

A half century of γ -aminobutyric acid

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Abstract

γ -aminobutyric acid has become one of the most widely known neurotransmitter molecules in the brain over the last 50 years, recognised for its pivotal role in inhibiting neural excitability. It emerged from studies of crustacean muscle and neurons before its significance to the mammalian nervous system was appreciated. Now, after five decades of investigation, we know that most neurons are γ -aminobutyric-acid-sensitive, it is a cornerstone of neural physiology and dysfunction to γ -aminobutyric acid signalling is increasingly documented in a range of neurological diseases. In this review, we briefly chart the neurodevelopment of γ -aminobutyric acid and its two major receptor subtypes: the γ -aminobutyric acid_A and γ -aminobutyric acid_B receptors, starting from the humble invertebrate origins of being an ‘interesting molecule’ acting at a single γ -aminobutyric acid receptor type, to one of the brain’s most important neurochemical components and vital drug targets for major therapeutic classes of drugs. We document the period of molecular cloning and the explosive influence this had on the field of neuroscience and pharmacology up to the present day and the production of atomic γ -aminobutyric acid_A and γ -aminobutyric acid_B receptor structures. γ -Aminobutyric acid is no longer a humble molecule but the instigator of rich and powerful signalling processes that are absolutely vital for healthy brain function.

Keywords

GABA, GABA_A receptor, GABA_B receptor, inhibition

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γ -Aminobutyric acid (GABA) is widely known today as the most important inhibitory neurotransmitter in the mammalian central nervous system, controlling the excitability of neurons and determining the temporal activity profiles of neural networks. To understand how GABA achieved this prominence in brain function, and initially how difficult and controversial this appeared to the early neuroscience field, we need to travel back to just over 50 years ago, to a time when the concept of ‘chemical transmission’ was in its infancy, and only acetylcholine and catecholamines were acknowledged as transmitter molecules. During this period, little thought had been given to amino acids performing such a transmitter role.

What causes the inhibition of neurons?

The discovery of GABA in the nervous system was first made in the 1950s when studies on mammalian neurons were significantly outnumbered by the use of more accessible and easier to prepare invertebrate systems with their excitatory and inhibitory control of muscle movement. It was the work of Stephen Kuffler (1954) and Ernst Florey (1954) that promoted the use of crustacean preparations as particularly good assay systems for identifying neuroactive substances extracted from mammalian brain. One such inhibitory factor (termed ‘Factor I’) possessed similar inhibitory properties on smooth muscle to that subsequently demonstrated with GABA (Florey and McLennan, 1959) and was later chemically identified as

GABA (Florey, 1991), even though Florey was sceptical as to whether GABA was actually a neurotransmitter. Identifying an endogenous substance from the mammalian brain, including its biosynthetic precursors (Roberts and Frankel, 1950), and also characterising its properties on invertebrate preparations were an important step but it did not prove unequivocally that GABA was an important inhibitory neurotransmitter. Addressing the latter aspect was to take up a large proportion of the 1960s.

The sixties – is GABA a neurotransmitter?

To award the status of ‘neurotransmitter’ to any molecule, the molecule has to fulfil a number of criteria. These have evolved over time and broadly include the following:

1. The molecule must be present, concentrated within the presynaptic neuron, preferably in synaptic vesicles.

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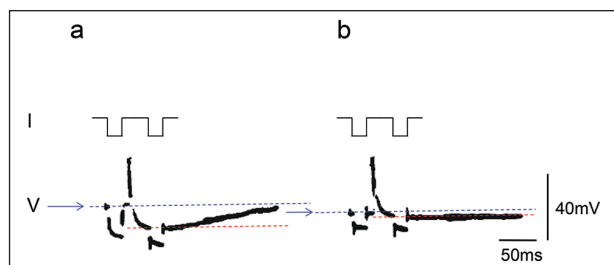


Figure 1. GABA-mediated inhibitory postsynaptic potentials (IPSPs) recorded from neocortical neurons: (a) Hyperpolarizing IPSP with two superimposed current steps (*I*, top trace) deflecting the membrane potential (*V*, arrow, blue dotted line, lower trace) before the IPSP and then at the peak IPSP. Note the reduction in the voltage step size during the IPSP, which is indicative of increased membrane conductance caused by synaptic release of GABA and the opening of GABA ion channels. Red dotted lines indicate the extent of hyperpolarisation. (b) Application of GABA also produces a hyperpolarisation of the membrane with increased membrane conductance (note that both voltage steps are now equally reduced due to GABA application). Note also the occlusion of the IPSP. Data in (a) and (b) are modified after being taken from Dreifuss et al (1969).

