below, cell surface expression of GABA_{B1} is in itself insufficient for GABA_B receptor functionality, and a correct physical association with GABA_{B2} is critical in order for the extracellular agonist-derived signal to be transduced into intracellular second messenger responses. Thus, if this trafficking checkpoint did not exist and GABA_{B1} was able to reach the plasma membrane in the absence of GABA_{B2}, the presence on the cell surface of non-signalling but GABA-binding GABA_{B1} subunits could severely compromise GABA_B physiology *in vivo*. However it should be noted here that, as mentioned earlier, a C-terminally truncated GABA_{B1} splice variant has been described, GABA_{B1e}, where this may in fact be the case, and the function of such a non-signalling form of the GABA_B receptor needs to be addressed further *in vivo* [90].

Molecular Determinants of GABA_B Receptor Signalling

Whilst the cloning of the GABA_{B2} subunit resolved the issue of non-functionality and intracellular retention of GABA_{B1} expressed alone, it also raised a number of questions about the role of GABA_{B2} in the receptor heterodimer. It seemed conceptually unlikely that the sole function of this seven-transmembrane homologue of GABA_{B1} was to mask a retention signal in the terminus of GABA_{B1} and thus facilitate its trafficking to the cell surface. This unique 'chaperone'-like role was also called into question by the finding that surface expression of GABA_{B1} was in itself insufficient to form a functional GABA_B receptor, and that co-expression of GABA_{B2} was still required. Mutation of the RSRR motif, or deletion of the portion of the C-terminus containing this sequence, results in strong cell surface expression of GABA_{B1}; however, these mutated proteins are still non-functional in the absence of GABA_{B2}, despite being able to bind GABA_B agonists [88, 119].

More recent experiments have provided compelling evidence for an additional crucial role of GABA_{B2} in the

functionality of the GABA_B receptor. Site-directed mutagenesis studies on specific residues in both the second (ic2) and third (ic3) intracellular loops of GABA_{B2} have demonstrated that these residues in GABA_{B2}, but not in GABA_{B1}, are necessary for correct signalling of the GABA_B receptor and the activation of downstream effectors (fig. 3). Mutation of a single amino acid in ic3 [122] or of three amino acids in ic2 [123] of GABA_{B2} results in the complete abolition of functional G protein coupling of the GABA_B receptor heterodimer, either in HEK-293 cells or in cultured neurones. The reciprocal mutations in GABA_{B1} have no functional effect on the receptor. These studies demonstrate that, in addition to its chaperone role in enabling the GABA_{B1} subunit to evade its intracellular retention mechanism by physical shielding of the RXRR motif, GABA_{B2} is crucial for the coupling of the GABA_B receptor to G proteins. It has also been shown that co-expression of GABA_{B2} and a chimeric subunit containing the extracellular domain of GABA_{B1} joined to the heptahelical domain of GABA_{B2} results in a functional GABA_B receptor, despite the lack of any intracellular sequences derived from GABA_{B1} [124]. Taken together, these studies strongly suggest a novel 'sequential' signalling mechanism for the GABA_B receptor, whereby agonist binding to the extracellular domain of GABA_{B1} results in a conformational change in the heptahelical region of GABA_{B2}, which in turn potentiates the binding of G proteins to GABA_{B2} and thus the activation of downstream signalling events. However it is worth noting that, although the intracellular domains of GABA_{B1} appear to be unnecessary to simply obtain a functional GABA_B receptor, some of the more subtle aspects of GABA_B signalling may well be influenced by sequences within GABA_{B1}. For example, it was recently demonstrated that agonist-independent, or constitutive, activity of the GABA_B receptor is strongly influenced by the coiled coil domains of both GABA_{B1} and GABA_{B2}, suggesting that these domains may be in part responsible for the stabilization of the active conformation of the receptor [89]. More detailed studies need to be carried out to further dissect the relative roles of GABA_{B1} and GABA_{B2} in receptor function.

Mechanisms of GABA_B Modulation and Interacting Proteins

Further complexity may be encoded by the possible existence of additional GABA_B receptor subunits which may help to explain the paradoxical discrepancy between an historically rich and diverse receptor pharmacology in

Fig. 3. Ligand binding to the GABA_{B1} subunit results in G protein binding and downstream signalling via the GABA_{B2} subunit resulting in novel 'sideways' signalling to effector systems. Agonists bind to the extracellular ligand-binding domain of the GABA_{B1} subunit (orange). Ligand binding results in conformational changes in the extracellular domains, as well as probably the transmembrane domains, of the GABA_B receptor heterodimer. These changes result in G protein recruitment to the GABA_{B2} subunit (green) via interaction of the G protein with amino acid residues in intracellular loops 2 and 3 of the GABA_{B2} subunit. Phosphorylation of C-terminal residues in both GABA_{B1} and GABA_{B2} subunits can also modulate receptor activity and downstream signalling pathways.

