GABAA receptors and plasticity of inhibitory neurotransmission in the central nervous system

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Abstract: GABAA receptors (GABAA Rs) are ligand-gated Cl(-) channels that mediate most of the fast inhibitory neurotransmission in the central nervous system (CNS). Multiple GABAA R 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 GABAA R 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. GABAA R 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 GABAA Rs, but also modifying the postsynaptic scaffold organised by gephyrin to anchor specific receptor subtypes at postsynaptic sites. The significance of GABAA R 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 GABAA R subtypes reveals their involvement in the pathophysiology of major CNS disorders, and opens novel perspectives for therapeutic intervention. In conclusion, GABAA R 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.

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GABA_{A} receptors and plasticity of inhibitory neurotransmission in the CNS

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Abstract

GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) are ligand-gated Cl<sup>-</sup> channels mediating most of fast inhibitory neurotransmission in the CNS. Multiple GABA<sub>A</sub>R 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<sub>A</sub>R subtypes represent distinct receptor populations with a specific spatio-temporal expression pattern in developing and adult CNS, being endowed with unique functional and pharmacological properties, as well as being differentially regulated at the (post-) transcriptional and translational levels. GABA<sub>A</sub>R 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 take place not only by changing the gating or kinetic properties of GABA<sub>A</sub>Rs, but also by modifying the postsynaptic scaffold organized by gephyrin to anchor specific receptor subtypes at postsynaptic sites. The significance of GABA<sub>A</sub>R heterogeneity is particularly evident during CNS development and adult neurogenesis, with different receptor subtypes fulfilling distinct steps of neuronal differentiation and maturation. Finally, the analysis of the specific role of GABA<sub>A</sub>R subtypes reveals their implication in the pathophysiology of major CNS disorders and opens novel perspectives for therapeutic intervention. In conclusion, GABA<sub>A</sub>R subtypes represent the substrate of a multi-faceted inhibitory neurotransmission system, which is dynamically regulated and performs multiple operations contributing globally to proper development, function and plasticity of the CNS.
Introduction

GABA_A receptors (GABA_A Rs) belong to the family of Cys-loop ligand-gated ion channels, along with nicotinic acetylcholine receptors, glycine receptors (GlyR), and serotonin type 3 receptors (5-HT_3), which form pentameric channels carrying two extracellular ligand binding sites (Olsen & Sieghart, 2008). Being permeable for Cl^- ions, GABA_A Rs mediate most of the inhibitory action of GABA in the CNS. By virtue of their ubiquitous expression in neurons (and possibly glial cells; (Passlick et al., 2013)), GABA_A Rs contribute to all CNS functions, including sensory and motor processing, central autonomic control, sleep-wakefulness, emotions, and cognition. Clinically, the main relevance of GABA_A Rs relates to their exclusive targeting by benzodiazepines (and other ligands with high affinity for the benzodiazepine binding site), used for their anxiolytic, sedative, anticonvulsant, and muscle relaxant properties. Benzodiazepine site ligands act as allosteric modulators, and, due to their extraordinary selectivity, produce no other direct action in the CNS. GABA_A Rs are also the target 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; Carver & Reddy, 2013)).

In the early ‘90s, the identification of 19 genes encoding GABA_A subunits (α1-α6, β1-β3, γ1-γ3, δ, ε, π, τ, ρ1-r3) in mammals, coupled with the demonstration that recombinant receptors assembled with an α, a β and the γ2 subunit variant were functionally and pharmacologically similar to native GABA_A Rs, raised the question of the significance of GABA_A heterogeneity. This question is still largely unresolved, but the evidence available suggests that GABA_A subtypes, differing in subunit composition, represent distinct functional entities, imparted with specific functions and pharmacological profile, and with a unique spatio-temporal mRNA and protein expression pattern. In this review, we will discuss this evidence, based on four fundamental observations made since the existence of GABA_A subtypes was established:

A first major thrust in understanding the function of GABA_A subtypes and the relevance of their molecular heterogeneity has been the recognition that they mediate two fundamentally distinct forms of inhibitory transmission, which depend on their localization, 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; Brickley & Mody, 2012)) (see Box 3). 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_A are molecularly
distinct, with postsynaptic receptors containing mainly the $\alpha_1$, $\alpha_2$, $\alpha_3$ subunit, along with $\beta$ subunit variants and the $\gamma_2$ subunit, and extrasynaptic receptors containing $\alpha_4$, $\alpha_5$, $\alpha_6$ subunit, often along with $\delta$ (instead of $\gamma_2$). This observation implies that mechanisms of subcellular targeting of GABA$_A$R subtypes are subunit-specific, and can vary between CNS regions and developmental stages. The crucial role played by the postsynaptic scaffold organized by gephyrin for post-synaptic targeting and confinement of some GABA$_A$Rs in GABAergic postsynaptic densities (PSDs) is now widely recognized, albeit poorly understood (Fritschy et al., 2012; Tretter et al., 2012). Further, there is ample evidence for modulation of both postsynaptic and extrasynaptic GABA$_A$Rs by multiple posttranslational mechanisms regulating single channel functional properties, trafficking (exo- and endocytosis, 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, using gene targeting techniques, that the spectrum of diazepam’s actions is elicited by distinct GABA$_A$R subtypes, distinguished by their $\alpha$ subunit variant (Rudolph & Möhler, 2004). The logical consequence of these findings is that these distinct GABA$_A$Rs are localized in different neuronal circuits, even when they are co-expressed within neurons or within specific brain areas (see Box 2). An important concept emerging along this line is that GABAergic interneurons are likewise specialized to control the activity of principal cells in a circuit-specific manner (Klausberger & Somogyi, 2008). This highly sophisticated organization raises the possibility that multiple forms of inhibitory neurotransmission, engaging specific interneurons and GABA$_A$R subtypes, operate in parallel in neuronal circuits involved in concurrent tasks. The possibility to probe interneuron function in vivo using optogenetic tools has substantially advanced our understanding of their role and functional specialization (Sohal et al., 2009; Pfeffer et al., 2013; Zhu et al., 2013).

A third fundamental insight into GABA$_A$R function is their dependence on ionic mechanisms, involving both Cl$^-$ and HCO$_3^-$ ions (Blaesse et al., 2009). Therefore, the effects of GABA$_A$Rs on the resting membrane potential, independently of their subunit composition, are determined by the action of KCl co-transporters and carbonic anhydrases (see Box 4). It has been recognized 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 depolarizing to hyperpolarizing GABA$_A$R actions during ontogeny (Ben-Ari, 2002), with wide ranging consequences to our understanding of GABA function, as well as clinical use of GABA$_A$R-modulating drugs in infants (Pavlov et al., 2013).

Fourth, it is now well established that GABA$_A$R-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; Dieni et al., 2013)). Accordingly, it is being increasingly recognized that perturbations of GABA\(_\alpha\)R 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\(_\alpha\)R heterogeneity for the regulation of GABAergic neurotransmission and GABAergic synaptic plasticity, and its potential impact for 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 understand the diversity of GABA\(_\alpha\)R regulation in health and disease. To do so, we will briefly introduce the molecular heterogeneity of GABAergic synapses, prior to discussing mechanisms regulating GABA\(_\alpha\)Rs in the context of GABAergic synaptic plasticity. Next, we will highlight the relevance of GABA\(_\alpha\)R subtypes for regulating neuronal development, and finally, for the pathophysiology of neurological and psychiatric diseases linked to abnormal GABAergic transmission.