2. Synthetic enzymes should be present in presynaptic terminals for generating the molecule.
3. Axonal depolarisation should transiently release molecules in high concentration into the synaptic cleft in a Ca^{2+} -dependent fashion.
4. Specific receptors that bind the molecule must be present on the postsynaptic cell membrane whose activation can be reproduced by exogenous application of the molecule to the preparation.
5. Enzymes and/or transporters should exist at the synapse to rapidly terminate the action of the molecule.

So in essence, in the early days of GABA research, its presence and release at synapses, and activation of postsynaptic receptors, was crucial for proving it was an inhibitory neurotransmitter. Providing solutions for the above conundrums took many years of the 1960s. Initial studies on cat spinal neurons using iontophoretic application of GABA showed promise and revealed inhibition of spiking activity (Curtis et al., 1959), but despite this observation, GABA did not gain traction as a neurotransmitter and the inhibition was considered to be a non-specific effect. In the cerebral cortex, also using iontophoresis, Krnjevic and Phillis (1963) demonstrated that GABA was an effective and rapidly acting inhibitor of neuronal excitation. Further studies on cortical neurons revealed that GABA hyperpolarised the membrane potential and caused a reduction in membrane resistance (Krnjevic and Schwartz, 1967), both key features expected of an inhibitory neurotransmitter. Moreover, application of GABA to the cortex also occluded the hyperpolarising inhibitory postsynaptic potential (IPSP; Figure 1), implying that GABA was indeed the inhibitory neurotransmitter (Dreifuss et al., 1969; Krnjevic, 2010). These findings indicated that GABA could reproduce the features of neuronal inhibition, but other criteria for a neurotransmitter remained unfulfilled at this time. The important aspect of terminating neurotransmitter action was eventually solved by the discovery of a GABA transporter (GAT) (uptake), which was proposed to be the main mode of inactivating the action of GABA

at inhibitory synapses (Iversen and Neal, 1968). Thus, towards the end of the sixties, GABA was becoming accepted as an inhibitory transmitter in the mammalian brain.

The seventies – GABA mapping and pharmacology

This new decade began with the important seminal discovery of a selective antagonist for GABA receptors, the plant alkaloid bicuculline (Curtis et al., 1970; Johnston, 2013). With such a selective compound, it became possible to pharmacologically isolate and analyse GABA-mediated inhibition in the nervous system. This discovery was facilitated by the use of a radiolabelled GABA agonist, [^3H]-muscimol, which could label GABA_A receptors in neuronal tissue, thus providing effective means to detect receptors *in vitro* (Beaumont et al., 1978). Consequently, GABA was now becoming increasingly recognised as a major inhibitory neurotransmitter in the brain. The 1970s marked a period when many began to define the characteristics of the receptor through which GABA was acting to cause inhibition. For example, ligands such as anaesthetics (e.g. barbiturates) were shown to potentiate the action of GABA (Scholfield, 1978, 1980), and most significant in terms of future therapeutics, the benzodiazepines appeared to act by potentiating GABA inhibition (Haefely et al., 1975). In tandem with early pharmacological exploration, others started to map the extent of GABA-containing cells and processes in the spinal cord by using the GABA synthesising enzyme, glutamate decarboxylase (GAD) (Barber et al., 1978), a procedure that is frequently used now in finding the locations of GABA-containing interneurons in the brain.

The eighties and nineties – GABA receptors: their functional and structural diversity

Just prior to these final decades of the 1900s, most researchers simplistically thought there was a single receptor type for GABA and that the pharmacological outlook was relatively simple. To provide evidence for this view, however, required the biochemical identification of the GABA receptor protein, but this proved to be quite elusive as there were no sources rich in GABA receptor protein. However, two developments helped to mitigate this, including the use of larger mammalian brains (e.g. bovine) to provide sufficient starting material for protein purification and the concurrent serendipitous use of benzodiazepine affinity column purification techniques (Sigel et al., 1982, 1983) to capture GABA receptor protein. These approaches led to the isolation of two different subunits termed α and β . Subsequent protein sequencing of the purified receptor and the use of selected peptide sequences to design oligonucleotide probes allowed the identification of (cloned) cDNAs encoding the α and β subunits. Significantly, these two subunits could be expressed outside the nervous system as a functional GABA receptor (Scholfield et al., 1987). This was achieved using a valuable heterologous expression vehicle, the *Xenopus laevis* oocyte, which was capable of expressing functional GABA_A receptors when injected with appropriate mRNA, cRNAs or cDNAs (Miledi et al., 1983; Smart et al., 1983, 1987).