Fig. 4. GABA_B receptor-interacting proteins and modulators – a putative 'signalosome' complex. Both GABA_{B1} and GABA_{B2} receptor subunits have now been shown to associate with a number of proteins both in vitro and in vivo. These interactions are mainly with the C-terminal tails of the subunits, although this almost certainly only reflects the nature of the protein-protein interaction studies that have been carried out. The functional association with mGluR1 is also shown; whether this is as a result of receptor juxtaposition remains to be demonstrated. This type of assembly could be described as a signalosome, and the presence or absence of different components could be responsible for elements of receptor diversity. Su1 and Su2 are the Sushi protein interaction domains present in GABA_{B1a}.

vivo on the one hand, and the existence of as yet only one functional GABA_B receptor, GABA_{B(1,2)}, on the other. However, to date, the best efforts from many groups in the field using diverse techniques such as database mining, yeast-2-hybrid screening and other proteomic-based tech-

nologies have failed to identify novel subunits. An example of a recently identified candidate gene is the GABA_B-like (GABA_{BL}) receptor, which has sequence homology to known GABA_B receptor subunits, but does not form functional GABA_B receptors with either GABA_{B1} or GABA_{B2}

and thus remains an orphan receptor with no known function [Calver and Pangalos, unpubl. results].

If candidate genes such as this, when expressed with the appropriate partners or chaperones, do not result in the identification of novel GABA_B receptors how can we explain the differences in pharmacology between recombinant and native GABA_B receptors? If we consider other GPCRs such as the adenosine A₁ receptor this exists as a single subunit receptor that couples to many of the signal transduction cascades activated/inhibited by GABA_B receptors. However, at pre- and postsynaptic loci this receptor exhibits different patterns of desensitization during chronic exposure to agonists and this difference is thought to be due to differences in the local environment of interacting proteins in the pre- and postsynaptic locations in which the receptors reside [125]. Thus, one possible solution to the GABA_B receptor pharmacology conundrum is that GABA_B receptor pharmacology is strongly influenced by intracellular enzymes or other receptor-associated proteins. Precedents for such associations can be found between mGluRs and Homer proteins [126], and calcitonin receptor-like receptors and RAMPs [127], the co-expression of which can dramatically alter the pharmacological profile of these receptors. Another candidate family of proteins that may fulfil this role is protein kinases. Kinases such as protein kinase A (PKA) and protein kinase C (PKC) have been shown by indirect methods to impinge on GABA_B receptor function [128–131], and interestingly the cloning of the GABA_B receptor subunits revealed that the C-termini of both GABA_{B1} and GABA_{B2} contained a number of consensus sequences for PKA, PKC and other protein kinases [132]. Subsequent to this, a recent study has directly shown that GABA_{B2} is a substrate for PKA both in vitro and in vivo, and that direct PKA activation or activation via another signalling pathway results in the enhancement of GABA_{B2} phosphorylation at a specific serine residue in the C-terminal tail [133]. More importantly, this phosphorylation results in a stabilization of receptor-effector coupling and a reduction in receptor desensitization, probably as a result of maintenance of the receptor on the plasma membrane. An interesting aspect of this study is that it is in stark contrast to the generally accepted tenet that GPCR phosphorylation results in receptor internalization via a β -arrestin-dependent pathway and thus negative modulation [134]. Preliminary evidence from another laboratory has in addition demonstrated that, upon agonist stimulation, GABA_B receptors are not phosphorylated by G protein receptor kinases, β -arrestin is not recruited, and the receptors are maintained on the cell surface, confirming that

GABA_B receptors do not appear to desensitize by the classical β -arrestin-mediated mechanism [135].

As mentioned above, both GABA_{B1} and GABA_{B2} C-termini contain a number of consensus sites for intracellular kinases, and in addition the consensus sites for many kinases are still unclear. It therefore remains to be seen if other kinases and/or phosphatases are able to interact with either of the GABA_B subunits and influence their activity in vivo, and in addition whether such chemical modifications may be able to explain any of the pharmacological diversity observed.

As has been shown by the studies described above, GABA_B receptors do not act in isolation, and in addition to the influence of PKA a number of other interactions have recently been described (fig. 4). These interactions have for the most part been discovered through the use of the yeast-2-hybrid system to detect protein-protein interactions, and therefore the functional relevance in vivo of a number of the interactions remains to be elucidated fully, although some clues can be gleaned from the known functions of the proteins involved.

Firstly, the intracellular C-terminus of GABA_{B1} (but not GABA_{B2}) has been shown to interact with specific members of the 14-3-3 family of intracellular proteins, and this interaction is able to interfere with the normal dimerization process between GABA_{B1} and GABA_{B2} [136]. 14-3-3 proteins have been implicated in a number of processes within the cell that are relevant to GABA_B receptor functionality, including GPCR signal transduction [137], interactions with phosphoproteins [138], and the regulation of synaptic transmission via potassium channels [139], although none of these functions has been directly demonstrated with respect to GABA_B receptors.