### Composition and localization of major GABA\(_\alpha\) receptor subtypes

The subunit composition of major GABA\(_\alpha\)R subtypes is well established on a regional level (immunoprecipitation using extracts from whole brain or a specific brain region), and there is general agreement that the most likely subunit stoichiometry is 2\(\alpha/2\beta/\gamma\) (the latter being sometimes substituted by \(\delta\) or \(\varepsilon\)) (Boileau et al., 2005; Olsen & Sieghart, 2008; Patel et al., 2013). The existence of receptors containing \(\alpha/\beta\) subunits only, as well as other stoichiometries (e.g. 2\(\alpha/2\beta/2\gamma\), 2\(\alpha/\beta/2\varepsilon\)) is probable (Jones & Henderson, 2013). Furthermore, the rules governing the formation of pentameric complexes are by far not fully elucidated. Functional GABA\(_\alpha\)Rs, with the pharmacological profile of native receptors, are formed by pentameric assembly of 2\(\alpha/2\beta/\gamma\), whereby the \(\alpha\) and \(\beta\) subunits can be either identical or different. The \(\gamma2\) subunit can be substituted by \(\gamma1\) or \(\gamma3\) (present at low abundance and/or with a restricted expression pattern) or by \(\delta\), and possibly \(\varepsilon\) subunits. There is consensus, therefore, that at least 3 dozen distinct GABA\(_\alpha\)R subtypes exist in CNS neurons (Olsen & Sieghart, 2008). In vitro expression of \(\beta/\gamma\) subunits only, or targeted deletion of an \(\alpha\) subunit gene in vivo, prevent assembly and/or surface targeting of a functional GABA\(_\alpha\)R complex, thereby providing the opportunity to remove specific GABA\(_\alpha\)R subtypes by inactivating a single \(\alpha\) subunit gene (see Box 1).

The most detailed information on the regional distribution of 18 GABA\(_\alpha\)R subunit mRNAs in mouse brain, determined by non-radioactive in situ hybridization with cellular resolution, is available in the
Allen Brain Atlas (http://mouse.brain-map.org). These data confirm original studies about the distribution of abundant subunits ($\alpha_1$-$\alpha_6$, $\beta_1$-$3$, $\gamma_2$) (Laurie et al., 1992; Wisden et al., 1992) and provide detailed information about subunits expressed either at low abundance in numerous regions throughout the neuraxis, such as $\gamma_3$, or at high abundance in specific regions, such as $\varepsilon$ in the amygdala, basal forebrain, locus coeruleus (and other noradrenergic cell groups). According to the Allen Brain Atlas, the $\rho_1$-$\rho_2$ mRNAs (corresponding to GABA$_C$ receptors) are restricted to the superficial layers of the superior colliculus and the $\pi$ subunit mRNA is undetectable in adult mouse brain.

Immunohistochemically, the distribution of ten subunits ($\alpha_1$-$\alpha_6$, $\beta_2$-$\beta_3$, $\gamma_2$, $\delta$) has been analyzed in detail, on 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 the $\beta_1$ and $\gamma_1$ subunit, which confirms in situ hybridization data. According to these studies, it is well apparent that the six $\alpha$ subunit variants largely correspond to distinct GABA$_A$R subtypes – notwithstanding the possibility to find two different $\alpha$ subunits in a substantial fraction of GABA$_A$Rs (Balic et al., 2009) – each with a specific distribution pattern that overlaps only partially with other $\alpha$ subunits (Figure 1). The same holds true for the $\beta$ subunit variants, with $\beta_2$ and $\beta_3$ overlapping to a large extent with the $\alpha_1$ and $\alpha_2$ subunits, respectively (whereas $\beta_1$ is expressed at lower abundance in numerous brain regions). The $\gamma_2$ subunit, in line with its association with the vast majority of GABA$_A$R subtypes, is ubiquitously expressed; in contrast, the $\gamma_1$ subunit appears to have a highly restricted distribution, being most abundant in hypothalamus, amygdala, and parts of basal ganglia, as well as the inferior olivary nucleus. Finally, the $\delta$ subunit, which forms GABA$_A$R located extrasynaptically (see Box 3), largely overlaps with the $\alpha_4$ subunit in the forebrain and with $\alpha_6$ in the cerebellum.

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

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* Box 1 approximately here *

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On the subcellular level, a distinction between postsynaptic and extra-synaptic GABA$_A$Rs can be made, based on the appearance of staining in weakly fixed tissue (see Box 1). Postsynaptic receptors
form brightly stained clusters, which co-localize with postsynaptic markers, such as gephyrin and neuroligin2, and are apposed to VGAT-positive presynaptic terminals (Fig. 2A). The reminder of the staining, besides these clusters, represents receptors dispersed at the cell surface, as well as the metabolic pool of receptors localized 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 exhibit no obvious relationship with the distribution of either gephyrin or GABAergic axon terminals (Fig. 2B). As discussed below, presynaptic receptors localized in axons and axon terminals represent a distinct subset of extrasynaptic receptors, with specialized functions.

Despite scant morphological evidence from immunohistochemical studies, there is strong functional support for the existence of GABA$\alpha$Rs 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$\alpha$Rs, readily detected immunohistochemically for the presence of $\alpha$2 subunit, located on the axon initial segment of cortical neurons, typically clustered in rows of synapses innervated by axo-axonic interneurons (Nusser et al., 1996; Fritschy et al., 1998; Panzanelli et al., 2011). Functionally, these GABA$\alpha$Rs correspond to postsynaptic receptors anchored by gephyrin, and their activation contributes to the genesis of $\gamma$-oscillations (Tukker et al., 2007). In contrast to axo-axonic synapses, most presynaptic GABA$\alpha$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 synchronization, regulation of transmitter release, and mediation of presynaptic afferent depolarization (Trigo et al., 2008; Long et al., 2009; Ruiz et al., 2010; Wakita et al., 2013). While 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 characterized recently a population of GABA$\alpha$R on primary afferent terminals in the spinal cord, containing $\alpha$2 and $\alpha$3 subunits, that are crucially involved in the anti-hyperalgesic action of diazepam (Witschi et al., 2011; Paul et al., 2012).