The primary sequence homology of these GABA α and β subunits clearly showed they belonged to a common class of

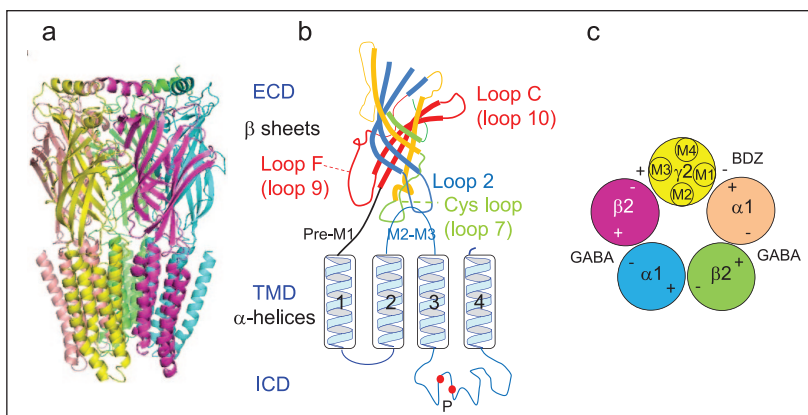


Figure 2. Structural architectures of GABA_A receptor subunits: (a) Representation of the GABA receptor $\beta 3$ homomer crystal structure showing the extracellular domain (ECD) and transmembrane domain (TMD) as seen from the plane of the cell membrane (PDB, 4cof, Miller and Aricescu, 2014). Each $\beta 3$ subunit comprising the pentamer is shown in a different colour. Secondary structures are shown – β sheets in the ECD and α helices in the TMD. (b) A flattened and simplified schematic of a typical GABA_A receptor subunit showing structures for the extracellular domain (ECD, β sheets), the transmembrane domain (TMD) with four α -helices, M1-M4, and the unknown structure of the intracellular domain (ICD) with phosphorylation sites (red circles). The key structures involved in receptor activation, loops 2, 7 and 9 are shown including loop C which is close to the GABA binding site. Modified from a schematic in Smart and Paoletti, 2012. (c) Plan view of a GABA_A receptor pentamer schematic showing subunit arrangement and the principal (+) and complementary (-) subunit interfaces as well as the position of the M1-M4 α -helices in the $\gamma 2$ subunit only. GABA and benzodiazepine interfacial binding locations are shown.

receptors, which included nicotinic acetylcholine and glycine receptors. They were initially characterised by a structural signature, a Cys loop, which is identified by two cysteine residues that engage in a disulphide bond encapsulating a loop of 13 amino acids (Barnard et al., 1987). From this structure, their name was derived – the Cys-loop receptors. Later, this family has been renamed as the pentameric ligand-gated ion channels and also includes serotonin type-3 receptors, Zn²⁺ activated cation channel, invertebrate channels activated by glutamate, serotonin or GABA, and bacterial homologues, *Gloeobacter violaceus* (GLIC) and *Erwinia chrysanthemi* (ELIC). All the eukaryotic receptors in this family possess a Cys-loop motif, while the prokaryotic counterparts do not (Smart and Paoletti, 2012).

Significantly, and surprisingly, given that GABA_A receptor protein was purified by benzodiazepine affinity chromatography, the cloned $\alpha\beta$ GABA receptor lacked sensitivity to benzodiazepines. This implied that other receptor subunits must exist to provide the full spectrum of pharmacological and physiological function. Indeed, this proved to be the case with the discovery of the γ subunit (Pritchett et al., 1989), and from the basis of single α , β and γ subunits, molecular cloning studies rapidly expanded the GABA receptor subunit portfolio (Seeburg et al., 1990), which eventually included new subunit families and some other families exhibiting multiple members, $\alpha(1-6)$, $\beta(1-3)$, $\gamma(1-3)$, δ , ϵ , π , θ and $\rho(1-3)$, eventually totalling 19 subunits, without including the increased diversity that arises from RNA alternative splicing affecting $\alpha 6$, $\beta 2$ and $\gamma 2$ (MacDonald and Olsen, 1994; Sieghart, 1995; Sigel and Steinmann, 2012; Smart, 2015; Stephenson, 1995).

