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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/145185 since 2017-01-12T10:48:17Z

Published version:

DOI:10.1111/ejn.12534

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17 August 2024

UNIVERSITÀ DEGLI STUDI DI TORINO

This is the accepted version of the following article:

GABA$_A$ receptors and plasticity of inhibitory neurotransmission in the central nervous system

European Journal of Neuroscience 39 (11) 2014 : 1845–1865

DOI: 10.1111/ejn.12534

which has been published in final form at

http://onlinelibrary.wiley.com/doi/10.1111/ejn.12534/full

$GABA_A$ receptors and plasticity of inhibitory neurotransmission in the central nervous system

Jean-Marc Fritschy[1,2,*], Patrizia Panzanelli[3]

[1]Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland
[2]Neuroscience Center Zurich, University of Zurich and ETH, Zurich, Switzerland
[3]Department of Neuroscience Rita Levi Montalcini, University of Turin, Turin, Italy

*Corresponding author: Jean-Marc Fritschy Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland. E-mail: fritschy@pharma.uzh.ch

Abstract

$GABA_A$ receptors ($GABA_ARs$) are ligand-gated $Cl^−$ channels that mediate most of the fast inhibitory neurotransmission in the central nervous system (CNS). Multiple $GABA_AR$ subtypes are assembled from a family of 19 subunit genes, raising the question of the significance of this heterogeneity. In this review, we discuss the evidence that $GABA_AR$ subtypes represent distinct receptor populations with a specific spatio-temporal expression pattern in the developing and adult CNS, being endowed with unique functional and pharmacological properties, as well as being differentially regulated at the transcriptional, post-transcriptional and translational levels. $GABA_AR$ subtypes are targeted to specific subcellular domains to mediate either synaptic or extrasynaptic transmission, and their action is dynamically regulated by a vast array of molecular mechanisms to adjust the strength of inhibition to the changing needs of neuronal networks. These adaptations involve not only changing the gating or kinetic properties of $GABA_ARs$, but also modifying the postsynaptic scaffold organised by gephyrin to anchor specific receptor subtypes at postsynaptic sites. The significance of $GABA_AR$ heterogeneity is particularly evident during CNS development and adult neurogenesis, with different receptor subtypes fulfilling distinct steps of neuronal differentiation and maturation. Finally, analysis of the specific roles of $GABA_AR$ subtypes reveals their involvement in the pathophysiology of major CNS disorders, and opens novel perspectives for therapeutic intervention. In conclusion, $GABA_AR$ subtypes represent the substrate of a multifaceted inhibitory neurotransmission system that is dynamically regulated and performs multiple operations, contributing globally to the proper development, function and plasticity of the CNS.

Keywords: diazepam, excitatory–inhibitory balance, gephyrin, neurogenesis, phosphorylation, post-synaptic density.

Introduction

$GABA_A$ receptors ($GABA_ARs$) belong to the family of Cys-loop ligand-gated ion channels, along with nicotinic acetylcholine receptors, glycine receptors (GlyRs), and serotonin type 3 receptors, which form pentameric channels carrying two extracellular ligand-binding sites (Olsen & Sieghart, 2008). Being permeable to chloride ions, $GABA_ARs$ mediate most of the inhibitory action of GABA in the central nervous system (CNS). By virtue of their ubiquitous expression in neurons (and possibly glial cells) (Passlick *et al*., 2013), $GABA_ARs$ contribute to all CNS functions, including sensory and motor processing, central autonomic control, sleep–wakefulness, emotions, and cognition. Clinically, the main relevance of $GABA_ARs$ relates to their exclusive targeting by benzodiazepines (and other ligands with high affinity for the benzodiazepine-binding site), which are used for their anxiolytic, sedative, anticonvulsant, and muscle relaxant properties. Benzodiazepine site ligands act as allosteric modulators, and, owing to their extraordinary selectivity, produce no other direct action in the CNS. $GABA_ARs$ are also the targets of general anesthetics (Rudolph & Antkowiak, 2004), ethanol, and endogenous modulators, notably endozepines (Christian *et al*., 2013) and neurosteroids (Hosie *et al*., 2006). These ligands, in large part derived from glial cells, are considered to be crucial regulators of neuronal function and excitability under both physiological and pathological conditions, and represent promising novel targets for specific neurological and psychiatric indications [reviewed in Belelli & Lambert (2005) and Carver & Reddy (2013)].

In the early 1990s, the identification of 19 genes encoding $GABA_AR$ subunits (α1–α6, β1–β3, γ1–γ3, δ, ε, π, τ, ρ1–ρ3) in mammals, coupled with the demonstration that recombinant receptors assembled with an α subunit, a β subunit and the γ2 subunit variant were functionally and pharmacologically similar to native $GABA_ARs$, raised the question of the significance of $GABA_AR$ heterogeneity. This question is still largely unresolved, but the evidence available suggests that $GABA_AR$ subtypes, differing in subunit composition, represent distinct entities with specific functions and pharmacological profiles, and with unique spatio-temporal mRNA and protein expression patterns (box 1). In this review, we will discuss this evidence, on the basis of four fundamental observations made since the existence of $GABA_AR$ subtypes was established.

A first major advance in understanding the function of $GABA_AR$ subtypes and the relevance of their molecular heterogeneity was the recognition that they mediate two fundamentally distinct forms of inhibitory transmission, which depend on their localisation, either postsynaptic (mediating fast, high-amplitude phasic currents upon quantal presynaptic GABA release) or extrasynaptic [mediating low-amplitude but persistent (tonic) currents activated by ambient GABA] [reviewed in Farrant & Nusser (2005), Belelli *et al*. (2009), and Brickley & Mody (2012)]. The significance of tonic inhibition, in particular for the control of neuronal excitability and plasticity, is now gaining widespread recognition. Importantly, these two major populations of $GABA_ARs$ are molecularly distinct, with postsynaptic receptors containing mainly the α1, α2 and α3 subunits, along with β subunit variants and the γ2 subunit, and extrasynaptic receptors containing the α4, α5 and α6 subunits, often along with the δ subunit (instead of the γ2 subunit). This observation implies that the mechanisms of subcellular targeting of $GABA_AR$ subtypes are subunit-specific, and can vary between CNS regions and developmental stages. The crucial role played by the postsynaptic scaffold organised by gephyrin for postsynaptic targeting and confinement of some $GABA_ARs$ in GABAergic postsynaptic densities (PSDs) is now widely recognised, albeit poorly understood (Fritschy *et al*., 2012; Tretter *et al*., 2012). Furthermore, there is ample evidence for modulation of both postsynaptic and extrasynaptic $GABA_ARs$ by multiple post-translational mechanisms regulating single-channel functional properties, trafficking (exocytosis, endocytosis, and degradation), cell surface mobility, and synaptic confinement. Thereby, these mechanisms have a major impact on the strength of GABAergic

transmission in response to changes in network function, and they represent a major facet of GABAergic synapse plasticity (Hines *et al*., 2011; Luscher *et al*., 2011a; Connelly *et al*., 2013a).

A second major advance was the demonstration, with gene targeting techniques, that the spectrum of diazepam's actions is elicited by distinct $GABA_AR$ subtypes, distinguished by their α subunit variant (Rudolph & Möhler, 2004). The logical consequence of these findings is that these distinct $GABA_ARs$ are localised in different neuronal circuits, even when they are coexpressed within neurons or within specific brain areas (Box 2). An important concept emerging in this regard is that GABAergic interneurons are likewise specialised to control the activity of principal cells in a circuit-specific manner (Klausberger & Somogyi, 2008). This highly sophisticated organisation raises the possibility that multiple forms of inhibitory neurotransmission, engaging specific interneurons and $GABA_AR$ subtypes, operate in parallel in neuronal circuits involved in concurrent tasks. The possibility of probing interneuron function *in vivo* with optogenetic tools has substantially advanced our understanding of their role and functional specialisation (Sohal *et al*., 2009; Pfeffer *et al*., 2013; Zhu *et al*., 2013).

A third fundamental insight into $GABA_AR$ function is their dependence on ionic mechanisms, involving both Cl^- and HCO_3^- (Blaesse *et al*., 2009). Therefore, the effects of $GABA_ARs$ on the resting membrane potential, independently of their subunit composition, are determined by the action of KCl co-transporters and carbonic anhydrases. It was recognised early that the expression of KCC2, the main Cl^- extrusion transporter in mature neurons (Kaila, 1994), is developmentally regulated, giving rise to the concept of a functional 'switch' from depolarising to hyperpolarising $GABA_AR$ actions during ontogeny (Ben-Ari, 2002), with wide-ranging consequences for our understanding of GABA function, as well as the clinical use of $GABA_AR$-modulating drugs in infants (Pavlov *et al*., 2013).

Fourth, it is now well established that $GABA_AR$-mediated transmission regulates multiple steps of neuronal development and maturation during ontogenesis and adult neurogenesis, including control of stem/precursor cell proliferation, cell fate decision, migration of precursor cells, survival of immature neurons, dendritic growth, and synaptogenesis [reviewed in Platel *et al*. (2007) and Dieni *et al*. (2013)]. Accordingly, it is being increasingly recognised that perturbations of $GABA_AR$ function during ontogeny or after a lesion, notably during critical periods of plasticity, can have long-lasting effects on CNS circuit structure and function, potentially contributing to the pathophysiology of neurological and psychiatric disorders, including epilepsies, chronic pain, neurodevelopmental disorders, mood disorders, and schizophrenia (Bavelier *et al*., 2010; Lewis, 2012).

Here, we will review the relevance of $GABA_AR$ heterogeneity for the regulation of GABAergic neurotransmission and GABAergic synaptic plasticity, and its potential impact on the pathophysiology of major neurological and psychiatric diseases, notably disorders linked to abnormal GABAergic function during brain development. We will highlight recent progress and discuss major roadblocks on the way to better understanding the diversity of $GABA_AR$ regulation in health and disease. To do so, we will briefly introduce the molecular heterogeneity of GABAergic synapses, prior to discussing mechanisms regulating $GABA_ARs$ in the context of GABAergic synaptic plasticity. Next, we will highlight the relevance of $GABA_AR$ subtypes for the regulation of neuronal development, and finally, their relevance for the pathophysiology of neurological and psychiatric diseases linked to abnormal GABAergic transmission.

Composition and localisation of major $GABA_AR$ subtypes

The subunit composition of major $GABA_AR$ subtypes is well established at a regional level (immunoprecipitation with extracts from the whole brain or a specific brain region), and there is general agreement that the most likely subunit stoichiometry is 2α/2β/γ (the last of these being sometimes substituted by δ or ε) (Boileau *et al*., 2005; Olsen & Sieghart, 2008; Patel *et al*., 2013). The existence of receptors containing α and β subunits only, as well as receptors with other stoichiometries (e.g. 2α/β/2γ and 2α/β/2ε), is probable (Jones & Henderson, 2013). Furthermore, the rules governing the formation of pentameric complexes are far from being fully elucidated. Functional $GABA_ARs$, with the pharmacological profile of native receptors, are formed by pentameric assembly of 2α/2β/γ2, where the α and β subunits can be either identical or different. The γ2 subunit can be substituted by γ1 or γ3 subunits (present at low levels and/or with a restricted expression pattern) or by δ, and possibly ε, subunits. There is consensus, therefore, that at least 36 distinct $GABA_AR$ subtypes exist in CNS neurons (Olsen & Sieghart, 2008). *In vitro* expression of β and γ subunits only, or targeted deletion of an α subunit gene *in vivo*, prevent the assembly and/or surface targeting of a functional $GABA_AR$ complex, thereby providing the opportunity to remove specific $GABA_AR$ subtypes by inactivating a single α subunit gene (Box 1).