*********Box 2 approximately here*********

Molecular organization of GABAergic synapses

The subcellular localization of GABA$\alpha$Rs is intimately linked to the molecular organization of GABAergic synapses, with specific proteins of the PSD contributing to trafficking and anchoring GABA$\alpha$Rs in a subtype-specific manner. It has been recognized early that GlyRs, which are homologous to GABA$\alpha$Rs, are localized 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_{A}Rs also are clustered with gephyrin took longer to be obtained (Sassô-Lepnetto et al., 1995; Sassô-Poggetto et al., 2000), in part because gephyrin was considered to be present only at glycinergic synapses, and in part because GABA_{A}Rs do not bind gephyrin with high affinity. Based on the evidence available to date, the \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \) subunits, as well as possibly \( \beta_2 \) and \( \beta_3 \), can interact directly with gephyrin via motives located in their main intracellular loop (Trettet et al., 2008; Mukherjee et al., 2011; Trettet et al., 2011; Kowalczyk et al., 2013), and, therefore, can be clustered postsynaptically. Conversely, GABA_{A}R containing the \( \alpha_4 \), \( \alpha_5 \), or \( \alpha_6 \) subunit are localized predominantly extrasynaptically (Nusser et al., 1998; Chandra et al., 2006; Panzanelli et al., 2011). The \( \alpha_4 \) and \( \alpha_6 \) subunits – as well as \( \alpha_1 \) in specific interneurons (Mann & Mody, 2010; Milenkovitch et al., 2013) – are frequently associated with the \( \delta \) subunit, which substitutes for \( \gamma_2 \) (see Box 3). However, extra- versus postsynaptic targeting of GABA_{A}Rs appear to depend on motifs present in \( \alpha \) subunit variants, as seen by comparing recombinant \( \alpha_2 \)- and \( \alpha_6 \)-GABA_{A}Rs containing either \( \gamma_2 \) or \( \delta \) subunit (Wu et al., 2012).

The finding that targeted deletion of the \( \gamma_2 \) subunit abolishes postsynaptic clustering of both, GABA_{A}Rs and gephyrin, highlighted the interdependence between receptors (rather than individual subunits) and the gephyrin scaffold for proper localization at the cell surface (Essrich et al., 1998; Schweizer et al., 2003). Likewise, targeted deletion of Gphn (encoding gephyrin) confirmed its key role for postsynaptic clustering of GlyRs and GABA_{A}Rs (Feng et al., 1998). These observations triggered intense research into elucidating the functions of gephyrin, and identifying proteins regulating GABA_{A}R trafficking and GABAergic synapse formation (reviewed in (Luscher et al., 2011a; Fritschy et al., 2012; Trettet et al., 2012)). These molecules include, in particular, collybistin, a Rho-GEF identified as direct binding partner of gephyrin, mediating its translocation to the cell surface (Kins et al., 2000), neurolig2 (NL2), which interacts transsynaptically with neurexin isoforms (Varoqueaux et al., 2004; Kang et al., 2008) (see Fig. 3), as well as an array of proteins regulating GABA_{A}R biogenesis, cell surface trafficking, endocytosis and degradation (Jacob et al., 2008; Luscher et al., 2011a; Vithlani et al., 2011).

**Gephyrin**: This phylogenetically ancient, highly conserved multi-functional molecule, is responsible throughout the living kingdom 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 posttranslational modifications and interaction with partner proteins (reviewed in (Fritschy et al., 2008)). The domain organization of mammalian gephyrin not only ensures optimal Moco synthesis, but also allowed the emergence of novel, neuron-specific functions for the regulation of GlyR and GABA_{A}R clustering (Belaidi &
Schwarz, 2013). Whereas the structure of gephyrin is not fully resolved, current evidence suggests formation of trimers (Sander et al., 2013), which organize themselves in a highly ordered supramolecular complex anchoring GABA$_A$Rs 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 glycinerergic synapses) represents a process fundamentally different from scaffold formation in the PSD of glutamatergic synapses, where PSD-95 and its homologues 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 only limited proteomic data of inhibitory PSDs yet (Heller et al., 2012), and the possibility exists that major players involved in scaffold formation and regulation at GABAergic synapses are not yet identified.

A major insight from recent studies was the finding that posttranslational 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 it depends strictly on the presence of GABA$_A$R subtypes with which gephyrin (and possibly additional proteins) can interact. Therefore, the loss of postsynaptic currents in $\alpha_1$- and $\alpha_2$-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$_A$R 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 localization. One of these mechanisms might be afforded by the dystrophin-glycoprotein complex (DGC), 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 $\alpha_2$-KO mice that postsynaptic clusters of $\alpha_1$-GABA$_A$Rs and NL2 remain selectively associated with the DGC, 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 synaptic scaffolding molecule (S-SCAM), which
interacts with both, dystrophin and NL2 (Sumita et al., 2007). Interestingly, the cell adhesion molecule IgSF9b, interacting with S-SCAM and NL2, has recently been identified to promote 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 analyze the molecular organization of inhibitory PSDs provided a first quantification of the number of gephyrin molecules present per synapse, and demonstrated that the number of both GlyRs and GABA_A Rs at the synapse depend on gephyrin abundance (Specht et al., 2013). A possible difference in the molecular organization 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 GlyRs.

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_A R and gephyrin clustering occurs in a cell type-specific manner in collybistin-KO mice, unraveling the essential function of this protein at GABAergic synapses (Papadopoulos et al., 2007; Papadopoulos et al., 2008). The effect can be reproduced in cultured neurons upon over-expression of a collybistin isoform 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 down-stream of collybistin (Tyagarajan et al., 2011a). However, elucidating the function(s) of collybistin and its effectors is rendered complicated by the existence of collybistin splice variants, carrying or lacking an N-terminal SH3 domain (collybistinSH3⁺ and collybistinSH3⁻, respectively), because collybistinSH3⁺ has been reported to be an inactive form that needs to be activated in order to contribute to gephyrin and GABA_A R 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 (Figure 3). It has been proposed, for example, that binding of GABA_A R 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 NLGN2 does not abolish gephyrin clustering and collybistin over-expression in neurons strongly stimulates gephyrin clustering independently of the presence/absence of the SH3 domain (Chiou et al., 2011; Tyagarajan et al., 2011a), suggesting alternative mechanisms. Among these, the small GTPase TC-10 was shown recently to activate collybistin upon interaction with the PH domain, thereby enhancing gephyrin clustering in cultured neurons (Mayer et al., 2013).
Neuroligin 2: 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 over-expression 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 NLGN4 at glycineergic synapses (Hoon et al., 2011); but see (Soto et al., 2011), whereas NLGN1 is selective for glutamatergic synapses (Song et al., 1999) and NLGN3 is found in both glutamatergic and GABAergic (Budreck & Scheiffele, 2007). NLGNs have raised 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 localization) are modulated by complex interaction with neurexin isoforms (Futai et al., 2013), post-translational modifications (Peixoto et al., 2012; Suzuki et al., 2012; Giannone et al., 2013), and homo- and heterodimerization (Poulopoulos et al., 2012; Shipman & Nicoll, 2012). In particular, it is not known whether NLGNs interact directly with GABA_{A}Rs. Nevertheless, NL2-KO exhibit specific reduction of perisomatic GABAergic synapses in principal neurons of the hippocampal formation, associated with reduction of inhibitory currents and increased network excitability (Jedlicka et al., 2011).

It should be noted that while NL2 is generally considered to be upstream in the chain of molecular events leading to 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} receptor 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 regards 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} receptor subunit expression: The gene structure and chromosomal localization 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 subject to
intense scrutiny (reviewed in (Steiger & Russek, 2004)). Still, little is known 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). A remarkable variability in the abundance of mRNAs encoding the 19 GABA<sub>A</sub>R subunits has been uncovered in both mice and human, 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<sub>A</sub>R 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; Grabenstatter et al., 2012)). A recent genetic study identified a chromosomal duplication in a locus encoding four GABA<sub>A</sub>R subunits (4p12; α2, α4, β1, γ1) 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<sub>A</sub>R subunit KO mice (see Box 1) reflects transcriptional control or is due to changes in mRNA stability and/or by 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 maintain homeostatic balance between excitation and inhibition in the mutant mice. Transcriptional control of GABA<sub>A</sub>R 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).