Another intriguing interaction reported with the C-termini of the GABA_{B1} receptor subunit is that of the basic leucine zipper (bZIP) transcription factor ATF-4, also known as CREB2. This association was described independently by three laboratories [140–142], and is interesting as it represents a novel putative signalling mechanism involving a direct interaction between a seven-transmembrane receptor and a transcription factor, reminiscent of the JAK-STAT-mediated transcriptional activation in response to various chemokines [143]. Interestingly, however, two of the groups report directly opposing effects of GABA_B activation on the cellular localization of ATF-4 in cultured neurons [141, 142]. White et al. [141] observed a significant increase in localization of ATF-4 to the nucleus upon stimulation with baclofen for 1 h, with a concomitant drop in cytoplasmic expression. However Vernon et al. [142] see the reverse effect; baclofen treatment

results in a dramatic translocation of ATF-4 out of the nucleus, presumably into the cytoplasm since the total cellular ATF-4 expression appears unchanged. It is difficult to explain these differences in the effect of baclofen, although it may depend on the nature of the neurons studied. White et al. also demonstrated a baclofen-mediated increase in reporter gene expression, where the reporter gene was under the control of the ATF-4-responsive Gadd153 promoter. It is of interest to note here too that Gadd153, also known as CHOP, has been reported to directly interact with the C-terminus of GABA_{B2}, and is also a bZIP transcription factor [144]. Therefore this and other compelling evidence described by the various groups strongly suggests a role for this receptor/transcription factor interaction *in vivo*, although the precise functional consequences of this association have yet to be fully elucidated.

In addition to the published studies described above for GABA_{B1}, a number of other GABA_{B2}-interacting proteins have been reported, albeit in abstract form. These include the multiple PDZ-domain-containing protein MUPP-1 [145], which has also been shown to directly interact with the C-terminal of the G protein-coupled serotonin receptor, 5-HT_{2C} [146]. Interestingly, the Homer family of PDZ-domain-containing proteins is known to interact with the mGluRs, also members of the group C GPCR family, and the GABA_{B2} subunit contains a sequence in its C-terminus, PPSFR, that is extremely reminiscent of the Homer-binding consensus PPXXFR [147]. However, work carried out in our laboratory has indicated that this motif within the GABA_{B2} sequence does not allow any interaction with the Homer family of proteins.

N-Ethylmaleimide-sensitive factor, or NSF, a protein critical for intracellular membrane trafficking events, has also been reported to interact with the C-terminal domain of GABA_{B2} [148]. Although the relevance of this association *in vivo* is unclear, it should be noted that NSF also indirectly binds to the ionotropic GABA_A receptor via the microtubule-associated protein GABARAP [149]. The final GABA_{B2}-associated protein reported thus far is the actin-binding protein β -filamin, which could therefore provide a physical link between the GABA_B receptor and the cytoskeleton [148].

All of the above proteins interact specifically with the intracellular C-terminal tail of either GABA_{B1} or GABA_{B2}, but this almost certainly simply reflects the nature of the yeast-2-hybrid screens that have been carried out. There will undoubtedly turn out to be many other, as yet unidentified, proteins that bind to or in some

way interact with other regions of the GABA_B receptor subunits. Indeed a recent report has been presented describing the interaction between the Sushi domains present in the N-terminal domain of GABA_{B1a} and the extracellular matrix protein fibulin [82]. In addition, a functional interaction has recently been demonstrated between the GABA_B receptor and the mGluR *in vivo*. This study showed that, in excitatory glutamatergic synapses in the mouse cerebellum, activation of GABA_B receptors by either exogenous baclofen or endogenous GABA resulted in an enhancement of a number of mGluR-mediated responses [150]. Although this is not necessarily as a result of the physical association of the two receptors, this is not an unreasonable possibility especially in light of a number of recent studies showing physical as well as functional associations between various GPCRs.

In summary, there is increasing evidence for multiple protein 'signalosome' complexes centred around the GABA_B receptor, possibly linking the receptor subunits with components of the relevant signalling pathways, structural and transport elements of the cellular architecture, and even other related receptors and/or ion channels (fig. 4). Consistent with this, it has recently been shown that in rat cerebellum the GABA_B receptor is associated with membrane lipid rafts, specific cholesterol-rich microdomains situated within the plasma membrane that have been suggested to act as assembly platforms for cell signalling complexes [151]. Therefore it is entirely conceivable that the receptor diversity observed for the GABA_B receptor *in vivo* could be explained not by the existence of novel receptor subunits, but by differences in the receptor environment and the associated proteins available to interact with the known receptor subunits.

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