The most detailed information on the regional distribution of 18 $GABA_AR$ subunit mRNAs in the mouse brain, determined by non-radioactive *in situ* hybridisation with cellular resolution, is available in the Allen Brain Atlas (http://mouse.brain-map.org). These data confirm the findings of original studies on the distribution of abundant subunits (α1–α6, β1–β3, and γ2) (Laurie *et al*., 1992; Wisden *et al*., 1992), and provide detailed information on subunits expressed either at low levels in numerous regions throughout the neuraxis, such as the γ3 subunit, or at high levels in specific regions, such as the ε subunit in the amygdala, basal forebrain, locus coeruleus, and other noradrenergic cell groups. According to the Allen Brain Atlas, the ρ1 and ρ2 subunit mRNAs (corresponding to $GABA_C$ receptors) are restricted to the superficial layers of the superior colliculus, and the π subunit mRNA is undetectable in the adult mouse brain.

Immunohistochemically, the distribution of 10 subunits (α1–α6, β2, β3, γ2, and δ) has been analysed in detail, at the regional and, sometimes, cellular level, and validated by targeted gene deletion (Fritschy & Mohler, 1995; Nusser *et al*., 1999; Peng *et al*., 2002; Chandra *et al*., 2006; Hörtnagl *et al*., 2013). There is also information available for β1 and γ1 subunits that confirms the *in situ* hybridisation data. According to these studies, it is very apparent that the six α subunit variants largely correspond to distinct $GABA_AR$ subtypes [notwithstanding the possibility of finding two different α subunits in a substantial fraction of $GABA_ARs$ (Balic *et al*., 2009)] – each with a specific distribution pattern that overlaps only partially with that of other α subunits (Fig. 1). The same holds true for the β subunit variants, with the β2 and β3 subunits overlapping to a large extent with the α1 and α2 subunits, respectively (whereas the β1 unit is expressed at lower levels in numerous brain regions). The γ2 subunit, in line with its association with the vast majority of $GABA_AR$ subtypes, is ubiquitously expressed; in contrast, the γ1 subunit appears to have a highly restricted distribution, being most abundant in the hypothalamus, amygdala, and parts of the basal ganglia, as well as the inferior olivary nucleus. Finally, the δ subunit, which forms $GABA_AR$ located extrasynaptically (Box 3), largely overlaps with the α4 subunit in the forebrain and with the α6 subunit in the cerebellum.

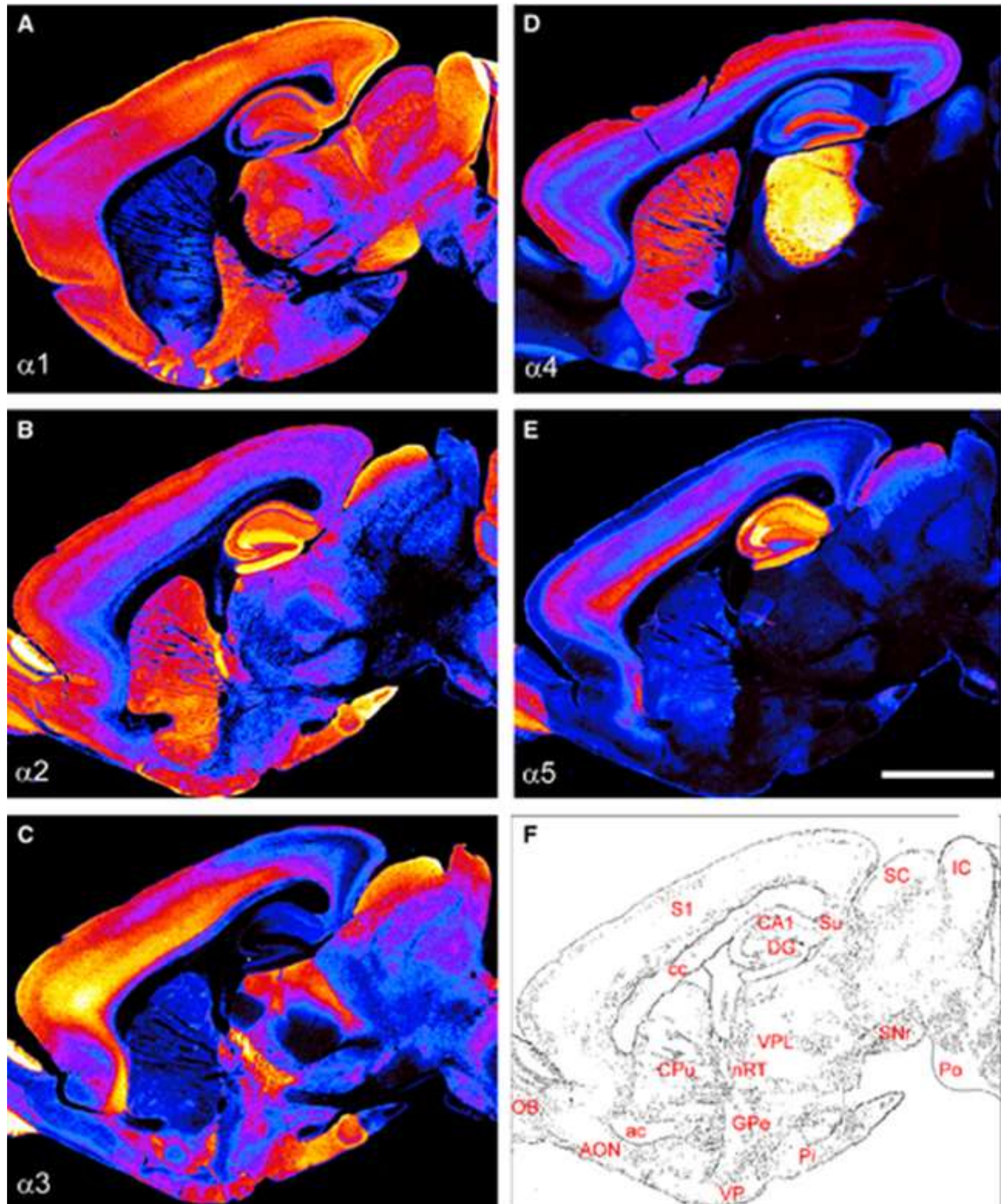

Figure 1. Differential distribution of GABA$_A$R α subunit variants in the adult mouse forebrain. (A–E) Each panel depicts in false colors (increasing intensity: dark blue, red, orange, yellow, and white) the relative staining intensity pattern of each subunit indicated, as determined by immunoperoxidase staining. The α6 subunit is not depicted, because it is not expressed in the forebrain. Note that each subunit has a unique distribution pattern, with partial overlap and complementarity to other α subunits. (F) Main anatomical structures present in the images of A–E. ac, anterior commissure; AON, anterior olfactory nucleus; CA1, CA1 region of the hippocampus; cc, corpus callosum; CPu, caudate nucleus and putamen (= striatum); DG, dentate gyrus; GPe, globus pallidus, external; IC, inferior colliculus; nRT, thalamic reticular nucleus; OB, olfactory bulb; Pi, piriform cortex; Po, pontine nuclei; S1, primary somatosensory cortex; SC, superior colliculus; SNr, substantia nigra, pars reticulata; Su, subiculum; VP, ventral pallidum; VPL, ventral postero-lateral thalamic nucleus. Scale bar: 2 mm. Adapted from Panzanelli *et al*. (2011).

So far, however, there are only a few CNS regions in which the $GABA_AR$ subunit repertoire and their cellular/subcellular distribution have been analysed in some detail by immunohistochemistry. These include the hippocampal formation (notably the CA1 subfield, which shows remarkable heterogeneity, with expression of at least 11 subunits), the neocortex, the olfactory bulb, parts of the thalamus (notably, the ventrobasal complex, lateral geniculate nucleus, and reticular nucleus), the cerebellum, and the spinal cord dorsal horn.

Box 1. ***Limitations of immunohistochemistry for studying $GABA_A$Rs***

Whereas $GABA_A$Rs are pentameric protein complexes, immunohistochemistry only allows the visualisation of individual subunits, each of which belongs to several subtypes. Furthermore, as the pentamer contains two α subunits and two β subunits, it is not possible in neurons expressing multiple α or β subunit variants to determine whether they belong to the same receptor, or to distinct receptors with a similar subcellular distribution. Therefore, the exact $GABA_AR$ subtype repertoire of any neuron type is not established [see, for example, Panzanelli *et al.* (2011)]. A further limitation is that, so far, there are no antibodies suitable for immunochemistry for some of the $GABA_AR$ subunits. In particular, very little information is available for the distribution of the γ1, γ3, ε, π and τ subunit proteins, or for the ρ1–ρ3 subunits, at either a regional, cellular or subcellular level. As a result, several $GABA_AR$ subtypes, probably with atypical pharmacology and a highly selective distribution, remain poorly characterised, and their contribution to inhibitory transmission in these specific CNS areas is unknown.

Besides these limitations, it should be emphasised that, although immunohistochemistry reveals the detailed distribution and relative staining intensity of each subunit across the CNS, this method does not allow quantitative comparisons between antibodies (and thus assessment of the abundance of a given subunit relative to other subunits), because the affinity of antibodies for their epitopes, and their accessibility in tissue sections prepared for histology, cannot be directly measured. Furthermore, in the light of evidence showing that the specificity and sensitivity of the immunohistochemical procedure critically depends on epitope accessibility (Lorincz & Nusser, 2008), each antibody should be tested with various antigen retrieval methods. This is particularly important to interpret a lack of staining for a specific antibody in some regions and to accurately determine the subcellular distribution of $GABA_A$Rs.

This latter point is of crucial relevance for understanding the functional organisation of the GABAergic system. Functional and biochemical studies differentiate among several pools of $GABA_A$Rs in neurons, being localised intracellularly (reflecting biosynthesis, metabolism, and the reserve pool) or at the cell surface, postsynaptically or extrasynaptically (Box 3). Immunohistochemical studies have shown that the detection of postsynaptic $GABA_A$Rs is greatly impaired by aldehyde fixation, and requires alternative methods to the classic transcardial perfusion. Therefore, immunohistochemical analysis of $GABA_AR$ subunit distribution provides different results according to the fixation method used. Furthermore, we had noted in our initial report (Fritschy & Mohler, 1995) that the cellular distribution pattern of $GABA_A$Rs revealed by each antibody across brain regions is highly variable, ranging from diffuse staining of the neuropil to 'Golgi-like' staining of a few neurons, outlining their entire dendritic tree. This feature makes it

impossible to quantify with a single densitometric parameter the abundance of a subunit in a given brain region, as it can vary widely between various cell types.

Therefore, analyses of regional distribution patterns, showing marked differences in subunit abundance across different anatomical structures of the CNS, need to be complemented with methods allowing cellular and subcellular resolution to map $GABA_AR$ subtypes in a synapse-specific manner, and to derive some quantitative measurement of relative receptor abundance. We have recently reported a method allowing concurrent visualisation of postsynaptic $GABA_ARs$ with high sensitivity and resolution and biochemical analysis of brain tissue from the same animal (Notter *et al*., 2013). In the future, new methods will have to be devised to unequivocally detect presynaptic $GABA_ARs$, whose existence is well established functionally, but whose distribution in axons remains largely elusive.

The limitations of immunohistochemistry are even more evident for ultrastructural studies, in which the sensitivity of the method is reduced by the strong fixation required for ultrastructural preservation. It was recognised early on that pre-embedding immune-electron microscopy is not suitable for the detection of $GABA_ARs$ at postsynaptic sites (Somogyi *et al*., 1989). Post-embedding techniques, notably following tissue embedding in Lowicryl, enabled this problem to be circumvented, but have low sensitivity and have been successful with only a limited number of subunits (Nusser *et al*., 1995; Somogyi *et al*., 1996; Panzanelli *et al*., 2004). More recently, the development of sodium dodecylsulfate digestion after freeze–fracture has enabled substantial progress by allowing the three-dimensional visualisation and quantification of $GABA_ARs$ in the plasma membrane (Kasugai *et al*., 2010), albeit with a limited number of antibodies.