**Posttranslational modifications of GABA<sub>A</sub> receptors:** Membrane-anchoring of GABA<sub>A</sub>Rs is regulated by palmitoylation of the γ2 subunit and this mechanism contributes to normal formation and function of GABAergic synapses (Fang et al., 2006). In addition, it is well established that multiple phosphorylation mechanisms, targeting various GABA<sub>A</sub>R 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 internalization of GABA<sub>A</sub>Rs (reviewed in (Jacob et al., 2008; Houston et al., 2009; Luscher et al., 2011a; Vithlani et al., 2011)). Combined with recent reports that gephyrin phosphorylation at residues S268 and S270 by GSK3β and ERK, respectively, is a negative regulator of GABAergic transmission (Tyagarajan et al., 2011b; Rui et al., 2013; Tyagarajan 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<sub>A</sub>Rs are subunit-specific, differential effects can be expected for various GABA<sub>A</sub>R subtypes, even within the same neuron. Furthermore, considering the tight functional coupling between the gephyrin scaffold and postsynaptic GABA<sub>A</sub>Rs, the question arises whether phosphorylation of gephyrin and GABA<sub>A</sub>Rs 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 characterization. In addition, it is conceivable that the gephyrin scaffold serves to anchor protein kinases (and phosphatases) acting on GABA$_A$Rs and therefore regulates the efficacy of receptor posttranslational modifications.

A major advance in our understanding of the in vivo significance of GABA$_A$R phosphorylation is provided by the generation of knock-in mice carrying point-mutations that abolish phosphorylation of residues known to be targeted by protein kinases in vitro. However, these studies have unraveled unexpectedly strong effects of the mutations, with $\gamma_2(Y365/367F)$ mutation being embryonically lethal, and inducing in heterozygous mice sex-specific increased in tonic inhibition (a$4/\delta$-GABAARs) to compensate for reduced neurosteroid sensitivity in the thalamus (Jurd & Moss, 2010; Nani et al., 2013).

**Regulation of GABA$_A$ receptor trafficking and cell-surface diffusion:** Membrane insertion (and internalization) of GABA$_A$Rs 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 reservoir pool of extrasynaptic receptors (Thomas et al., 2005). Furthermore, preventing docking of GABA$_A$Rs at endocytotic zones in the plasma membrane – by interfering with a binding motif located in the intracellular loop of the $\beta_3$ subunit – blocked GABA$_A$R internalization, as well as down-regulation following oxygen-glucose deprivation, a model of ischemia in vitro (Smith et al., 2012). Such observations underscore that regulated diffusion of GABA$_A$Rs in the plasma membrane is of prime relevance under physiological and pathophysiological conditions.

Single-particle tracking studies also revealed that GABA$_A$R 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$_A$Rs have similar diffusion rates in the plasma membrane, but the former remain confined longer in GABAergic postsynaptic sites and their trapping depended 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 mIPSCs, due to dispersion of GABA$_A$R 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$_A$Rs and gephyrin occur with different time scales, partially independently of each other (Niwa et al., 2012). Taken 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$_A$Rs and the postsynaptic scaffold. Much remains to be determined how these phenomena observed in vitro are mediated in vivo, and whether they selectively affect specific GABA$_A$R subtypes in cortical principal cells or cause global effects across various types of synapses.
Regulation of GABA\(_A\)-R-mediated transmission by neurotrophins and metabolic factors:

Neurotrophins, such as 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\(_A\)-R-mediated transmission, by acting on both GABAergic synapse formation (Chen et al., 2011) and plasticity: BDNF produces bi-phasic effects on GABA\(_A\)-R-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 internalization of \(\alpha1\)-GABA\(_A\)-Rs in the amygdala by causing rapid gephyrin degradation (Mou et al., 2013). However, a long-lasting enhancement of GABA\(_A\)-R-mediated transmission in the hippocampus, due to enhanced cell surface expression, has been shown to arise from phosphorylation of the tyrosine residues Y657 and Y367 in the \(\gamma2\) subunit (Vithlani et al., 2013); the significance of this modulation, as tested in \(\gamma2(Y365/367F)\) knock-in mice, is anti-depressant behavioral phenotype and increased hippocampal neurogenesis, raising the possibility that GABA\(_A\)-R phosphorylation regulates the anti-depressant action of BDNF.

We have recently uncovered a novel mode of GABA\(_A\)-R 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 in cerebellar interneurons, caused a gradual increase in the frequency of mIPSCs, due to the appearance of additional low amplitude currents with slow decay kinetics. While the majority of mIPSCs in these cells are mediated by \(\alpha1\)-GABA\(_A\)-Rs, these newly induced currents had kinetic properties of \(\alpha3\)-GABA\(_A\)-Rs, and, indeed, depended on expression of the \(\alpha3\) subunit in these cells, as tested in \(\alpha3\)-KO mice (whereas deletion of the \(\alpha1\) subunit had no effect on this phenomenon). Along with evidence that the effects of ROS elevation are due 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 \(\alpha3\)-GABA\(_A\)-R to either “silent” synapses, or to de novo formed synaptic contacts from terminals known to form multiple release sites (Accardi et al., 2014). In line with these results, insulin acutely increases GABA\(_A\)-R cell-surface expression in vitro (Wan et al., 1997), but it is not established whether the same mechanism is involved.

Significance for GABAergic synapse plasticity: The main conclusion derived from studies of GABA\(_A\)-R (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 to changes in their environment. These regulatory adjustments concern both post- 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.

Posttranslational regulation of GABA$_A$Rs (and gephyrin) is implicated in 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 depolarization of Purkinje cells and involves Ca$^{2+}$ influx and activation of calcium-calmodulin kinase 2 (Kano et al., 1996). Rebound potentiation involves trafficking of GABA$_A$Rs 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., 2013). In addition to functional plasticity, regulation of GABA$_A$Rs 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 $\alpha5$-GABA$_A$R negative allosteric modulator reduced the density of GABAergic synapses in the hippocampal formation and normalized behavior in these mutants (Martínez-Cué et al., 2013).

**GABA$_A$ receptor heterogeneity in times of change: CNS development and adult neurogenesis**

*Developmental changes in GABA$_A$ receptor subunit expression:* GABA$_A$Rs 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 depolarization (reviewed in (Represa & Ben-Ari, 2005; Cellot & Cherubini, 2013; Lu et al., 2013)) (see box 4). Accordingly, one might expect developmental deficits in the CNS of GABA$_A$R subunit knock-out mice, notably with regard to subunits highly expressed in fetal brain. However, among the targeted deletions analyzed so far ($\alpha1-\alpha6$, $\beta2$, $\beta3$, $\delta$, $\gamma2$) 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$_A$Rs are dispensable for regulating brain development. Rather, they might be so important that functional redundancy has been developed to prevent deleterious effects in case of dysfunction of a given subtype.