Core features of the pentameric receptor structure

Once the GABA_A receptor genes were known and functional receptors could be expressed in cell lines for exploratory experiments,

there followed a period of intense scrutiny of the GABA receptor's structure and its functional properties. The pentameric ligand-gated receptor family retains a characteristic structural signature (Ernst et al., 2005). They possess a large extracellular domain (ECD) that incorporates the neurotransmitter (orthosteric) binding site (Lummiss, 2009) located at interfaces between $\beta^+-\alpha^-$ subunits, and allosteric binding sites for modulators, such as the benzodiazepines at the $\alpha^+-\gamma^-$ subunit interface (Sigel, 2002) and barbiturates at the $\gamma^+-\alpha^-$ interface (Jayakar et al., 2015) (Figure 2(a) and (c)). The signature Cys loop is evident in all receptors and appeared to interact with residues in the transmembrane domain (TMD) M2-M3 region (Figure 2(b)).

A linker connects the ECD to the start of four α -helical TMDs (M1-M4, per subunit), of which the M2 subunit forms the lining of an ion channel pore that selects for anion (mostly Cl⁻) permeation (Figure 2(b) and (c)). This domain also incorporates a number of allosteric binding sites for a variety of ligands including loreclezole (Wafford et al., 1994), neurosteroids (Hosie et al., 2006) and some anaesthetics (Franks, 2015). At the receptor's intracellular surface, there are two peptide loops, the M1-M2 linker and the much larger M3-M4 linker that incorporates binding sites for receptor-associated molecules such as the synaptic scaffold protein, gephyrin, and also consensus sites for protein kinase phosphorylation (Jacob et al., 2008; Luscher et al., 2011; Moss and Smart, 2001). These GABA receptors were clearly ionotropic in nature capable of enabling rapid flux of Cl⁻ across the cell membrane.

The GABA_B receptor

Towards the end of the 1970s and early 1980s, Norman Bowery and colleagues were studying how GABA affected the release of noradrenaline in the heart and they identified another type of GABA receptor. This receptor was insensitive to the specific

Tentatively identified	Probables	Possibles
$\alpha 1\beta 2\gamma 2$	$\alpha 1\beta 3\gamma 2$	$\alpha\beta\gamma 1$
$\alpha 2\beta\gamma 2$	$\alpha 1\beta\delta$	$\alpha\beta\gamma 3$
$\alpha 3\beta\gamma 2$	$\alpha 5\beta 3\gamma 2$	$\alpha\beta\theta$
$\alpha 4\beta\gamma 2$	$\alpha\beta 1\gamma$	$\alpha\beta\varepsilon$
$\alpha 4\beta 2\delta$	$\alpha\beta 1\delta$	$\alpha\beta\pi$
$\alpha 4\beta 3\delta$	$\alpha\beta$	$\alpha x\beta\gamma\gamma 2$
$\alpha 5\beta 2\gamma 2$	$\alpha 1\alpha\beta\beta\gamma$	
$\alpha 6\beta 2\gamma 2$	$\alpha 1\alpha\beta\delta$	
$\alpha 6\beta 2\delta$		
$\alpha 6\beta 3\delta$		
$\rho 1-3$		

Figure 3. Subunit composition of native GABA_A receptors.

The three lists are designed with minor modifications in terms of assignments from Table 3 in Olsen and Sieghart (2008) showing the likeliest combinations of GABA_A receptors subunits that are thought to exist in the mammalian brain. The probability of existence of a receptor in the brain is considered highest in the left panel and less so in the middle to right panels. x and y signify that different copies of α and β subunits may co-assemble in the same receptor pentamer.

GABA receptor antagonist, bicuculline, and could be activated by the selective agonist, baclofen (Bowery et al., 1980). These receptors were clearly different to the ionotropic GABA receptors, and they were classed as GABA_B receptors (Bowery, 1993) and assigned to the class C G-protein-coupled receptor (GPCR) superfamily in comparison to the ionotropic receptors that were designated as GABA_A receptors.