At the subcellular level, a distinction between postsynaptic and extrasynaptic $GABA_ARs$ can be made, based on the appearance of staining in weakly fixed tissue (Box 1). Postsynaptic receptors form brightly stained clusters, which co-localise with postsynaptic markers, such as gephyrin and neuroligin 2 (NL2), and are apposed to vesicular GABA transporter (VGAT)-positive presynaptic terminals (Fig. 2A). The remainder of the staining, besides these clusters, represents receptors dispersed at the cell surface, as well as the metabolic pool of receptors localised in the cytoplasm. Extrasynaptic receptors fail to form clusters, and the staining is of uniform intensity, with a ‘powdery’ appearance in the neuropil, suggesting a widespread distribution on dendritic branches (Fig. 2B). Accordingly, these structures show no obvious relationship with the distribution of either gephyrin or GABAergic axon terminals (Fig. 2B). As discussed below, presynaptic receptors localised in axons and axon terminals represent a distinct subset of extrasynaptic receptors, with specialised functions.

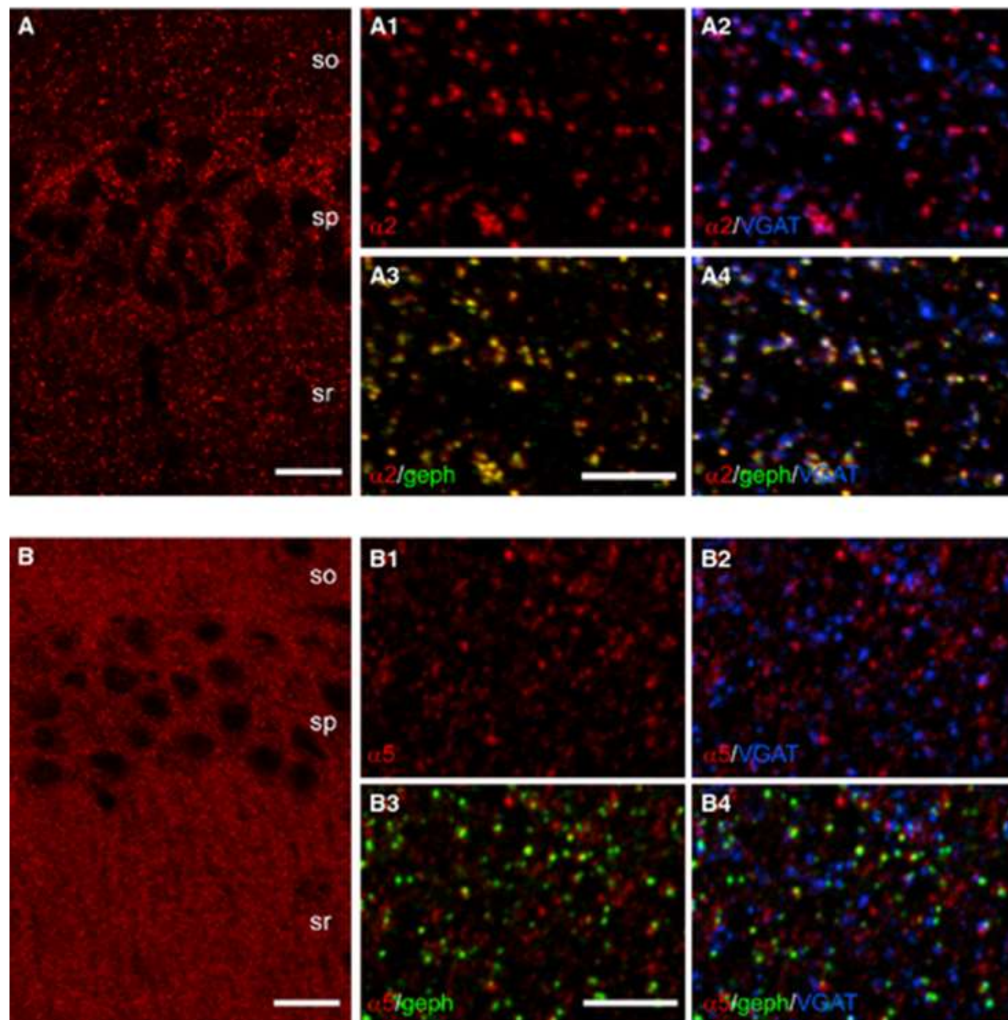

Figure 2. Distinction between postsynaptic and extrasynaptic $GABA_ARs$ by immunofluorescence staining and confocal laser scanning microscopy. The subcellular localisation is based on the identification of postsynaptic sites positive for gephyrin (geph) and presynaptic GABAergic terminals positive for VGAT. (A) α2 subunit staining in the adult mouse CA1, revealing numerous brightly stained clusters around pyramidal cell bodies in the stratum pyramidale (sp) and on their dendrites in the stratum oriens (so) and radiatum (sr). (A1–A4) High-magnification images taken from a section triple-stained for the α2 subunit (red), gephyrin (yellow) and VGAT (blue) in the stratum radiatum, showing, with single, double and triple staining, that α2 clusters are co-localised with gephyrin (A3; yellow) and apposed to VGAT-positive terminals (A2). (B) α5 subunit staining in the adult mouse CA1, showing the granular appearance of the staining, with pyramidal cells in the stratum pyramidale appearing as lightly stained structures with an unstained nucleus. (B1–B4) At higher magnification, the granular staining lacks bright clusters (as seen for α2 in A1) and shows no obvious relationship with gephyrin clusters (B3; green) or VGAT-positive terminals (B4; blue). Scale bars: A and B, 20 µm; A1–A4 and B1–B4, 5 µm. Adapted from Panzanelli *et al*. (2011).

Despite scant morphological evidence from immunohistochemical studies, there is strong functional support for the existence of $GABA_ARs$ located on axons and presynaptic terminals (Grasshoff *et al*., 2007; Trigo *et al*., 2008; Long *et al*., 2009; Witschi *et al*., 2011). A prominent exception is a population of $GABA_ARs$, readily detected immunohistochemically for the presence of the α2 subunit, located on the axon initial segment of cortical neurons, typically clustered in rows of synapses innervated by axo-axonic interneurons (Nusser *et al*., 1996; Fritschy *et al*., 1998; Panzanelli *et al*., 2011). Functionally, these $GABA_ARs$ correspond to postsynaptic receptors anchored by gephyrin, and their activation contributes to the genesis of γ-oscillations (Tukker *et al*., 2007). In contrast to

those on axo-axonic synapses, most presynaptic $GABA_A$Rs located on distal axons and terminals are hardly detectable by immunohistochemistry. They nevertheless play a key role in the control of axon potential transmission, neuronal synchronisation, regulation of transmitter release, and mediation of presynaptic afferent depolarisation (Trigo *et al*., 2008; Long *et al*., 2009; Ruiz *et al*., 2010; Wakita *et al*., 2013). Although their subunit composition is unknown, it might be inferred from the repertoire of subunit mRNAs expressed by the cell of origin of these axons. We have recently characterised a population of $GABA_A$Rs on primary afferent terminals in the spinal cord, containing α2 and α3 subunits, that are crucially involved in the anti-hyperalgesic action of diazepam (Witschi *et al*., 2011; Paul *et al*., 2012).

Box 2. ***Lessons from GABRA-KO and GABRA-knock-in mice: $GABA_A$R subtypes have specific roles***

Targeted deletion of a $GABA_A$R subunit gene, especially when constitutive, can lead to important changes in the distribution and expression pattern of the remaining subunits, suggestive of compensatory adaptations. For example, *GABRA1*-KO (α1-KO) mice show upregulation of α2-$GABA_A$Rs and α3-$GABA_A$Rs in regions where the α1 subunit is abundant (Kralic *et al*., 2006; Zeller *et al*., 2008); δ-KO mice show increased α4 subunit expression, associated with the γ2 subunit, and with an altered subcellular distribution (Peng *et al*., 2002). Typically, however, the receptor subtype that is missing as a consequence of the deletion is not merely ‘replaced’ by another subtype present in the same cell. This feature is particularly striking in neurons expressing a mixture of postsynaptic and extrasynaptic receptors. Deletion of the α subunit variant present in the postsynaptic receptors leads to their disappearance (and a corresponding loss of postsynaptic currents), whereas the extrasynaptic receptors remain either unchanged, or increased (Kralic *et al*., 2006; Peden *et al*., 2008). Therefore, the inability of α4-$GABA_A$Rs to cluster at postsynaptic sites is not attributable to competition with other receptor subtypes. In neurons expressing multiple postsynaptic receptors, synapse-specific rearrangements occur, but there is no replacement of the missing receptor, as seen, for example, in CA1 pyramidal cells of α2-KO mice, where α1-$GABA_A$Rs remain unaffected in perisomatic synapses, but disappear from the axon initial segment (Panzanelli *et al*., 2011). Some striking forms of compensation have been reported, which remain unexplained. Thus, in thalamic reticular neurons of α3-KO mice, immunohistochemistry reveals the apparent loss of postsynaptic $GABA_A$Rs and gephyrin, but these mutants show larger postsynaptic currents than wild-type mice (Schofield *et al*., 2009).

These observations lend support to the contention that $GABA_A$R subtypes, defined by their subunit composition, are unique functional entities, fulfilling specific roles, and are not interchangeable within a given type of neuron. This contention received further support from the analysis of H101R knock-in mice, engineered to remove the diazepam-binding site located at the α/γ interface of the pentameric complex, without affecting assembly, cell surface trafficking, regulation or gating of the receptor (Rudolph *et al*., 1999; Löw *et al*., 2000; Crestani *et al*., 2002; Yee *et al*., 2005). Behavioral analysis of histidine to arginine knock-in mice for each the four α subunit variants assembled in diazepam-sensitive $GABA_A$Rs revealed a loss of specific effects of diazepam, which allowed classification of the contribution of each subtype to the spectrum of diazepam's effects. Thus, sedation involves only α1-$GABA_A$Rs, whereas anxiolysis occurs upon allosteric modulation of α2-$GABA_A$Rs, and, when stress is involved, partially α3-$GABA_A$Rs [reviewed in Rudolph &

Möhler (2004)]. More recent studies have shown a corresponding segregation of other effects of diazepam (and midazolam), including benzodiazepine addiction (Tan *et al.*, 2010), tachypnea (Masneuf *et al.*, 2012), and anti-hyperalgesia (Knabl *et al.*, 2008), to specific $GABA_AR$ subtypes. Importantly, electrophysiological analyses confirmed that the point mutations are functionally silent. However, in α5(H105R) mutants, the mutation leads to decreased expression of α5-$GABA_ARs$, which is behaviorally significant (Prut *et al.*, 2010) (Box 3).

Distinction between $GABA_AR$ subtypes based on the β subunit variants is less straightforward, in particular because each β subunit can be associated with various α subunits. Nevertheless, β3 subunit-containing $GABA_ARs$ selectively mediate the action of intravenous general anesthetics, as well as some of the effects of pentobarbital, as shown in β3(N265M) mutant mice (Jurd *et al.*, 2003; Zeller *et al.*, 2007). In addition, neuron-specific deletion of these receptors curtails survival beyond early postnatal age in the majority of mutant mice (Ferguson *et al.*, 2007).

Taken together, these findings are of fundamental relevance not only for the development of efficacious benzodiazepine site ligands devoid of unwanted side-effects (in particular, sedation), but also to investigate how neuronal circuits are assembled during brain development and regulated by plasticity mechanisms in adulthood.