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* Box 4 approximately here *

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It is well established that the subunit composition of predominant GABA$_A$R subtypes changes during the period of synaptogenesis, accounting in part for the distinct functional and pharmacological
properties of GABA\(_A\)Rs 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 about the expression pattern of GABA\(_A\)R subunits in neural precursor cells and immature neurons. mRNA in situ hybridization data available in the Allen Brain Atlas (http://mouse.brain-map.org) show predominance of \(\alpha_2, \alpha_3, \alpha_5, \beta_2, \beta_3, \gamma_2\) subunits at E18.5. As this stage precedes synaptogenesis, one might assume that these receptors are mainly engaged in tonic, possibly depolarizing, GABAergic transmission. In particular, the \(\alpha_5\) subunit is strongly expressed perinatally and decreases during synaptogenesis, except in regions where it remains abundant in adult brain (hippocampal formation, olfactory bulb, brainstem). Of note, down-regulation of the \(\alpha_5\) subunit in layer 4 of the neocortex and its replacement by the \(\alpha_1\) subunit, have been shown to be dependent on the integrity of thalamocortical projection (Paysan et al., 1997), providing an attractive model to study the mechanisms underlying (post-)transcriptional control of subunit expression. In contrast to \(\alpha_5\), other subunits defining the main extrasynaptic GABA\(_A\)Rs of the adult brain (\(\alpha_4, \alpha_6, \delta\)) are absent or expressed at low levels in fetal brain, and are upregulated when neuronal maturation nears completion. In human neocortex, the GABAergic system develops during the second half of pregnancy and infancy (Xu et al., 2011), and the expression of GABA\(_A\)R subunit genes appears to be coordinated based on their respective chromosomal localization (Fillman et al., 2010), with distinct up- and down regulation patterns suggestive of differential expression of specific GABA\(_A\)R subtypes.

To elucidate why the pattern of GABA\(_A\)R subunit expression evolves during ontogeny, considerable attention was given to \(\alpha_1\)-GABA\(_A\)Rs, which are upregulated in a region-specific manner during the phase of synaptogenesis to become the predominant GABA\(_A\)R subtype present in adult CNS (Fritschy et al., 1994; Paysan et al., 1994; Hashimoto et al., 2009). As these receptors typically display fast decay kinetics and generate large amplitude events (Eyre et al., 2012), their gradual appearance was taken as evidence for a 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, \(\alpha_1\)-GABA\(_A\)Rs 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 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 do not depend solely on the subunit composition of GABA\(_A\)Rs (Koksma et al., 2003; Koksma et al., 2005; Peden et al., 2008). Rather, post-translational modifications mechanisms affecting GABA\(_A\)R 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$_A$Rs shapes the functional properties of GABAergic transmission, it will be essential to determine, as well, the developmental GABA$_A$R expression profile in interneurons, as these receptors will have a key role on the maturation and determine the firing properties of input cells controlling synaptic circuits.

**GABA$_A$ receptor 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$_A$R-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 $\alpha_1$-GABA$_A$Rs (reviewed in (Hensch, 2005)). The latter piece of the puzzle was brought about by the demonstration that diazepam is unable to advance the opening of a critical period window in $\alpha_1$(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 reopening 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 determinant for setting the opening and closing of critical period windows (Morishita *et al.*, 2010).

The relevance of parvalbumin-positive basket cells for setting network configurations permissive for structural and functional plasticity, 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 VIP-positive interneurons is low in mice exposed to conditions permissive for learning (e.g., enriched environment) and high, either when a new task is acquired or when adverse conditions (e.g., fear conditioning) lead to memory retention. Remarkably, 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 developing brain (and during permissive phases of plasticity critical for learning and circuit refinement) is regulated by
sophisticated mechanisms, and mediated by specific circuits containing defined GABA$_A$R subtypes, such as $\alpha_1$-GABA$_A$Rs in synapses formed on principal cells by parvalbumin-positive basket cells.

**Regulation of adult neurogenesis:** In analogy to brain development, GABA$_A$Rs expressed by stem cells, neural precursor cells and immature neurons contribute to proliferation, migration, differentiation and synaptic integration of adult-born neurons (reviewed by (Overstreet *et al.*, 2005; Ge *et al.*, 2007; Sernagor *et al.*, 2010; Nissant & Pallotto, 2011)). Likewise, as in developing neurons, GABA initially exerts depolarizing effects on precursor cells (see 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, analyzing 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 versus asymmetric) as well as neuronal versus glial fate (Song *et al.*, 2012); furthermore, the pharmacological profile of these receptors (zolpidem-insensitive) is compatible with expression of $\alpha_5$-GABA$_A$R in these stem cells. Along the same line, investigating the role of extrasynaptic ($\alpha_4$, $\delta$) and postsynaptic ($\alpha_2$) 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 a negative control on neural precursor cell proliferation (Platel *et al.*, 2007), $\alpha_4$-KO mice exhibited increased neurogenesis. No phenotype was seen in $\delta$-KO mice, as expected from the delayed expression of this subunit during ontogeny (but see (Whissell *et al.*, 2013)). Ablation of $\alpha_4$-GABA$_A$Rs also impaired dendritic growth and final positioning of adult-born granule cells; in contrast, $\alpha_2$-KO newborn neurons exhibited 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 $\alpha_2$ subunit inactivation in adult-born olfactory bulb granule cells (Pallotto *et al.*, 2012), which represent the main interneuron subtype of the olfactory bulb, continuously generated from mitotic precursor cells in the subventricular zone (Carleton *et al.*, 2003). As $\alpha_2$-GABA$_A$Rs provide most synaptic inhibition to these cells, their inactivation profoundly altered dendritic development, spine formation, and maturation of glutamatergic inputs. Also, modulation of dendritic differentiation by environmental enrichment or
deprivation, which is prominent in wildtype 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 GABA₆R in regulating neuronal differentiation. Moreover, these results taken together provide an exquisite demonstration that GABA₆R subtypes are specialized to fulfill specific tasks, with considerable spatio-temporal specificity.

**Significance for CNS disorders**

Elucidation of the molecular organization and regulation of GABA₆R 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 GABA₆R-mediated transmission in the pathophysiology of CNS disorders lie in their contribution to developmental processes and dependence on Cl⁻ and HCO₃⁻ fluxes (see Box 4). In turn, GABA₆R dysfunction can be determined genetically, and/or depend on abnormal regulation and trafficking. Here, we briefly discuss these mechanisms at the light of a few selected examples.

GABA₆R subunit mutations are typically associated with generalized, mostly idiopathic epilepsies and Dravet syndrome. They have been described in α subunits, β3, as well as γ2 and δ. While data are somewhat controversial, the majority of these mutations impair trafficking and cell surface expression of GABA₆Rs, as well as their diffusion dynamics in the plasma membrane (hence, their postsynaptic clustering (Bouthour et al., 2012)) (reviewed in (Galanopoulou, 2010; Macdonald et al., 2010). Some of these effects have been proposed to be temperature-dependent, hence providing a plausible cause for febrile seizures. However, the general picture emerging from these studies is that there is that GABA₆R mutations cause multiple molecular and biochemical alterations, which are not easily related to specific symptoms of disease, notably epileptogenesis and seizure occurrence; thereby reflecting the complexity of mechanisms underlying epileptic syndromes.