We now know that the GABA_B receptor once activated by GABA or other agonists, for example, baclofen, signals via opening inwardly rectifying K⁺ channels, by closing voltage-gated Ca²⁺ channels, and by modulating adenylate cyclase activity. To do so, it must assemble from two distinct subunits that were subsequently cloned (Jones et al., 1998; Kaupmann et al., 1997, 1998; White et al., 1998). The R1 and R2 subunits co-assemble as an obligate heterodimer to ensure trafficking to the cell surface. The R1 subunit exists in several alternatively spliced versions, of which two major forms, R1a and R1b, are considered to be physiologically important and differ by the insertion of two Sushi domains in the N-terminal of the R1a subunit (Bettler et al., 2004).

Similar to the structural and functional diversity displayed by GABA_{A/B} receptors, heterogeneity of GABA transporters (GAT) is also evident (Kanner and Bendahan, 1990). Molecular approaches have confirmed this diversity (Borden et al., 1992; Guastella et al., 1990), and GATs are designated to belong to a family of neurotransmitter–Na⁺ symporters (Scimemi, 2014). The function of GATs can be regulated by both post-translational modification (Quick et al., 2004; Whitworth and Quick, 2001) and selective ligands, such as nipecotic acid (Krause and Schwarz, 2005). Although six transporters are known, it is GAT 1-3 that are prominent. GABA transporter Type 1 (GAT1) and 3 (GAT3) are the major transporters expressed in the brain, whereas GAT2 is expressed at lower levels (Scimemi, 2014). There is also a vesicular GABA transporter (VGAT), which is also known as a vesicular inhibitory amino acid transporter (VIAAT) (Dumoulin et al., 1999; Sagne et al., 1997). Targeting of GATs is an alternative and valuable target for the manipulation of GABAergic neurotransmission for disorders in which dysfunction is apparent (e.g. epilepsy, see below).

The 21st century and into the future

The discovery of a rich array of subunits available for assembly into GABA_A receptors was unexpected, and raised many questions that we are still addressing today. These can be loosely grouped into the following categories:

1. In terms of *physiology and structure* – Are there rules for subunit assembly? How many different receptor subtypes are there and what roles do they play? Are GABA receptors differentially expressed across the brain and even in single neurons? Why is there receptor heterogeneity? Such basic questions for which we have limited information.
2. With regard to *pharmacology and therapeutics* – Can drugs selectively modulate some GABA receptors but not others? Do we know where drugs bind on GABA receptors? Can these sites be exploited for future therapeutics?
3. And in terms of *neurology and psychiatry* – Do aberrant inhibition and dysfunctional GABA receptors underlie specific neurological/neuropsychiatric diseases?

Some answers to these questions are emerging. There are rules governing receptor subunit assembly since the number of subunits available would suggest in excess of 150,000 different GABA receptors are possible, and yet in the brain, this is likely to be highly restricted to around ~50 or so tentatively determined using subunit-selective antisera and functional expression experiments (Olsen and Sieghart, 2009) (Figure 3). Typically, many physiologically relevant GABA receptors are thought to be mainly composed of $\alpha\beta\gamma$ subunits usually in a stoichiometry of 2:2:1 with identical α and β subunit pairs; however, there is also evidence that α and β subunit pairs can differ in some receptors (e.g. $\alpha 2$ with $\alpha 3$) (Duggan et al., 1991; McKernan et al., 1991; McKernan and Whiting, 1996). Thus, the precise subunit composition of many native GABA_A receptors still needs to be verified.

The physiological roles that GABA_A receptors play are multifarious. At early stages of development, there are roles in neurogenesis and synaptogenesis promoting neurodevelopment and neuronal excitation (Ben Ari, 2002; Ben Ari et al., 2007), although this has been challenged (Bregestovski and Bernard, 2012; Valeeva et al., 2016). However, their pre-eminent role is to provide neural inhibition to control network excitability (Farrant and Kaila, 2007). This effect proceeds via GABA_A receptors associated with two distinct membrane domains: inhibitory synapses, where rapid but brief, quantal release of GABA causes intense inhibition via activation of mostly $\alpha\beta\gamma$ subunit-containing receptors; and at extrasynaptic sites, where ambient low levels of GABA levels initiate tonic inhibition, via $\alpha\beta\gamma$, $\alpha\beta\delta$ and $\alpha\beta$ containing receptors, which performs a less intense but more persistent inhibitory role (Farrant and Nusser, 2005; Mody, 2001). The effectiveness of inhibition depends upon the location of GABA synapses and the subunit composition of individual GABA_A receptors, and crucially on the timing of activation with regard to concurrent excitation. All are critical aspects in determining the high-order level of neural network excitation.