Molecular organisation of GABAergic synapses

The subcellular localisation of $GABA_ARs$ is intimately linked to the molecular organisation of GABAergic synapses, with specific proteins of the PSD contributing to trafficking and anchoring $GABA_ARs$ in a subtype-specific manner. It was recognised early that GlyRs, which are homologous to $GABA_ARs$, are localised at postsynaptic sites (Triller *et al.*, 1985), owing to their high-affinity binding to the scaffolding protein gephyrin (Pfeiffer *et al.*, 1982; Kirsch *et al.*, 1993), which interacts with the cytoskeleton. The demonstration that the majority of postsynaptic $GABA_ARs$ also are clustered with gephyrin took longer (Sassoè-Pognetto *et al.*, 1995, 2000), in part because gephyrin was considered to be present only at glycinergic synapses, and in part because $GABA_ARs$ do not bind gephyrin with high affinity. On the basis of the evidence available to date, the α1, α2 and α3 subunits, as well as possibly the β2 and β3 subunits, can interact directly with gephyrin via motifs located in their main intracellular loop (Tretter *et al.*, 2008, 2011; Mukherjee *et al.*, 2011; Kowalczyk *et al.*, 2013), and can therefore be clustered postsynaptically. Conversely, $GABA_ARs$ containing the α4, α5 or α6 subunit are localised predominantly extrasynaptically (Nusser *et al.*, 1998; Chandra *et al.*, 2006; Panzanelli *et al.*, 2011). The α4 and α6 subunits – as well as the α1 subunit in specific interneurons (Mann & Mody, 2010; Milenkovic *et al.*, 2013) – are frequently associated with the δ subunit, which substitutes for the γ2 subunit (Box 3). However, extrasynaptic vs. postsynaptic targeting of $GABA_ARs$ appears to depend on motifs present in α subunit variants, as seen by comparing recombinant α2-$GABA_ARs$ and α6-$GABA_ARs$ containing either γ2 or δ subunits (Wu *et al.*, 2012).

The finding that targeted deletion of the γ2 subunit abolishes postsynaptic clustering of both $GABA_ARs$ and gephyrin highlighted the interdependence between receptors (rather than individual subunits) and the gephyrin scaffold in proper localisation at the cell surface (Essrich *et al.*, 1998; Schweizer *et al.*, 2003). Likewise, targeted deletion of *Gphn* (encoding gephyrin) confirmed its key role in postsynaptic clustering of GlyRs and $GABA_ARs$ (Feng *et al.*, 1998). These observations triggered intense research on the functions of gephyrin and the identification of proteins regulating $GABA_AR$ trafficking and GABAergic synapse formation [reviewed in Luscher *et al.* (2011a),

Fritschy *et al*. (2012), and Tretter *et al*. (2012)]. These molecules include, in particular, collybistin, a Rho-GEF identified as a direct binding partner of gephyrin, mediating its translocation to the cell surface (Kins *et al*., 2000), NL2, which interacts trans-synaptically with neurexin isoforms (Varoqueaux *et al*., 2004; Kang *et al*., 2008) (Fig. 3), and an array of proteins regulating $GABA_AR$ biogenesis, cell surface trafficking, endocytosis, and degradation (Jacob *et al*., 2008; Luscher *et al*., 2011a; Vithlani *et al*., 2011).

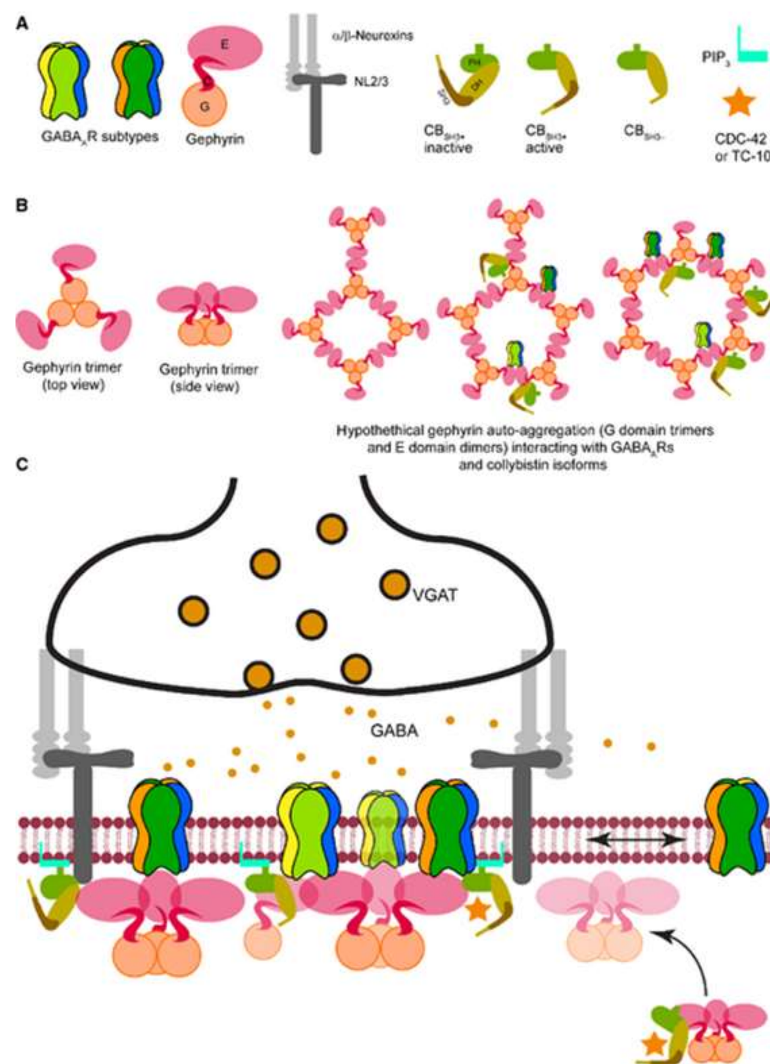

Figure 3. Schematic depiction of major postsynaptic proteins interacting with $GABA_ARs$ and their putative organisation in the PSD of a GABAergic synapse (see main text for details). (A) Schematic depiction of key molecules of GABAergic synapses. (B) Possible arrangement of gephyrin molecules, forming trimers as proposed from structural analysis (Sander *et al*., 2013), and models of the scaffolding assembly, to which $GABA_ARs$ and collybistin bind. (C) Basic molecular organisation of the GABAergic PSD, showing the presence of NL2 (interacting with presynaptic neurexin isoforms and with gephyrin), collybistin splice variants, in both active and inactive conformations, and interacting with the small GTPases CDC-42 and/or TC-10, in addition to gephyrin and $GABA_ARs$. The exact roles of collybistin, and its enzymatic activity, remain hypothetical; the scheme shows a proposed function for gephyrin submembrane targeting, along with possible effects within the PSD itself to facilitate recruitment of $GABA_ARs$ moving via lateral diffusion in the membrane. The size of each molecule is depicted roughly relative to its molecular mass. CB, collybistin; PIP_3, phosphatidylinositol 3,4,5-trisphosphate.

Gephyrin

This phylogenetically ancient, highly conserved multifunctional molecule is responsible throughout the kingdom of life for Moco biosynthesis (Stallmeyer *et al*., 1999). Moco activates molybdenum enzymes, whose functions are essential for survival (Schwarz *et al*., 2009). Gephyrin comprises two main catalytic domains (G and E), which, in bacteria, are encoded by two distinct genes (*MogA* and *MoeA*); in plants, the domains are fused, and in vertebrates, they are interconnected by a flexible linker (or central domain), which carries numerous sites for post-translational modifications and interaction with partner proteins [reviewed in Fritschy *et al*. (2008)]. The domain organisation of mammalian gephyrin not only ensures optimal Moco synthesis, but has also allowed the emergence of novel, neuron-specific functions for the regulation of GlyR and $GABA_AR$ clustering (Belaidi & Schwarz, 2013). Whereas the structure of gephyrin is not fully resolved, current evidence suggests the formation of trimers (Sander *et al*., 2013), which organise themselves in a highly ordered supramolecular complex anchoring $GABA_ARs$ in the plasma membrane and interacting with effector proteins (including NL2 and collybistin) and the cytoskeleton (Fig. 3) [reviewed in Tyagarajan & Fritschy (2014)].

Postsynaptic clustering of gephyrin by auto-aggregation at GABAergic (and glycinergic synapses) represents a process that is fundamentally different from scaffold formation in the PSD of glutamatergic synapses, where PSD-95 and its homologs assemble a modular scaffold and interact with their partners by means of the PDZ interaction domain (Kennedy, 2000; Sheng & Sala, 2001). In GABAergic PSDs, most proteins lack a PDZ domain; therefore, there must be molecular mechanisms specifically enabling gephyrin scaffold formation at postsynaptic sites. Identifying these mechanisms is complicated by the fact that biochemical purification of GABAergic PSDs has not been achieved so far, whereas glutamatergic PSDs are highly enriched in synaptosomal fractions (Husi & Grant, 2001). Therefore, there are, as yet, only limited proteomic data on inhibitory PSDs (Heller *et al*., 2012), and the possibility exists that major players involved in scaffold formation and regulation at GABAergic synapses have not yet been identified.

A major insight from recent studies was the finding that post-translational modifications of gephyrin, mainly via phosphorylation, regulate its clustering properties, and thereby the structural and functional properties of GABAergic synapses (Tyagarajan & Fritschy, 2014). This regulation is coupled to complex interactions with collybistin and NL2 (see below), contributing to GABAergic synapse formation and their dynamic regulation by activity-dependent mechanisms. Therefore, gephyrin emerges as a key element of a signaling hub regulating GABAergic function and plasticity in health and disease.

It is of note that postsynaptic gephyrin clustering is not an intrinsic property of this protein, but depends strictly on the presence of $GABA_AR$ subtypes with which gephyrin (and possibly additional proteins) can interact. Therefore, the loss of postsynaptic currents in α1-knockout (KO) and α2-KO mice also leads to disruption of gephyrin postsynaptic clustering (Fritschy *et al*., 2006; Kralic *et al*., 2006; Pallotto *et al*., 2012). Despite these crucial bidirectional interactions, gephyrin-independent $GABA_AR$ clustering at postsynaptic sites has been observed, both *in vitro* and *in vivo* (Kneussel *et al*., 2001; Levi *et al*., 2002; Panzanelli *et al*., 2011), pointing to alternative pathways regulating their synaptic localisation. One of these mechanisms might be afforded by the dystrophin–glycoprotein complex, which is present selectively in a subset of GABAergic synapses in cortical neurons and cerebellar Purkinje cells [reviewed in Fritschy *et al*. (2012)]. Indeed, we have shown in α2-KO mice that postsynaptic clusters of α1-$GABA_ARs$ and NL2 remain selectively associated with the dystrophin–glycoprotein complex, but not with gephyrin, in perisomatic synapses of CA1 pyramidal cells (Panzanelli *et al*., 2011). A possible molecular link holding these proteins together is provided by the synaptic scaffolding molecule, which interacts with both dystrophin and NL2 (Sumita *et al*., 2007). Interestingly, the cell adhesion molecule IgSF9b, which interacts with the synaptic scaffolding

molecule and NL2, has recently been shown to promote the formation of GABAergic synapses, in particular in cortical interneurons, where it is most strongly expressed (Woo *et al.*, 2013).

A recent study using super-resolution microscopy to analyse the molecular organisation of inhibitory PSDs provided a first quantification of the number of gephyrin molecules present per synapse, and demonstrated that the numbers of both GlyRs and $GABA_A$Rs at the synapse depend on gephyrin abundance (Specht *et al.*, 2013). A possible difference in the molecular organisation of these synapses emerged from the observation that silencing neuronal cultures with tetrodotoxin for 48 h affected the synaptic enrichment of $GABA_A$Rs, but not of GlyRs.