GABA₆R mutations have also been associated with other CNS pathologies. For example, although the complex interactions between ethanol intoxication or ethanol dependence and GABA₆Rs 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 openings 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 GABA₆R was confirmed by a second mutation, which caused similar behavioral phenotype. These data underscore the main contention of this review, namely how dysfunction of specific GABA₆R 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, due to over-expression or reduction of specific GABA$_A$R subtypes (reviewed in (Hines et al., 2011; Grabenstatter et al., 2012; Houser et al., 2012)). In a seminal report 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 expression of extrasynaptic GABA$_A$Rs, promoted functional recovery (Clarkson et al., 2010). These data go well in line with evidence that GABAergic transmission regulates neuronal plasticity by setting inhibitory-excitatory balance in neuronal networks.

GABA$_A$R-mediated transmission during brain development has been linked to the emergence of neurodevelopmental disorders, as well as adult-onset diseases that depend on 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, as well as impair sensory-motor and cognitive development; importantly, understanding the underlying mechanisms provides cues 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$_A$R-mediated transmission and inhibitory-excitatory balance regulates key steps of neuronal migration and differentiation, the effects can be enduring. Thus, conditional inactivation of one $\gamma_2$ subunit allele at defined stages of brain maturation induces either depression-like or anxiety-like behaviors in adult mice (Shen et al., 2012). While the underlying mechanisms are not yet fully elucidated, they comprise alteration of adult neurogenesis in the dentate gyrus, as well as changes in synaptic connectivity and function of specific interneurons, notably fast-spiking parvalbumin-positive basket cells and somatostatin-positive interneurons. The crucial role played by interneurons for 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 formation of axo-axonic synapses and synchronization between prefrontal cortex and hippocampal formation, leading to schizophrenia-like phenotype (Del Pino et al., 2013). In human, a corresponding deficit in axo-axonic synapses in prefrontal cortex is selectively found in schizophrenia but not bipolar disorder, and is accompanied by compensatory upregulation of the $\alpha_2$ subunit in the axon initial segment of pyramidal cells (reviewed in (Lewis & Hashimoto, 2007)).

The analysis of knock-in mice expressing diazepam-insensitive GABA$_A$R subtypes (see Box 2) has noticeably expanded the catalogue 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_A Rs in a broader set of diseases than those treated with classical benzodiazepine site-ligands. Thus, α2-GABA_A Rs not only mediate diazepam anxiolysis, but they 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_A Rs 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_A Rs, 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 also have 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_A R-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_A Rs 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 level 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 organized 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 multi-facetted process fundamental to proper brain development, function, and plasticity. The analysis of the specific role of GABA_A R subtypes reveals their implication in the pathophysiology of major CNS disorders and open novel perspectives for therapeutic intervention; notably based on subtype-specific ligands, and/or targeting specific signaling pathways regulating GABAergic synapse function.

While the concept of GABA_A R subtype, with 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 auto- or paracrine mechanisms. In particular, there are no behavioral readouts to probe the
consequences of altered GABA$_{\alpha}$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 to the contention that GABA$_{\alpha}$R subtypes are tailor-made to modulate highly specific steps of neuronal differentiation and circuit formation during CNS ontogeny.

Several GABA$_{\alpha}$R subtypes, encoded by “rare” subunits ($\gamma$1, $\gamma$3, $\varepsilon$, $\pi$, $\tau$), remain to be characterized. Their restricted localization in specific brain regions (notably hypothalamus and basal forebrain), coupled with a 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$_{\alpha}$R subtypes.

The major focus given recently to “extrasynaptic” receptors, notably those containing the $\delta$ subunit, follows the same logic to exploit receptors possessing non-conventional pharmacological profile 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 how these receptors are regulated and how they interact with membrane-proteins and intracellular effectors. The other major population of extrasynaptic GABA$_{\alpha}$Rs, containing the $\alpha$5 subunit, also offers promising perspectives as a target for improving intellectual disabilities, memory functions, and cognition. However, as seen with the analysis of $\alpha$5(H105R) mutant mice (see Box 3), care has to be taken in the interpretation of behavioral performance in mice.

The molecular heterogeneity of GABA$_{\alpha}$R subtypes provides the substrate for differential transcriptional and translational regulation. Much remains to be learned how post-translational regulation impacts on trafficking and function of specific receptor subtypes, as well as how the presence/absence of a defined subunit changes this regulation. One might speculate, for example, that the heterogeneity of $\beta$ subunits, which are associated with multiple $\alpha$ subunits, adds to the regulation of GABA$_{\alpha}$R function, because the $\beta$ subunits are differentially targeted by protein kinases and phosphatases (Houston et al., 2008). In contrast, phosphorylation of the $\gamma$2 subunit might represent a mechanism common to multiple GABA$_{\alpha}$R subtypes. Our recent observation that $\alpha$3-, but not $\alpha$1-GABA$_{\alpha}$Rs, are selectively targeted to postsynaptic sites to enhance GABAergic transmission when intracellular levels of ROS are increased (Accardi et al., 2014) provides a striking example for a GABA$_{\alpha}$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 understanding the difference between α3- versus α1-GABA_ARs. Furthermore, considering that α1-, α2, and α3-GABA_ARs comprise 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 characterization 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.
Acknowledgments

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List of abbreviations

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<th>Abbreviation</th>
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<tr>
<td>DGC</td>
<td>dystrophin-glycoprotein complex</td>
</tr>
<tr>
<td>GABA$_A$R</td>
<td>GABA$_A$ receptor</td>
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<td>GlyR</td>
<td>Glycine receptor</td>
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<td>NLGN</td>
<td>Neuroligin gene</td>
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<tr>
<td>NL2</td>
<td>Neuroligin2</td>
</tr>
<tr>
<td>PSD</td>
<td>Postsynaptic density</td>
</tr>
<tr>
<td>ROS</td>
<td>radical oxygen species</td>
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Box 1: Limitations of immunohistochemistry for studying GABA<sub>A</sub> receptors

Whereas GABA<sub>A</sub>R are pentameric protein complexes, immunohistochemistry only allows the visualization of individual subunits, each of which belongs to several subtypes. Furthermore, as the pentamer contains 2α and 2β 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 similar subcellular distribution. Therefore, the exact GABA<sub>A</sub>R 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 immunohistochemistry for some of the GABA<sub>A</sub>R subunits. In particular, very little information is available for the distribution of the γ1, γ3, ε, π, and ε subunit proteins, as well as ρ1-ρ3 subunits, either at a regional, cellular, or subcellular level. As a result, several GABA<sub>A</sub>R subtypes, probably with atypical pharmacology and a highly selective distribution, remain poorly characterized and their contribution to inhibitory transmission in these specific CNS areas is unknown.

Besides these limitations, it should be emphasized 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 assessing the abundance of a given subunit relative to other subunits), because the affinity of antibodies to their epitope, and its 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 lack of staining for a specific antibody in some regions and to accurately determine the subcellular distribution of GABA<sub>A</sub>Rs.