The diversity of receptor subunits spawned many studies investigating the types of GABA_A receptors expressed in neurons across the central nervous system. This has revealed distinct

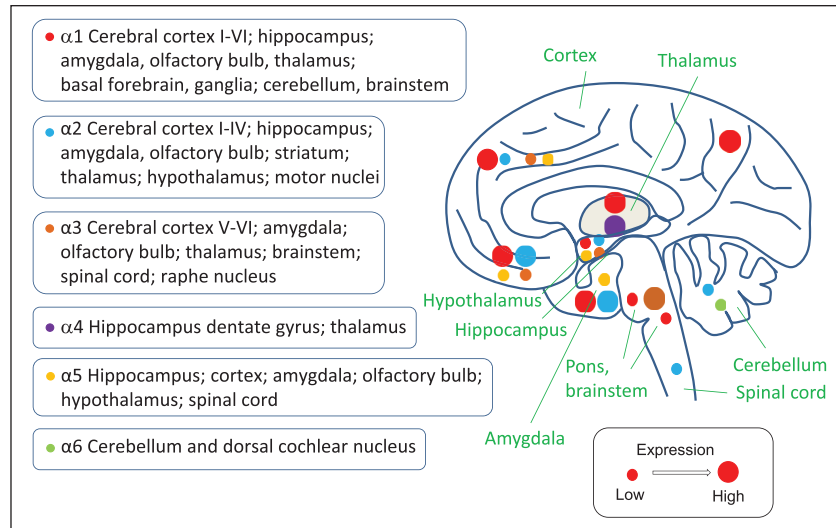


Figure 4. Cellular expression profiles of GABA_A receptors subunits.

The text boxes show the major sites of expression of GABA_A receptor α subunits that are also depicted in diagrammatic form in the schematic parasagittal section through the brain. The relative sizes of the spheres indicate the extent of GABA_A receptor α subunit expression. Data have been accrued from numerous immunofluorescence studies, and mostly taken as a collation from (Fritschy and Brunig, 2003).

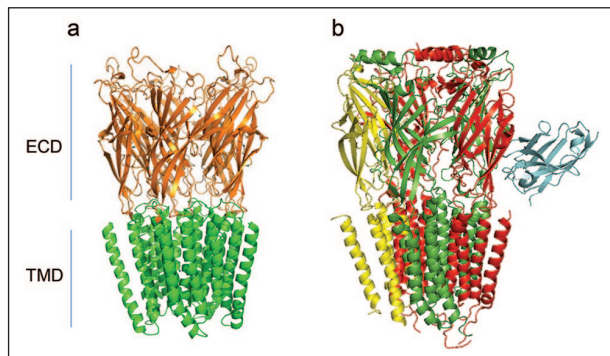


Figure 5. Structures of the GABA_A receptor: (a) Shows a chimeric GABA_A receptor composed of an extracellular domain (orange) from GLIC and transmembrane domain from the GABA_A receptor $\alpha 1$ subunit (green). Note GLIC is a homomer and allows the expression of a pentamer of $\alpha 1$ subunit TMDs. Schematic based on study by Laverty et al., 2017. (b) Cryo-EM structure of an $\alpha 1\beta 3\gamma 2L$ GABA_A receptor in ribbon format. The $\alpha 1$ subunits are red, $\beta 3$ subunits are green and the $\gamma 2L$ subunit is yellow; the nanobody used to isolate the receptor protein (blue). Note the resolution of the initial part of the ICD. Images are taken from the protein databank (PDB 6I53) (Laverty et al., 2019).

expression profiles for selected subunits (Figure 4), such as for example, $\alpha 6$ in cerebellar granule cells, and $\alpha 5$ subunits predominant in the hippocampus and selected cortical areas. The differential expression patterns imply that there are physiological roles underlying GABA_A receptor heterogeneity. This is supported by other studies showing that the subunit composition of the receptor can have profound effects on its functional profile, including the potency of GABA, the kinetics of receptor activation and desensitisation and the pharmacological phenotype (Gingrich et al., 1995; Korpi et al., 2002; Lavoie et al., 1997; Mortensen et al., 2011; Picton and Fisher, 2007).