Box 3. ***Extrasynaptic $GABA_A$Rs***

Tonic inhibition, mediated by persistent activation of extrasynaptic $GABA_A$Rs, is an important determinant of neuronal excitability, and is increasingly being recognised as playing a key role in mediating the effects of neurosteroids, and contributing to the pathophysiology of major disease states [reviewed in Belelli *et al.* (2009), Gunn *et al.* (2011), and Brickley & Mody (2012)]. Although tonic inhibition is widely considered to reflect receptor activation by ambient GABA, this concept has been questioned by evidence that spontaneous opening of $GABA_A$Rs might contribute to most of the tonic currents that can be recorded in dentate gyrus granule cells (Wlodarczyk *et al.*, 2013). Extrasynaptic receptors containing the δ subunit have a very high affinity for GABA, and mediate most actions of neurosteroids, thus contributing to the regulatation of brain activity under circumstances when their synthesis is increased, including stress, delivery, and ethanol intoxication (Sarkar *et al.*, 2011; Carver & Reddy, 2013). These receptors are selectively modulated by the super-agonist gaboxadol (but are insensitive to classic benzodiazepine agonists) (Mortensen *et al.*, 2010). Until recently, it was unclear how the expression and cell surface expression of extrasynaptic receptors is regulated. Evidence now indicates that tonic inhibition in the dentate gyrus and thalamus is modulated by protein kinase A and protein kinase C (PKC) activity (targeting α4-$GABA_A$R) (Connelly *et al.*, 2013a), e.g. upon stimulation of $GABA_B$Rs (Connelly *et al.*, 2013b; Tao *et al.*, 2013). Specifically, PKC-mediated phosphorylation of Ser443 in the α4 subunit was shown to enhance the cell surface expression and activity of these receptors (Abramian *et al.*, 2010). However, another study contended that PKC activation reduces tonic inhibition in the thalamus by targeting the β2 subunit (Bright & Smart, 2013). As tonic inhibition is a major determinant of neuronal excitability, these data indicate novel, albeit contradictory, mechanisms that potentially have major effects on network activity.

Besides α4/β/δ receptors, α5-$GABA_A$Rs (most probably composed of α5, β3 and γ2 subunits) also form a prominent population of extrasynaptic receptors in the hippocampal formation, olfactory bulb, and cerebral cortex. These receptors are modulated by diazepam, but are insensitive to zolpidem. Interest in these receptors was triggered by the observations in α5(H105R) mutant mice (carrying diazepam-insensitive α5-$GABA_A$Rs; see Box 2) that they do not develop tolerance to the sedative (i.e. motor-impairing) action of diazepam (van Rijnsoever *et al.*, 2004). Furthermore, α5(H105R) mutant mice showed an approximately 30% reduction in α5-$GABA_A$Rs but showed improved performance in a hippocampus-dependent memory task (trace fear conditioning) as compared with wild-type mice (Crestani *et al.*, 2002). This observation opened the tantalising perspective that reducing the function of α5-$GABA_A$Rs might be exploited to reverse disease-related deficits in cognition and memory performance, and triggered the search for inverse agonists

acting selectively at these receptors [reviewed in Rudolph & Möhler (2013)]. However, a more systematic analysis of α5(H105R) mutant mice revealed increased basal locomotion and altered memory for the location of objects, indicative of hippocampal dysfunction (Prut *et al.*, 2010). Therefore, the reduced expression of α5-$GABA_AR$ induces complex bidirectional changes in behavioral performance in these mutants.

Other receptors, presumably containing the γ2 subunit, as revealed by their sensitivity to diazepam, are located extrasynaptically and mediate tonic inhibition. They include, in particular, α3-$GABA_ARs$ in the basolateral amygdala (Marowsky *et al.*, 2012) and the inferior olivary nucleus (Devor *et al.*, 2001). The rules governing the extrasynaptic localisation of these receptors are not understood. The majority of α3-$GABA_ARs$, notably in thalamic reticular neurons or in hippocampal or cerebellar interneurons, form postsynaptic clusters associated with gephyrin (Studer *et al.*, 2006; Schneider Gasser *et al.*, 2007; Notter *et al.*, 2013). Whereas interactions with gephyrin are thought to be crucial for postsynaptic receptors, other mechanisms, remaining to be explored, might supersede them to determine (and maintain) α3-$GABA_ARs$ at extrasynaptic sites in specific neuron populations. A similar dichotomy exists for α5-$GABA_ARs$, which are not strictly extrasynaptic in the hippocampal formation (Serwanski *et al.*, 2006); those located extrasynaptically were shown to interact with radixin, a phosphoprotein belonging to the ezrin–radixin–meosin protein family and interacting with the actin cytoskeleton (Loebrich *et al.*, 2006).

Collybistin

Collybistin is a neuron-specific guanine nucleotide exchange factor that activates the small Rho GTPases CDC-42 and TC-10 (Mayer *et al.*, 2013), and binds gephyrin at an identified site. Loss of $GABA_AR$ and gephyrin clustering occurs in a cell type-specific manner in collybistin-KO mice, showing the essential function of this protein at GABAergic synapses (Papadopoulos *et al.*, 2007, 2008). The effect can be reproduced in cultured neurons upon overexpression of a collybistin isoform that is unable to interact with membrane phospholipids (via its PH domain) (Reddy-Alla *et al.*, 2010; Tyagarajan *et al.*, 2011a). In these experiments, expression of constitutively active CDC-42 restored gephyrin clustering, indicating that it probably operates downstream of collybistin (Tyagarajan *et al.*, 2011a). However, elucidating the function(s) of collybistin and its effectors is complicated by the existence of collybistin splice variants, carrying or lacking an N-terminal SH3 domain ($collybistin_{SH3+}$ and $collybistin_{SH3-}$, respectively), because $collybistin_{SH3+}$ has been reported to be an inactive form that needs to be activated in order to contribute to gephyrin and $GABA_AR$ clustering (Poulopoulos *et al.*, 2009). The nature of this activation presumably involves a conformational change that enhances binding of the PH domain to membrane phospholipids (Fig. 3). It has been proposed, for example, that binding of the $GABA_AR$ α2 or α3 subunit to collybistin, in conjunction with gephyrin, facilitates gephyrin cluster formation (Saiepour *et al.*, 2010). Likewise, binding of NL2 to the SH3 domain was suggested to activate collybistin and thereby enable gephyrin clustering (Poulopoulos *et al.*, 2009). However, targeted deletion of the NL2 gene (*NLGN2*) does not abolish gephyrin clustering, and collybistin overexpression in neurons strongly stimulates gephyrin clustering independently of the presence or absence of the SH3 domain (Chiou *et al.*, 2011; Tyagarajan *et al.*, 2011a), suggesting alternative mechanisms. Among these, the small GTPase TC-10 was recently shown to activate collybistin upon interaction with the PH domain, thereby enhancing gephyrin clustering in cultured neurons (Mayer *et al.*, 2013).

NL2

There are four neuroligin isoforms encoded by distinct genes (*NLGN1–NLGN4*) (Bolliger *et al.*, 2001). These proteins, anchored postsynaptically by means of a single transmembrane domain, interact with presynaptic neurexins. This interaction is strongly synaptogenic, even in non-neuronal cells, upon overexpression *in vitro* (Scheiffele *et al.*, 2000; Graf *et al.*, 2004; Chih *et al.*, 2005). NL2 is selectively located at GABAergic synapses (Varoqueaux *et al.*, 2004) and neuroligin 4 at glycinergic synapses (Hoon *et al.*, 2011) [but see Soto *et al.* (2011)], whereas neuroligin 1 is selective for glutamatergic synapses (Song *et al.*, 1999), and neuroligin 3 is found in both glutamatergic and GABAergic synpases (Budreck & Scheiffele, 2007). Neuroligins have drawn considerable attention because they are associated with autism-spectrum disorders and other forms of mental retardation [reviewed in Südhof (2008)], as well as schizophrenia (Sun *et al.*, 2011). Their specific *in vivo* role is not yet fully elucidated, in part because of functional redundancy with other synaptogenic molecules (Varoqueaux *et al.*, 2006), and because their functions (and localisation) are modulated by complex interactions with neurexin isoforms (Futai *et al.*, 2013), post-translational modifications (Peixoto *et al.*, 2012; Suzuki *et al.*, 2012; Giannone *et al.*, 2013), homodimerisation, and heterodimerisation (Poulopoulos *et al.*, 2012; Shipman & Nicoll, 2012). In particular, it is not known whether neuroligins interact directly with $GABA_A$Rs. Nevertheless, NL2-KO mice show a specific reduction in the number of perisomatic GABAergic synapses in principal neurons of the hippocampal formation, associated with a decrease in inhibitory currents and increased network excitability (Jedlicka *et al.*, 2011).

It should be noted that, whereas NL2 is generally considered to be upstream in the chain of molecular events leading to the formation of GABAergic synapses (Dong *et al.*, 2007; Poulopoulos *et al.*, 2009), *in vitro* evidence indicates that overexpression of $GABA_A$R alone in non-neuronal cells (lacking collybistin and, presumably, gephyrin) is sufficient to generate functional contacts, generating inhibitory postsynaptic currents (Fuchs *et al.*, 2013).

Functional regulation of $GABA_A$R subtypes: significance for GABAergic synapse plasticity

$GABA_A$Rs are regulated by ubiquitous transcriptional and post-translational processes, as well as by multiple protein–protein interactions. As discussed above with regard to their interactions with gephyrin, there is considerable subtype specificity in the regulation of $GABA_A$Rs, which allows neurons expressing several receptor subtypes to make fine, synapse-specific adjustments in response to a large array of extrinsic and intrinsic signals. Here, we will discuss the regulation of $GABA_A$R subtypes in mature neurons, focusing on four major aspects.

Transcriptional control of $GABA_A$R subunit expression

The gene structure and chromosomal localisation of human (and rodent) $GABA_A$Rs are well established (Simon *et al.*, 2004), and their promoter sequences and binding sites for transcription factors and regulatory elements are being subjected to intense scrutiny [reviewed in Steiger & Russek (2004)]. However, little is known about how the subunit repertoire of any given neuron is determined during development, although models explaining the coordinated expression of subunits located in gene clusters (e.g. β2–α1–γ2–α6) have been proposed (Uusi-Oukari *et al.*, 2000; Joyce, 2007). Remarkable variability in the abundance of mRNAs encoding the 19 $GABA_A$R subunits has been found in both mice and humans, with considerable regional specificity, and being under the control of multiple gene regulatory mechanisms (Mulligan *et al.*, 2012). In addition, there is strong evidence

for transcriptional regulation of $GABA_AR$ subunits by neurosteroids, as well as in a number of pathological conditions, including epilepsy, ethanol intoxication, Alzheimer's disease, and schizophrenia [reviewed in Steiger & Russek (2004) and Grabenstatter *et al.* (2012)]. A recent genetic study identified a chromosomal duplication in a locus encoding four $GABA_AR$ subunits (4p12: α2, α4, β1, and γ1) that is associated with neurodevelopmental disorders (Polan *et al.*, 2013). In contrast, it is not well established whether the compensatory increase in subunit expression observed in some $GABA_AR$ subunit-KO mice (Box 1) reflects transcriptional control or is attributable to changes in mRNA stability and/or post-translational mechanisms (Peng *et al.*, 2002; Kralic *et al.*, 2006; Ogris *et al.*, 2006). The issue is of relevance, because these compensatory changes contribute to the maintenance of homeostatic balance between excitation and inhibition in the mutant mice. Transcriptional control of $GABA_AR$ subunit expression would probably involve activity-dependent mechanisms targeting specific transcription factors (or possible non-coding RNAs), and would need to deal with the regulation of local translation in dendrites (Cajigas *et al.*, 2012).

Post-translational modifications of $GABA_ARs$

Membrane anchoring of $GABA_ARs$ is regulated by palmitoylation of the γ2 subunit, and this mechanism contributes to the normal formation and function of GABAergic synapses (Fang *et al.*, 2006). In addition, it is well established that multiple phosphorylation mechanisms, targeting various $GABA_AR$ subunits, play a key role in modulating the efficacy of GABAergic transmission, either by changing single-channel gating or kinetic properties, or by regulating stability, cell surface delivery, or internalisation of $GABA_ARs$ [reviewed in Jacob *et al.* (2008), Houston *et al.* (2009), Luscher *et al.* (2011a), and Vithlani *et al.* (2011)]. Combined with recent reports that gephyrin phosphorylation at Ser268 and Ser270 by glycogen synthase kinase-3β and extracellular signal-related kinase, respectively, is a negative regulator of GABAergic transmission (Tyagarajan *et al.*, 2011b, 2013; Rui *et al.*, 2013), these data indicate that multiple signaling pathways can dynamically modulate neuronal excitability by activating protein kinases or phosphatases, as well as their downstream effectors. As phosphorylation events on $GABA_ARs$ are subunit-specific, differential effects can be expected for various $GABA_AR$ subtypes, even within the same neuron. Furthermore, considering the tight functional coupling between the gephyrin scaffold and postsynaptic $GABA_ARs$, the question arises of whether phosphorylation of gephyrin and $GABA_ARs$ is coordinated. As gephyrin carries multiple consensus sites for phosphorylation (and other post-translational modifications, such as acetylation and SUMOylation) (Tyagarajan & Fritschy, 2014), the response to this question awaits their further characterisation. In addition, it is conceivable that the gephyrin scaffold serves to anchor protein kinases (and phosphatases) acting on $GABA_ARs$, and therefore regulates the efficacy of receptor post-translational modifications.