This latter point is of crucial relevance for understanding the functional organization of the GABAergic system. Functional and biochemical studies differentiate among several pools of GABA<sub>A</sub>Rs in neurons, being localized intracellularly (reflecting biosynthesis, metabolism, reserve pool) or at the cell surface, post- or extrasynaptically (see Box 3). Immunohistochemical studies have shown that the detection of postsynaptic GABA<sub>A</sub>Rs is largely impaired by aldehyde fixation and requires alternative methods to the classical transcardial perfusion. Therefore, immunohistochemical analysis of GABA<sub>A</sub>R subunit distribution provides distinct results depending on the fixation method used. Furthermore, we had noted in our initial report (Fritschy & Mohler, 1995) that the cellular distribution pattern of GABA<sub>A</sub>R 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 be widely different for 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<sub>A</sub>R subtypes in a synapse-specific manner, and to derive some quantitative measurement of relative receptor abundance. We have recently reported a method allowing concurrent visualization of postsynaptic GABA<sub>A</sub>Rs with high sensitivity and resolution and biochemical analysis using brain tissue from the same animal (Notter et al., 2013). In
the future, new methods will have to be devised to unequivocally detect pre-synaptic GABA$_A$Rs, 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 recognized early on that pre-embedding immune-electron microscopy is not suitable for the detection of GABA$_A$Rs at postsynaptic sites (Somogyi et al., 1989). Post-embedding techniques, notably following tissue embedding in Lowicryl permitted to circumvent this problem, 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 SDS-digestion after freeze-fracture has enabled substantial progress by allowing the 3-D visualization and quantification of GABA$_A$Rs in the plasma membrane (Kasugai et al., 2010), albeit working only with a limited number of antibodies.
Box 2: Lessons from Gabra knock-out and knock-in mice: GABA_A receptor subtypes fulfill specific tasks

Targeted deletion of a GABA_A 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 knock-out (α1-KO) mice exhibit upregulation of α2- and α3-GABA_ARs in regions where the α1 subunit is abundant (Kralic et al., 2006; Zeller et al., 2008); δ-KO mice exhibit increased α4 subunit expression, associated with the γ2 subunit, and with 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 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_ARs to cluster at postsynaptic sites is not due 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_ARs 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 apparent loss of postsynaptic GABA_ARs and gephyrin, but these mutants exhibit larger postsynaptic currents than wildtype mice (Schofield et al., 2009).

These observations lend to the contention that GABA_A subtypes, defined by their subunit composition, are unique functional entities, fulfilling specific tasks, without being inter-changeable 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, and gating of the receptor (Rudolph et al., 1999; Löw et al., 2000; Crestani et al., 2002; Yee et al., 2005). Behavioral analysis of H/R knock-in mice for each the four α subunit variants assembled in diazepam-sensitive GABA_ARs revealed loss of specific effects of diazepam, which allowed to classify the contribution of each subtype to the spectrum of diazepam’s effects. Thus, sedation only involves α1-GABA_ARs, whereas anxiolysis occurs upon allosteric modulation of α2-GABA_ARs, and when stress is involved, partially α3-GABA_ARs (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_A subtypes. Importantly, electrophysiological analyses confirmed that the point-mutations are functionally silent. However, in α5(H105R)-mutants, it leads to decreased expression of α5-GABA_ARs, which is behaviorally significant (Prut et al., 2010) (see Box 3).
A distinction of GABA$_A$R subtypes based on the $\beta$ subunit variants is less straightforward, in particular because each $\beta$ subunit can be associated with various $\alpha$ subunits. Nevertheless, $\beta3$ subunit-containing GABA$_A$Rs selectively mediate the action of intravenous general anesthetics, as well as part of the effects of pentobarbital, as shown in $\beta3$(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 being assembled during brain development and regulated by plasticity mechanisms in adulthood.
Box 3: Extrasynaptic GABA\(_A\) receptors

Tonic inhibition, mediated by persistent activation of extrasynaptic GABA\(_A\)Rs, is an important determinant of neuronal excitability and is increasingly recognized to play a key role in mediating effects of neurosteroids, as well as to contribute to pathophysiology of major disease states (reviewed in (Belelli \textit{et al.}, 2009; Gunn \textit{et al.}, 2011; Brickley & Mody, 2012)). While tonic inhibition is widely considered to reflect receptor activation by ambient GABA, this vision has been questioned by evidence that spontaneous openings of GABA\(_A\)Rs might contribute to most of tonic currents that can be recorded in dentate gyrus granule cells (Wlodarczyk \textit{et al.}, 2013). Extrasynaptic receptors containing the \(\delta\) subunit have a very high affinity to GABA, and mediate most actions of neurosteroids, and thus contribute to regulate brain activity under circumstances when their synthesis is increased, including stress, delivery, ethanol intoxication (Sarkar \textit{et al.}, 2011; Carver & Reddy, 2013). These receptors are selectively modulated by the super-agonist gaboxadol (but insensitive to classical benzodiazepine agonists) (Mortensen \textit{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 PKA and PKC activity (targeting \(\alpha_4\)-GABA\(_A\)R) (Connelly \textit{et al.}, 2013a), for example upon stimulation of GABA\(_B\) receptors (Connelly \textit{et al.}, 2013b; Tao \textit{et al.}, 2013). Specifically, PKC-mediated phosphorylation of Ser443 in the \(\alpha_4\) subunit was shown to enhance cell surface expression and activity of these receptors (Abramian \textit{et al.}, 2010). However, another study contends that PKC activation reduces tonic inhibition in the thalamus by targeting the \(\beta_2\) subunit (Bright & Smart, 2013). As tonic inhibition is a major determinant of neuronal excitability, these data unravel novel, albeit contradictory, mechanisms that potentially have major effects on network activity.

Besides \(\alpha_4/\beta/\delta\) receptors, \(\alpha_5\)-GABA\(_A\)Rs (most probably composed of \(\alpha_5/\beta_3/\gamma_2\) 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 insensitive to zolpidem. Interest in these receptors was triggered by the observations in \(\alpha_5\)(H105R)-mutant mice (carrying diazepam-insensitive \(\alpha_5\)-GABA\(_A\)Rs, see Box 2) that they do not develop tolerance to the sedative (i.e., motor impairing) action of diazepam (van Rijnsoever \textit{et al.}, 2004). Furthermore, \(\alpha_5\)(H105R)-mutant mice exhibited \(\sim\)30% reduction in \(\alpha_5\)-GABA\(_A\)Rs but displayed improved performance in a hippocampus-dependent memory task (trace fear conditioning) compared to wildtype mice (Crestani \textit{et al.}, 2002). This observation opened the tantalizing perspective that reducing the function of \(\alpha_5\)-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 \(\alpha_5\)(H105R)-mutant mice revealed increased basal locomotion and altered memory for location of objects, indicative of hippocampal dysfunction (Prut \textit{et al.}, 2010). Therefore, the reduced expression of \(\alpha_5\)-GABA\(_A\)R 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_{A}Rs in the basolateral amygdala (Marowsky et al., 2012) and the inferior olivary nucleus (Devor et al., 2001). The rules governing the extrasynaptic localization of these receptors are not understood. The majority of α3-GABA_{A}Rs, 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_{A}Rs at extrasynaptic sites in specific neuron populations. A similar dichotomy also exist for α5-GABA_{A}Rs, which are not strictly extrasynaptic in the hippocampal formation (Serwanski et al., 2006); those located extrasynaptically were shown to interact with radixin, a phospho-protein belonging to the ezrin-radixin-moesin protein family and interacting with the actin cytoskeleton (Loebrich et al., 2006).
Box 4: GABA<sub>A</sub> receptor-mediated “excitation”