Recently, our understanding of the structure of GABA_A receptors has undergone marked changes from our early perception of circular ‘doughnut-shaped’ entities, labelled with approximate positions of drug/modulator binding sites, to detailed structural homology models. These models were based on other receptors from the same superfamily whose atomic structures had been resolved. Later, high-resolution crystal structures of initially homomeric receptors and chimeric (bacterial homologue-GABA_A receptor subunit) receptors (Figure 5) became available, and now most recently, we have some atomic structures for heteromeric GABA_A receptors that are expressed in the brain, composed of α , β and γ subunits with and without bound ligands. This rapid structural development initially relied on a structure for the $\beta 3$ homomeric GABA receptor (Miller and Aricescu, 2014), then GLIC-GABA_AR $\alpha 1$ subunit chimera (Laverty et al., 2017), revealing both the modular structure of the receptor and some important modulatory binding sites, for example, for the modulatory neurosteroids (Laverty et al., 2017) reported simultaneously for a $\beta 3$ - $\alpha 5$ chimeric GABA receptor structure (Miller et al., 2017). Together, these structures, and particularly the seminal cryo-electron microscopy (EM) structures of GABA_A receptors that followed, revealed the ECD and associated GABA binding loops (A-F), the central anion-conducting pore region in the TMDs, and identified roles for several subdomains of the receptor in the process of receptor activation, including the channel activation gate, the deep lying desensitisation gate in the TMD, and the linker regions between the ECD and TMD (Corringer et al., 2012; Laverty et al., 2019; Masiulis et al., 2019; Phulera et al., 2018; Smart and Paoletti, 2012; Zhu et al., 2018). Despite this level of new knowledge, one area is notable by its absence from these structures and this concerns the intracellular domain (ICD) represented largely by the M3-M4 linker, the site of receptor phosphorylation and binding of receptor-associated molecules such as gephyrin (Luscher et al., 2011) and the GARHLs (Yamasaki et al., 2017). This domain is invariably removed from receptor structures due to its perceived ability to hamper crystallisation and structural determination by cryo-EM,

which may reflect difficulties in forming a stable structure. Thus, we have little or no understanding of the shape of the ICD, apart from what can be extrapolated from the *Torpedo* nicotinic acetylcholine receptor (Unwin, 2005). It is possible that future development of cryo-EM methods will enable the resolution of this important area.

For many years, there has been a concerted effort to determine how drugs affect the function of GABA_A receptors and to deduce where they bind (Korpi et al., 2002). The earliest example concerned the benzodiazepines since it was clear from the first cloning paper that GABA receptors composed of α and β subunits lacked high sensitivity to the benzodiazepines. Only the later incorporation of the γ subunit in the receptor resolved this conundrum. Despite this important observation, there are very few drugs that can be truly classed as GABA_A receptor subtype specific. Many ligands may exhibit some preference for modulating specific GABA receptors, but at higher concentrations or doses, they frequently lose their selectivity. Achieving a high degree of receptor selectivity is an important goal, and worthy of pursuit for future therapeutic gain. This important concept has been realised by the creative use of mouse genetics to either remove (knock-out) or, more subtly, replace (knock-in) wild-type GABA receptor subunits with mutant counterparts that lack sensitivity to particular drugs. Generally, the knock-out approach is no longer favoured, given the propensity for adaptive compensatory changes and sometimes a severe phenotype that confounds interpretation. Using the knock-in approach, a seminal study targeted a key residue (histidine 101) in receptor α subunits, which was necessary for benzodiazepine action (Rudolph et al., 1999), and demonstrated that their classic quadruple effects (anxiolysis, sedation, muscle relaxation and amnesia) are likely associated with neural circuits involving GABA_A receptors composed of specific receptor subtypes. For example, removing benzodiazepine sensitivity from receptor α 2 subunits resulted in reduced anxiolysis without affecting sedation by benzodiazepines (Rudolph et al., 1999). Similarly, α 5 subunits were associated with benzodiazepine-induced amnesia (Rudolph and Mohler, 2004). These, among others, are sites that are being exploited for therapeutic purposes to provide anxiolytics that are non-sedative (α 2, α 3), and to provide cognitive enhancers that may be useful in some conditions of neurodegeneration (α 5). Extending this approach to receptor β 2 and β 3 subunits and the activity of general anaesthetics revealed another important residue in the TMD that distinguishes between the modulation of GABA_A receptors by various intravenous anaesthetics (Rudolph and Antkowiak, 2004). Similar approaches have been adopted to establish that GABA receptors in the spinal cord play a significant role in pain processing and modulation of these receptors may offer possibilities for new analgesics (Zeilhofer et al., 2009).