A major advance in our understanding of the *in vivo* significance of $GABA_AR$ phosphorylation is provided by the generation of knock-in mice carrying point mutations that abolish the phosphorylation of residues known to be targeted by protein kinases *in vitro*. However, these studies have shown unexpectedly strong effects of the mutations, with the γ2 Y365F/Y367F mutation being embryonically lethal, and inducing in heterozygous mice sex-specific increases in tonic inhibition (α4/δ-$GABA_ARs$) to compensate for reduced neurosteroid sensitivity in the thalamus (Jurd & Moss, 2010; Nani *et al.*, 2013).

Regulation of $GABA_AR$ trafficking and cell surface diffusion

Membrane insertion (and internalisation) of $GABA_ARs$ occurs at extrasynaptic sites, followed by lateral diffusion and reversible trapping in the postsynaptic membrane (Bogdanov *et al.*, 2006), suggesting dynamic regulation of phasic inhibition from a reserve pool of extrasynaptic receptors (Thomas *et al.*, 2005). Furthermore, preventing docking of $GABA_ARs$ at endocytotic zones in the

plasma membrane – by interfering with a binding motif located in the intracellular loop of the β3 subunit – blocked $GABA_AR$ internalisation, as well as downregulation following oxygen–glucose deprivation, which is a model of ischemia *in vitro* (Smith *et al*., 2012). Such observations underscore the fact that regulated diffusion of $GABA_ARs$ in the plasma membrane is of prime relevance under physiological and pathophysiological conditions.

Single-particle tracking studies have also revealed that $GABA_AR$ mobility at the cell surface and at postsynaptic sites is tightly regulated by activity-dependent mechanisms and differential interactions with gephyrin (Bannai *et al*., 2009; Shrivastava *et al*., 2011; Niwa *et al*., 2012). Thus, postsynaptic and extrasynaptic $GABA_ARs$ have similar diffusion rates in the plasma membrane, but the former remain confined in GABAergic postsynaptic sites for longer, and their trapping depends on the presence of the gephyrin scaffold (Mukherjee *et al*., 2011; Renner *et al*., 2012). Increasing synaptic activity, leading to Ca^{2+} influx, reduced the amplitude of miniature inhibitory postsynaptic currents (mIPSCs), owing to dispersion of $GABA_ARs$ to extrasynaptic sites by a mechanism involving the protein phosphatase calcineurin (Bannai *et al*., 2009). Recent evidence indicates, however, that this activity-dependent Ca^{2+} influx also affects the gephyrin scaffold, although dispersion of $GABA_ARs$ and dispersion of gephyrin occur on different time scales, partially independently of each other (Niwa *et al*., 2012). Together with previous evidence (Muir *et al*., 2010), these results indicate that cross-talk between excitatory and inhibitory transmission occurs via the activation of Ca^{2+}-dependent signaling events that impinge on both $GABA_ARs$ and the postsynaptic scaffold. Much remains to be determined about how these phenomena observed *in vitro* are mediated *in vivo*, and whether they selectively affect specific $GABA_AR$ subtypes in cortical principal cells or cause global effects across various types of synapse.

Regulation of $GABA_AR$-mediated transmission by neurotrophins and metabolic factors

Neurotrophins, such as brain-derived neurotrophic factor (BDNF), have major effects on excitatory synaptic plasticity, mediated by multiple signaling pathways downstream of TrkB. It is therefore no surprise that BDNF also regulates the strength of $GABA_AR$-mediated transmission, by acting on both GABAergic synapse formation (Chen *et al*., 2011) and plasticity: BDNF produces bi-phasic effects on $GABA_AR$-mediated transmission, reflecting its action on cell surface expression (Brünig *et al*., 2001; Jovanovic *et al*., 2004). In a recent study, the possibility has been raised that BDNF acutely causes internalisation of α1-$GABA_ARs$ in the amygdala by causing rapid gephyrin degradation (Mou *et al*., 2013). However, long-lasting enhancement of $GABA_AR$-mediated transmission in the hippocampus, owing to enhanced cell surface expression, has been shown to arise from phosphorylation of Tyr657 and Tyr367 in the γ2 subunit (Vithlani *et al*., 2013); the significance of this modulation, as tested in γ2(Y365F/Y367F) knock-in mice, is an anti-depressant behavioral phenotype and increased hippocampal neurogenesis, raising the possibility that $GABA_AR$ phosphorylation regulates the anti-depressant action of BDNF.

We have recently uncovered a novel mode of $GABA_AR$ regulation, activated by reactive oxygen species (ROS), and therefore by cellular metabolism (Accardi *et al*., 2014). In this study, blocking the mitochondrial respiratory chain or elevating intracellular ROS levels in cerebellar interneurons caused a gradual increase in the frequency of mIPSCs, owing to the appearance of additional low-amplitude currents with slow decay kinetics. Whereas the majority of mIPSCs in these cells are mediated by α1-$GABA_ARs$, these newly induced currents had the kinetic properties of α3-$GABA_ARs$, and, indeed, depended on expression of the α3 subunit in these cells, as tested in α3-KO mice (whereas deletion of the α1 subunit had no effect on this phenomenon). Along with evidence that the effects of ROS elevation are attributable to postsynaptic adaptations, rather than presynaptic changes in transmitter release, these results suggested that ROS activate a signaling cascade leading to the selective recruitment of α3-$GABA_ARs$ to either 'silent' synapses, or to synaptic contacts formed *de novo* from terminals known to form multiple release sites (Accardi *et al*., 2014). In line with these

results, insulin acutely increases $GABA_AR$ cell surface expression *in vitro* (Wan *et al*., 1997), but it has not been established whether the same mechanism is involved.

Significance for GABAergic synapse plasticity

The main conclusion derived from studies of $GABA_AR$ (and gephyrin) post-translational regulation is that GABAergic synapses represent dynamic entities regulated by multiple mechanisms to homeostatically adjust the responsiveness and function of neuronal networks according to changes in their environment. These regulatory adjustments concern both postsynaptic and extrasynaptic receptors, and involve multiple intracellular signaling cascades. We have speculated elsewhere that, by means of its role as scaffolding protein, gephyrin might interact with various effectors to adjust the structure and function of GABAergic synapses over a considerable dynamic activity range (Tyagarajan & Fritschy, 2014), thereby ensuring homeostatic synaptic plasticity in mature neuronal circuits.

Post-translational regulation of $GABA_ARs$ (and gephyrin) is implicated in the functional plasticity of GABAergic synapses, as demonstrated in several systems [reviewed in Kullmann *et al*. (2012)]. For example, a well-studied model is rebound potentiation at inhibitory synapses of cerebellar Purkinje cells, which is induced by depolarisation of Purkinje cells, and involves Ca^{2+} influx and activation of Ca^{2+}/calmodulin kinase 2 (Kano *et al*., 1996). Rebound potentiation involves trafficking of $GABA_ARs$ to enhance their surface expression, and is necessary for adaptation of the vestibule-ocular reflex, a form of Purkinje cell-dependent motor learning (Kawaguchi & Hirano, 2007; Tanaka *et al.*). In addition to functional plasticity, regulation of $GABA_ARs$ also contributes to structural plasticity by inducing changes in GABAergic synaptic connectivity. For example, chronic treatment of Ts65Dn mouse mutants, a model of Down syndrome, with a selective α5-$GABA_AR$ negative allosteric modulator reduced the density of GABAergic synapses in the hippocampal formation and normalised behavior (Martínez-Cué *et al*., 2013).

$GABA_AR$ heterogeneity in times of change: CNS development and adult neurogenesis

Developmental changes in $GABA_AR$ subunit expression

$GABA_ARs$ are expressed at early stages of fetal brain development by neural precursor cells and during neuronal differentiation, and have been proposed to regulate cell proliferation, migration, and differentiation, possibly through Ca^{2+}-mediated signals activated by neuronal depolarisation [reviewed in Represa & Ben-Ari (2005), Cellot & Cherubini (2013), and Lu *et al*. (2014)] (Box 4). Accordingly, one might expect developmental deficits in the CNS of $GABA_AR$ subunit-KO mice, notably with regard to subunits that are highly expressed in fetal brain. However, among the targeted deletions analysed so far (α1–α6, β2, β3, δ, and γ2), no detectable alterations in brain general architecture at birth have been reported, suggesting the existence of compensatory mechanisms substituting for the missing receptor subtype. Therefore, the absence of phenotype in these mutant mice should not be taken as evidence that $GABA_ARs$ are dispensable for regulating brain development. Rather, they might be so important that functional redundancy has been developed to prevent deleterious effects in the case of dysfunction of a given subtype.

Box 4. ***GABA$_A$R-mediated 'excitation'***

As GABA$_A$Rs are selectively permeable for Cl^- and HCO_3^- (Kaila, 1994), there is now a large consensus that elevation of the intracellular concentration of either species might result in a depolarising current upon GABA$_A$R activation, and thereby potentially 'excite' this neuron (Blaesse *et al*., 2009). There is ample evidence for GABA$_A$R-mediated depolarisation of immature (and mature) neurons and NG2 cells, leading to Ca^{2+} influx, opening the door to speculation about the role of Ca^{2+} as a second messenger in these cells, in particular to control the cell cycle and differentiation mechanisms (Tanaka *et al*., 2009; Merz *et al*., 2011; Young *et al*., 2012). It has also been proposed that GABA$_A$R-mediated excitation precedes (and is replaced by) glutamatergic transmission during the maturation of cortical neurons (Hennou *et al*., 2002), and that 'excitatory' GABA drives giant depolarising potentials, which are network phenomena thought to contribute to proper axonal wiring of the developing CNS [reviewed in Dehorter *et al*. (2012)].

The reality of GABA$_A$R-mediated excitation *in vivo* has been much debated, as have its functional significance and the main ion species responsible for it. In particular, there is often confusion about the excitatory effects of depolarising GABA, considering that one of the main effects of GABA$_A$R activation is a net increase in membrane conductance, opposing the depolarising effect of positive charge influx induced by any other neurotransmitter. Therefore, whereas a neuron can be depolarised by GABA, this does not necessarily translate into it being 'excited'.

The prevalent view is that GABA-induced depolarisation is attributable to high expression of the co-transporter NKCC1 and low expression of KCC2, which exert opposing actions on intracellular Cl^-. Nevertheless, in mature neurons (expressing high levels of KCC2), intense GABA$_A$R stimulation can lead eventually to neuronal depolarisation caused by KCC2-mediated K^+ efflux (Viitanen *et al*.,2010). This bi-phasic response, which involves short-lasting changes in the ionic driving force of GABA$_A$R and reduces the efficacy of diazepam (Deeb *et al*., 2013), has been coined 'short-term ionic plasticity' (Raimondo *et al*., 2012). Despite the importance of NKCC1 and KCC2 for regulating GABA function, in particular under pathological conditions such as epilepsy and chronic pain, little is known about their precise subcellular localisation and functional regulation. Evidence is now emerging that transcriptional and post-translational mechanisms, involving, among other factors, BDNF signaling, have major impacts on the availability and cell surface expression of KCC2 (Yeo *et al*., 2009; Lee *et al*., 2011; Puskarjov *et al*., 2012; Chamma *et al*., 2013; Sun *et al*., 2013). Furthermore, it is now being recognised that, besides NKCC1 and KCC2, the developmental maturation of carbonic anhydrases is a major determinant of the driving force of GABA$_A$Rs in the immature brain (Rivera *et al*., 2005). Thus, the absence of carbonic anhydrase (upon targeted gene deletion) enhances the depolarising action of GABA and induces seizures in neonatal mice (Ruusuvuori *et al*., 2013). Finally, it should be emphasised that, despite initial speculations that the depolarising and hyperpolarising effects of GABA might be mediated by distinct GABA$_A$R subtypes, there is no evidence supporting this possibility. These speculations were triggered, in part, by observations that the subunit composition of major GABA$_A$R subtypes changes drastically, in particular in the neocortex and thalamus, during the phase of synaptogenesis (see main text). However, this subunit switch appears to be unrelated to GABA depolarisation, and its significance remains a matter of speculation.