GABA<sub>A</sub>Rs being selectively permeable for Cl<sup>−</sup> and HCO<sub>3</sub>− ions (Kaila, 1994), there is now a large consensus that elevation of the intracellular concentration of either species might result in a depolarizing current upon GABA<sub>A</sub>R activation, and thereby potentially “excite” this neuron (Blaesse et al., 2009). There is ample evidence for GABA<sub>A</sub>R-mediated depolarization of immature (and mature) neurons and NG2 cells, leading to Ca<sup>2+</sup>-influx; opening the door to speculating about the roles of Ca<sup>2+</sup> as 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<sub>A</sub>R-mediated excitation precedes (and is replaced by) glutamatergic transmission during maturation of cortical neurons (Hennou et al., 2002), and that “excitatory” GABA drives giant depolarizing 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<sub>A</sub>R-mediated excitation in vivo has been much debated, as well as its functional significance and the main ion species responsible for it. In particular, there is often confusion about the excitatory effects of depolarizing GABA. Considering that one of the main effect of GABA<sub>A</sub>R activation is a net increase in membrane conductance, opposing the depolarizing effect of positive charge influx induced by any other neurotransmitter. Therefore, while a neuron can be depolarized by GABA, this does not necessarily translate as being “excited”.

The prevalent view is that GABA-induced depolarization is due to high expression of the co-transporter NKCC1 and low expression of KCC2, which exert opposing action on intracellular Cl<sup>−</sup>. Nevertheless, in mature neurons (expressing high levels of KCC2), intense GABA<sub>A</sub>R stimulation can lead eventually to neuronal depolarization due to KCC2-mediated K<sup>+</sup> efflux (Viitanen et al., 2010). This biphasic response, which involves short-lasting changes in ionic driving force of GABA<sub>A</sub>R and reduces the efficacy of diazepam (Deeb et al., 2013), has been coined as “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 localization and functional regulation. Evidence is now emerging that transcriptional and post-translational mechanisms, involving among others BDNF signaling, have major impact 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 recognized that, besides NKCC1 and KCC2, the developmental maturation of carbonic anhydrases is a major determinant of the driving force of GABA<sub>A</sub>Rs in immature brain (Rivera et al., 2005). Thus, absence of carbonic anhydrase (upon targeted gene deletion) enhances depolarizing action of GABA and induces seizures in neonatal mice (Ruusuvuori et al., 2013). Finally, it should be emphasized that, unlike initial speculations that the depolarizing and hyperpolarizing effects of GABA might be mediated by distinct GABA<sub>A</sub>R subtypes, there is no evidence supporting this possibility. These speculations were triggered, in part, by observations that the subunit composition of major GABA<sub>A</sub>R subtypes changes drastically, in particular in neocortex and thalamus, during the phase of synaptogenesis (see main text). However,
this subunit switch appears to be unrelated to GABA depolarization, and its significance remains a matter of speculation.
References


oxytocin neurons caused by switch in GABA\(_\alpha\) receptor subunit expression. *Neuron*, 19, 1103-1114.


aminobutyric acid type A (GABA_A) receptors by collybistin isoforms. *J. Biol. Chem.*, **286**, 22456-22468.


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

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

Figure 1
Differential distribution of GABA\(_{\alpha}\)R \(\alpha\) subunit variants in the adult mouse forebrain. **A-E:** Each panel depicts in false colors, ranging from dark blue to red, orange, yellow, and white for maximal intensity, the relative staining intensity pattern of each subunit indicated, as determined by immunoperoxidase staining. \(\alpha6\) 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 \(\alpha\) subunits. **F:** Main anatomical structures present in the images of panels A-E (ac, anterior commissure; cc, corpus callosum; AON, anterior olfactory nucleus; CA1, CA1 region of the hippocampus; 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 somato sensory cortex; SC, superior colliculus; SNc, 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).

Figure 2
Distinction of postsynaptic and extrasynaptic GABA\(_{\alpha}\)Rs detected by immunofluorescence staining and confocal laser scanning microscopy. The subcellular localization is based on the identification of postsynaptic sites positive for gephyrin and presynaptic GABAergic terminals positive for VGAT. **A:** \(\alpha2\) subunit staining in adult mouse CA1, revealing numerous brightly stained clusters around pyramidal cell bodies in the stratum pyramidale (sp) and on their dendrites in stratum oriens (so) and radiatum (sr). **A1-A4:** High magnification images taken from a section triple stained for \(\alpha2\) (red), gephyrin (yellow), and VGAT (blue) in stratum radiatum, depicting in single, double, and triple staining that \(\alpha2\) clusters are colocalized with gephyrin (A3; yellow) and apposed to VGAT-positive terminals (A2). **B:** \(\alpha5\) subunit staining in adult mouse CA1, depicting the granular appearance of the staining, with pyramidal cells in sp appearing as lightly stained structures with an unstained nucleus. **B1-B4:** at higher magnification, the granular staining lacks bright clusters (as seen for \(\alpha2\) in panel A1) and shows no obvious relationship to gephyrin clusters (B3; green) or VGAT-positive terminals (B4; blue). Scale bars: A-B, 20 \(\mu\)m; A1-A4, B1-B4, 5 \(\mu\)m. Adapted from (Panzanelli *et al.*, 2011).
Figure 3
Schematic depiction of major postsynaptic proteins interacting with GABA_A Rs and their putative organization 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 scaffolding assembly, to which GABA_A Rs and collybistin bind. C: Basic molecular organization of the GABAergic PSD, depicting the presence of NL2 (interacting with presynaptic neurexin isoforms and with gephyrin), collybistin splice variants, in both active and inactive conformation, and interacting with the small GTPases CDC-42 and/or TC-10, in addition to gephyrin and GABA_A Rs. 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_A Rs moving via lateral diffusion in the membrane. The size of each molecule is depicted roughly relative to its molecular weight.
Graphical abstract

GABA\(_A\) receptor heterogeneity arises through combinatorial assembly of a large family of subunits to generate multiple receptor subtypes. It is an important facet of the variety of GABAergic signaling in adult and developing CNS, and a key factor underlying GABAergic synaptic plasticity underlying excitatory/inhibitory balance in neuronal circuits. This review presents and discusses recent progress in elucidating the relevance of GABA\(_A\) receptor heterogeneity for CNS function in health and disease.
Figure 1
Figure 2
Figure 3

A. GABA<sub>\text{A}</sub> R subtypes and gephyrin. GABA<sub>\text{A}</sub> Rs and collybistin isoforms interacting with gephyrin trimers (top view) and hypothetical gephyrin auto-aggregation (G domain trimers and E domain dimers) interacting with GABA<sub>\text{A}</sub> Rs and collybistin isoforms.

B. Geophyrin trimer (top view) and (side view).

C. Geophyrin trimer (top view) and hypothetical gephyrin auto-aggregation (G domain trimers and E domain dimers) interacting with GABA<sub>\text{A}</sub> Rs and collybistin isoforms.

Figure 3