Defining the precise location of binding sites on high-resolution GABA receptor structures will provide an excellent opportunity for new drug design. To date, most ligand docking has been performed with general anaesthetic agents either making use of photoactive derivatives to covalently bind to the receptor (Li et al., 2006) or solving crystal structures with anaesthetics bound in situ (Yip et al., 2013). However, this is set to change with the advent of high-resolution GABA_A receptor structures (Laverty et al., 2017; Masiulis et al., 2019).

The increased understanding of the cellular, molecular and structural properties of GABA_A receptors has revealed

the likelihood of their playing multiple roles in neurological and psychiatric diseases, in addition to identifying new potential drug targets in the receptor protein. Dysfunctional inhibition and GABA_A receptors have been associated with, for example, anxiety, depression, epilepsy, autism, Angelman's syndrome, Fragile-X syndrome, schizophrenia and Down syndrome (Braat and Kooy, 2015; Hines et al., 2012; Macdonald et al., 2010; Rudolph and Mohler, 2014; Yuan et al., 2015). The involvement of GABA inhibitory systems invariably takes the form of gain-of-function or loss-of-function at the receptor level, but of course, the consequential network effects can be far more complex. For epilepsy, there is an increasing diversity of genetic mutations that associate with multiple clinical phenotypes directly correlating receptor dysfunction with disease (Macdonald et al., 2010). While, for epilepsy, this often involves a loss-of-function, there are circumstances where a gain-of-function can be problematic such as in some forms of absence epilepsy, where no underlying mutations are apparent in the receptor protein, but tonic inhibition is increased due to aberrant GABA transport (Cope et al., 2009).

Increased GABA inhibition is also apparent in Down syndrome and its reversal using either a GABA antagonist or α 5 benzodiazepine inverse agonists appears to reverse the cognitive decline that is associated with the Down phenotype in animal models (Fernandez et al., 2007). What has also become apparent with some neurodevelopmental disorders, such as schizophrenia, are changes that occur to receptor subunit levels, such as the δ and α 5 subunits, that could lead to deficits in tonic, and for other subunits, synaptic inhibition. A similar outcome has also been noted associating depression with GABA inhibition, with GABAergic deficits (e.g. low GABA levels in the brain) thought to be one cause since these can be reversed by anti-depressant agents (Rudolph and Mohler, 2014). Thus, the field of GABA therapeutics is expanding and becoming prominent and argues for increased development of subtype-selective agents to combat central nervous system diseases. It is also encouraging that relatively new unexplored sites for drug binding are being revealed, evident from the largely unstudied α^+ - β^- interface, where novel drugs can exert modulatory actions on GABA_A receptors (Varagic et al., 2013) at a site that shares homology with the benzodiazepine binding site at the α^+ - γ^- subunit interface.

And so to the future

Manipulation of GABA inhibition in animal models and humans will become ever more sophisticated with the use of new subtype-selective drugs that target known binding sites as these become identified by new structural studies using crystallography and cryo-EM. To confer specificity, greater use of antibody fragments (variable domain of new antigen receptors (VNARs) and nanobodies) will enable the delivery of less specific drugs to particular receptor isoforms, especially where such binding sites are defined at receptor subunit interfaces.

Other methods to control inhibition will rely on interneuron manipulation driven by using optogenetics and chemogenetic approaches, supported by optical neurobiological methods coupled to photochemical probes targeting the GABA_A receptor (Lin et al., 2015; Mortensen et al., 2014; Yue et al., 2012). These latter agents will have in-built light-driven 'molecular switches' (e.g. azobenzenes) to flip molecular structure and functionality from neutral to active in terms of therapeutic effect in vivo. The

challenge will lie in being able, in intact brain structures, to deliver light at the appropriate wavelength to the correct neural circuits. Nevertheless, the ability to dynamically manipulate inhibition will have significant impact on a range of neurological diseases and is a goal that is worthy of our aspiration.

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