It is well established that the subunit composition of the predominant $GABA_AR$ subtypes changes during the period of synaptogenesis, accounting in part for the distinct functional and pharmacological properties of $GABA_ARs$ in neonatal and mature brain (Fritschy *et al.*, 1994; Paysan *et al.*, 1997; Hutcheon *et al.*, 2000; Bosman *et al.*, 2002; Fagiolini *et al.*, 2004; Peden *et al.*, 2008; Hashimoto *et al.*, 2009). Nevertheless, there is only fragmentary information on the expression pattern of $GABA_AR$ subunits in neural precursor cells and immature neurons. mRNA *in situ* hybridisation data available in the Allen Brain Atlas (http://mouse.brain-map.org) show a predominance of α2, α3, α5, β2, β3 and γ2 subunits at embryonic day 18.5. As this stage precedes synaptogenesis, one might assume that these receptors are mainly engaged in tonic, possibly depolarising, GABAergic transmission. In particular, the α5 subunit is strongly expressed perinatally, and its expression decreases during synaptogenesis, except in regions where it remains abundant in the adult brain (hippocampal formation, olfactory bulb, and brainstem). It is of note that downregulation of the α5 subunit in layer 4 of the neocortex and its replacement by the α1 subunit have been shown to be dependent on the integrity of thalamocortical projections (Paysan *et al.*, 1997), providing an attractive model with which to study the mechanisms underlying transcriptional and post-transcriptional control of subunit expression. In contrast to the α5 subunit, other subunits defining the main extrasynaptic $GABA_ARs$ of the adult brain (α4, α6, and δ) are absent or expressed at low levels in the fetal brain, and are upregulated when neuronal maturation nears completion. In the human neocortex, the GABAergic system develops during the second half of pregnancy and in infancy (Xu *et al.*, 2011), and the expression of $GABA_AR$ subunit genes appears to be coordinated on the basis of their respective chromosomal localisations (Fillman *et al.*, 2010), with distinct upregulation and downregulation patterns suggestive of differential expression of specific $GABA_AR$ subtypes.

To elucidate why the pattern of $GABA_AR$ subunit expression evolves during ontogeny, considerable attention has been given to α1-$GABA_ARs$, which are upregulated in a region-specific manner during the phase of synaptogenesis to become the predominant $GABA_AR$ subtype present in the adult CNS (Fritschy *et al.*, 1994; Paysan *et al.*, 1994; Hashimoto *et al.*, 2009). As these receptors typically show fast decay kinetics and generate large-amplitude events (Eyre *et al.*, 2012), their gradual appearance was taken as evidence for maturation of GABAergic function (Vicini *et al.*, 2001; Bosman *et al.*, 2002), in particular to endow postsynaptic neurons with fast-acting receptors matching the high firing rate of certain interneurons. Furthermore, as discussed in the next paragraph, α1-$GABA_ARs$ on perisomatic synapses of cortical pyramidal cells enable critical windows of plasticity (Fagiolini *et al.*, 2004). Similar, but reversible, changes in subunit expression have been proposed to account for the plasticity of GABAergic transmission in the supraoptic nucleus during gestation and after delivery (Brussaard *et al.*, 1997). However, there is evidence from several electrophysiological studies that the acceleration of mIPSC kinetics occurring during brain development or following hormonal fluctuations does not depend solely on the subunit composition of $GABA_ARs$ (Koksma *et al.*, 2003, 2005; Peden *et al.*, 2008). Rather, post-translational modifications mechanisms affecting $GABA_AR$ gating properties, and possibly their trafficking and interactions with scaffolding proteins, might also play a role in defining their gating properties.

To fully understand how differential expression of $GABA_ARs$ shapes the functional properties of GABAergic transmission, it will be essential to also determine the developmental $GABA_AR$ expression profile in interneurons, as these receptors will have a key role in the maturation and determine the firing properties of input cells controlling synaptic circuits.

$GABA_AR$ subtype setting critical periods of plasticity

The seminal observation that the time of opening of critical period windows (during which sensory deprivation causes lasting structural and functional alterations) can be delayed or advanced by reducing or enhancing GABAergic transmission provided direct evidence for the fundamental role played by $GABA_AR$-mediated transmission in regulating cortical development (Hensch *et al.*, 1998;

Fagiolini & Hensch, 2000). Further investigations unambiguously showed that this effect requires a highly specific cortical circuit (Katagiri *et al*., 2007), involving a central role for large parvalbumin-positive basket cells, which control the output of principal cells by activating α1-$GABA_A$Rs [reviewed in Hensch (2005)]. Evidence for the key role played by these receptors was provided by the demonstration that diazepam is unable to advance the opening of a critical period window in α1(H101R) mice (Fagiolini *et al*., 2004).

GABA is not the only neurotransmitter involved in this process, as modulation of nicotinic acetylcholine receptors by targeted deletion of Lynx1, a membrane-anchored prototoxin that negatively regulates nicotinic acetylcholine receptor function (Ibañez-Tallon *et al*., 2002), allows re-opening of critical period windows in adulthood; remarkably, this effect of Lynx1 deletion can be blocked by co-application of diazepam, demonstrating that a balance between excitation and inhibition, rather than the action of a single transmitter, is the determinant for setting the opening and closing of critical period windows (Morishita *et al*., 2010).

The relevance of parvalbumin-positive basket cells in setting network configurations that are permissive for structural and functional plasticity, and that are required for learning and memory acquisition, is not restricted to critical period windows, but appears to be a fundamental principle of brain plasticity (Donato *et al*., 2013), involving a canonical pattern of interconnections between interneurons (Pfeffer *et al*., 2013). According to these experiments, inhibitory control of parvalbumin-positive basket cells by vasoactive intestinal peptide-positive interneurons is low in mice exposed to conditions permissive for learning (e.g. enriched environment) and high when either a new task is acquired or when adverse conditions (e.g. fear conditioning) lead to memory retention. Remarkably, the maturation and strength of inhibitory control of parvalbumin-positive basket cells is regulated by the transcription factor Otx2. This secreted molecule requires binding to a specific receptor in perineuronal nets – which selectively surround parvalbumin-positive interneurons – for cell penetration and activation of gene transcription (Beurdeley *et al*., 2012).

Taken together, these results underscore the fact that GABAergic transmission in the developing brain (and during permissive phases of plasticity that are critical for learning and circuit refinement) is regulated by sophisticated mechanisms, and mediated by specific circuits containing defined $GABA_A$R subtypes, such as α1-$GABA_A$Rs in synapses formed on principal cells by parvalbumin-positive basket cells.

Regulation of adult neurogenesis

By analogy with brain development, $GABA_A$Rs expressed by stem cells, neural precursor cells and immature neurons contribute to the proliferation, migration, differentiation and synaptic integration of adult-born neurons [reviewed by Overstreet *et al*. (2005), Ge *et al*. (2007), Sernagor *et al*. (2010), and Nissant & Pallotto (2011)]. Likewise, as in developing neurons, GABA initially exerts depolarising effects on precursor cells (Box 4), activating Ca^{2+}-dependent mechanisms that have enduring effects on precursor cell migration, cell survival, and subsequent neuronal maturation (Overstreet *et al*., 2005; Ge *et al*., 2006; Jagasia *et al*., 2009; Chancey *et al*., 2013).

Adult neurogenesis, taking place in the subventricular zone of the lateral ventricles and in the subgranular zone of the dentate gyrus, represents an important facet of brain plasticity regulated by GABAergic mechanisms. Experimentally, it offers an attractive paradigm for investigating the role of $GABA_A$R-mediated regulation of neuronal maturation and functional integration into pre-existing synaptic circuits. Thus, analysis of the consequences of $GABA_A$R inactivation in radial glia-like stem cells revealed that local parvalbumin-positive fast-spiking interneurons in the dentate gyrus determine their mode of division (symmetric vs. asymmetric) as well as neuronal vs. glial fate (Song *et al*., 2012); furthermore, the pharmacological profile of these receptors (zolpidem-insensitive) is compatible with expression of α5-$GABA_A$R in these stem cells. In relation to this, investigating the

role of extrasynaptic (α4 and δ) and postsynaptic (α2) $GABA_A$Rs inactivated by gene targeting, we have provided evidence that these $GABA_A$R subtypes regulate distinct phases of adult neurogenesis in the dentate gyrus (Duveau *et al*., 2011). In line with findings that GABA exerts negative control on neural precursor cell proliferation (Platel *et al*., 2007), α4-KO mice showed increased neurogenesis. No phenotype was seen in δ-KO mice, as expected from the delayed expression of this subunit during ontogeny [but see Whissell *et al*. (2013)]. Ablation of α4-$GABA_A$Rs also impaired dendritic growth and the final positioning of adult-born granule cells; in contrast, α2-KO newborn neurons showed delayed pruning of dendritic branches, presumably to maintain inhibitory–excitatory balance upon maturation of glutamatergic inputs (Duveau *et al*., 2011).

A more dramatic phenotype was observed upon selective α2 subunit inactivation in adult-born olfactory bulb granule cells (Pallotto *et al*., 2012), which represent the main interneuron subtype of the olfactory bulb, being continuously generated from mitotic precursor cells in the subventricular zone (Carleton *et al*., 2003). As α2-$GABA_A$Rs provide most synaptic inhibition to these cells, their inactivation profoundly altered dendritic development, spine formation, and the maturation of glutamatergic inputs. Also, modulation of dendritic differentiation by environmental enrichment or deprivation, which is prominent in wild-type adult-born granule cells (Saghatelyan *et al*., 2005), was abrogated in adult-born α2-KO granule cells (Pallotto *et al*., 2012). The severity of these effects underscores the central role played by $GABA_A$Rs in regulating neuronal differentiation. Moreover, these results, taken together, provide an exquisite demonstration that $GABA_A$R subtypes are specialised for specific tasks, with considerable spatio-temporal specificity.

Significance for CNS disorders

Elucidation of the molecular organisation and regulation of $GABA_A$R subtypes opens new perspectives for understanding pathophysiological mechanisms in neurological and psychiatric diseases, and for developing treatment approaches that go beyond symptomatic relief. Key aspects of the possible involvement of $GABA_A$R-mediated transmission in the pathophysiology of CNS disorders lie in their contribution to developmental processes and dependence on Cl^- and HCO_3^- fluxes (Box 4). In turn, $GABA_A$R dysfunction can be determined genetically, and/or depend on abnormal regulation and trafficking. Here, we briefly discuss these mechanisms in the light of a few selected examples.

$GABA_A$R subunit mutations are typically associated with generalised, mostly idiopathic epilepsies and Dravet syndrome. They have been described in α, β3, γ2 and δ subunits. Although the data are somewhat controversial, the majority of these mutations impair the trafficking and cell surface expression of $GABA_A$Rs, as well as their diffusion dynamics in the plasma membrane, and hence their postsynaptic clustering (Bouthour *et al*., 2012) [reviewed in Galanopoulou (2010) and Macdonald *et al*. (2010)]. Some of these effects have been proposed to be temperature-dependent, hence providing a plausible cause of febrile seizures. However, the general picture emerging from these studies is that $GABA_A$R mutations cause multiple molecular and biochemical alterations that are not easily related to specific symptoms of disease, notably epileptogenesis and seizure occurrence, thereby reflecting the complexity of the mechanisms underlying epileptic syndromes.

$GABA_A$R mutations have also been associated with other CNS pathologies. For example, although the complex interactions between ethanol intoxication or ethanol dependence and $GABA_A$Rs go beyond the scope of this review, it is worth mentioning that a dominant point mutation in the β1 subunit (L285R), which causes spontaneous channel opening and strongly enhances tonic inhibition in the nucleus accumbens, was shown recently to induce severe spontaneous ethanol consumption in mice (Anstee *et al*., 2013). In the same study, the selective contribution of β1-containing $GABA_A$Rs

was confirmed by a second mutation, which resulted in a similar behavioral phenotype. These data underscore the main contention of this review, namely how dysfunction of specific $GABA_AR$ subtypes, affecting a minor subpopulation of receptors, can cause strong behaviorally relevant effects.

Brain lesions, such as stroke or temporal lobe epilepsy with hippocampal sclerosis, lead to pathological alterations in GABAergic tonic inhibition, owing to overexpression or a reduction in expression of specific $GABA_AR$ subtypes [reviewed in Hines *et al*. (2011), Grabenstatter *et al*. (2012), and Houser *et al*. (2012)]. In a seminal study investigating the relevance of tonic inhibition in stroke, Clarkson *et al*. showed that reducing tonic inhibition in the peri-infarct area, a zone that is of crucial importance for functional recovery, with infusion of a benzodiazepine inverse agonist, or by genetically reducing the expression of extrasynaptic $GABA_ARs$, promoted functional recovery (Clarkson *et al*., 2010). These data are well in line with evidence that GABAergic transmission regulates neuronal plasticity by setting the inhibitory–excitatory balance in neuronal networks.

$GABA_AR$-mediated transmission during brain development has been linked to the emergence of neurodevelopmental disorders, as well as adult-onset diseases that depend on the proper formation of neuronal circuits, such as schizophrenia and depression (Luscher *et al*., 2011b; Lewis, 2012; Marín, 2012). Similarly, abnormal GABAergic transmission during critical periods of development can cause severe sensory deficits, such as amblyopia, and can impair sensorimotor and cognitive development; importantly, understanding the underlying mechanisms provides clues for therapeutic intervention (Bavelier *et al*., 2010). Table 1 lists four principal mechanisms through which altered GABAergic transmission during CNS development and maturation has been implicated in brain diseases. In most cases, alterations can be traced back to mutations affecting neuronal maturation, synapse formation, and/or signaling cascades. As $GABA_AR$-mediated transmission and inhibitory–excitatory balance regulate key steps of neuronal migration and differentiation, the effects can be enduring. Thus, conditional inactivation of one γ2 subunit allele at defined stages of brain maturation induces either depression-like or anxiety-like behaviors in adult mice (Shen *et al*., 2012). Although the underlying mechanisms are not yet fully elucidated, they comprise alterations in adult neurogenesis in the dentate gyrus, and changes in the synaptic connectivity and function of specific interneurons, notably fast-spiking parvalbumin-positive basket cells and somatostatin-positive interneurons. The crucial role played by interneurons in the proper development of GABAergic synaptic connections is underscored by the long-ranging consequences of cell type-specific conditional gene deletions, such as inactivation of ErbB4 in parvalbumin-positive cortical interneurons, which affects the formation of axo-axonic synapses and synchronisation between the prefrontal cortex and the hippocampal formation, leading to a schizophrenia-like phenotype (Del Pino *et al*., 2013). In humans, a corresponding deficit in axo-axonic synapses in the prefrontal cortex is selectively found in schizophrenia but not bipolar disorder, and is accompanied by compensatory upregulation of the α2 subunit in the axon initial segment of pyramidal cells [reviewed in Lewis & Hashimoto (2007)].

Table 1. Consequences of altered GABAergic function for neurodevelopmental and psychiatric disorders

Possible causes	Functional consequences	Functional deficit, disorder	Selected references
Reduced GABA synthesis, defective maturation of interneurons, altered excitatory–inhibitory balance	Abnormal opening/duration of critical windows of plasticity	Sensory, motor or cognitive (e.g. language) deficits	Hensch (2005), Ehninger *et al.* (2008), Bavelier *et al.* (2010)
Mutations affecting extracellular matrix proteins, synapse formation, and transcription factors; defective neurotrophin signaling	Abnormal neuronal migration, interneuron differentiation, synapse formation (e.g. axon initial segment)	Epilepsy, schizophrenia	Lewis *et al.* (2005), Galanopoulou (2010); Heinrich *et al.* (2011), Marín (2012)
Mutations affecting genes involved in synaptogenesis (e.g. *NLGN* genes), defective postsynaptic scaffold formation and intracellular signaling, impaired excitatory–inhibitory balance	Altered intracellular signaling, impaired dendrite development and spine maturation, reduced synaptic plasticity	Intellectual disabilities, Angelman syndrome, autism-spectrum disorders	Südhof (2008), Blundell *et al.* (2009), Shen & Scheiffele (2010), Pizzarelli & Cherubini (2011)
Altered expression of $GABA_AR$ subunit genes, early-life stress, defective BDNF signaling	Abnormal $GABA_AR$ function at critical stages of brain development	Anxiety disorders, major depression	Hong *et al.* (2008), Maguire & Mody (2009), Shen *et al.* (2012), Smith (2013), Vithlani *et al.* (2013)

The analysis of knock-in mice expressing diazepam-insensitive $GABA_AR$ subtypes (Box 2) has noticeably expanded the catalog of potential therapeutic applications of benzodiazepine site ligands, provided that subtype specificity and differential efficacy can be achieved with novel compounds [reviewed in Rudolph & Möhler (2013)]. These studies also implicate potential dysfunction of $GABA_ARs$ in a broader set of diseases than those treated with classic benzodiazepine site ligands. Thus, α2-$GABA_ARs$ not only mediate diazepam anxiolysis, but also contribute to anxiety-related behaviors elicited by exposure to novelty and mild threat, as shown in α2-KO mice (Koester *et al.*, 2013). These receptors also contribute to mood disorders and chronic pain, and polymorphisms in *GABRA2* have been linked to alcohol dependence and drug abuse (Engin *et al.*, 2012). In chronic pain, the anti-hyperalgesic action of benzodiazepine site ligands devoid of sedative liability occurs primarily via stimulation of α2-$GABA_ARs$ in primary afferents and in the spinal cord dorsal horn, without involving *supra*-spinal sites (Witschi *et al.*, 2011; Paul *et al.*, 2013). α5-$GABA_ARs$, as noted in Box 3, regulate learning and memory, as well as hippocampal neurogenesis, and represent a promising target for improving cognitive performance in Down syndrome patients. These receptors have also been implicated in memory deficits associated with acute neuroinflammation, possibly because interleukin-1β increases their cell surface expression in hippocampal neurons (Wang *et al.*, 2012). Considering the multiple post-translational mechanisms regulating $GABA_AR$-mediated transmission, the latter finding opens the possibility that multiple chemokines and cytokines might affect GABAergic transmission by activating the underlying signaling pathways. Therefore, one might speculate that $GABA_ARs$ contribute extensively to the mediation of neuro-immune interactions.

Conclusions and perspectives

This review discusses the evidence that $GABA_A$Rs form multiple subtypes, endowed with specific functional and pharmacological properties and being differentially regulated by multiple mechanisms, at the levels of both gene expression and protein modification. Furthermore, we underscore that this regulation does not operate in isolation, but is intimately linked to the regulation of the postsynaptic scaffold organised by gephyrin, thereby vastly enlarging the repertoire of mechanisms that dynamically contribute to fine-tuning GABAergic transmission in response to various extracellular and intracellular signals. From this perspective, $GABA_A$R-mediated transmission appears as a multifaceted process that is fundamental to proper brain development, function, and plasticity. The analysis of the specific role of $GABA_A$R subtypes reveals their involvement in the pathophysiology of major CNS disorders, and opens novel perspectives for therapeutic intervention, notably based on subtype-specific ligands, and/or targeting specific signaling pathways regulating GABAergic synapse function.

Whereas the concept of $GABA_A$R subtype, with a well-defined subunit composition and functional properties, holds well in the adult brain, it is more difficult to define (and test) during brain development, when synaptic transmission is not yet present, and most effects of GABA are mediated by autocrine or paracrine mechanisms. In particular, there are no behavioral readouts with which to probe the consequences of altered $GABA_A$R function in developing animals, and the significance of the major changes in subunit expression taking place during synaptogenesis remains unexplored. Nevertheless, the evidence available, in particular from studies of critical window plasticity and of adult neurogenesis (during which developmental processes are re-initiated in adult brain), provides strong support for the contention that $GABA_A$R subtypes are tailor-made to modulate highly specific steps of neuronal differentiation and circuit formation during CNS ontogeny.

Several $GABA_A$R subtypes, composed of 'rare' subunits (γ1, γ3, ε, π, and τ), remain to be characterised. Their restricted localisation in specific brain regions (notably the hypothalamus and the basal forebrain) and their non-conventional pharmacological profile offer opportunities for selective intervention to regulate specific brain functions, notably related to the neuro-endocrine axis, sleep–wake regulation, and central autonomic function. However, these distant perspectives will first require the development of analytical tools (and genetically engineered mice) to probe the function of these so far overlooked $GABA_A$R subtypes.

The major focus given recently to 'extrasynaptic' receptors, notably those containing the δ subunit, follows the same logic to exploit receptors possessing non-conventional pharmacological profiles and unique regulatory mechanisms for improved therapeutic intervention. These perspectives are broad, ranging from stress-related disabilities to the treatment of stroke, epilepsy, alcohol intoxication, and drug dependency. However, much remains to be learned about how these receptors are regulated and how they interact with membrane proteins and intracellular effectors. The other major population of extrasynaptic $GABA_A$Rs, containing the α5 subunit, also offer promise as a target for improving intellectual disabilities, memory functions, and cognition. However, as seen with the analysis of α5(H105R) mutant mice (Box 3), care has to be taken in the interpretation of behavioral performance in mice.

The molecular heterogeneity of $GABA_A$R subtypes provides the substrate for differential transcriptional and translational regulation. Much remains to be learned about how post-translational regulation impacts on the trafficking and function of specific receptor subtypes, and how the presence or absence of a defined subunit changes this regulation. One might speculate, for example, that the heterogeneity of β subunits, which are associated with multiple α subunits, adds to the regulation of $GABA_A$R function, because the β subunits are differentially targeted by protein kinases and phosphatases (Houston *et al.*, 2008). In contrast, phosphorylation of the γ2 subunit might represent a mechanism that is common to multiple $GABA_A$R subtypes. Our recent observation that α3-

GABA$_A$Rs, but not α1-GABA$_A$Rs, are selectively targeted to postsynaptic sites to enhance GABAergic transmission when intracellular levels of ROS are increased (Accardi *et al.*, 2014) provides a striking example of GABA$_A$R subtype-specific regulation to adjust the strength of inhibition in response to a specific stimulus. Uncovering the underlying mechanisms of this specific adaptation will help in understanding the difference between α3-GABA$_A$Rs and α1-GABA$_A$Rs. Furthermore, considering that α1-GABA$_A$Rs, α2-GABA$_A$Rs and α3-GABA$_A$Rs constitute the vast majority of postsynaptic GABA$_A$Rs, it will be essential to unravel their distinguishing features, which require them to be differentially expressed and targeted to distinct subcellular sites. As a first step towards this goal, a proteomics analysis of GABAergic PSDs, and/or the characterisation of the interactome of each main GABA$_A$R subtype, would provide an exhaustive list of signaling pathways involved in their trafficking and synaptic function.

Acknowledgements

The work of J. M. Fritschy is supported by the Swiss National Science Foundation.

Abbreviations

BDNF, brain-derived neurotrophic factor

CNS, central nervous system

GABA$_A$R, GABA$_A$ receptor

GlyR, glycine receptor

KO, knockout

mIPSC, miniature inhibitory postsynaptic current

NL2, neuroligin 2

PKC, protein kinase C

PSD, postsynaptic density

ROS, reactive oxygen species

VGAT, vesicular GABA transporter

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