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Regulation of $GABA_A$ receptors by prolonged exposure to endogenous and exogenous ligands

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ABSTRACT

$GABA_A$ receptors mediate most of the fast inhibitory transmissions in the central nervous system. These receptors are pentameric complexes that exhibit high structural and pharmacological heterogeneity, as they can be constructed from 19 distinct subunits. $GABA_A$ receptors are the targets of numerous clinically relevant drugs used to treat various disorders such as anxiety, insomnia and epilepsy. These receptors are also the targets of many volatile anesthetics and drugs of abuse, such as alcohol. This review is focused on the effect of long-term treatment with GABA, and the positive allosteric modulators benzodiazepines, neurosteroids and ethanol on $GABA_A$ receptors. Prolonged exposure of $GABA_A$ receptors to these compounds triggers several adaptive mechanisms that lead to changes in the structure, function and localization of receptors. These changes include $GABA_A$ receptor subunit expression, intracellular trafficking and phosphorylation. These adaptations are relevant to different physiological, pathological and pharmacological conditions and, in most cases, are associated with the development of tolerance. Understanding the molecular mechanisms underlying these regulatory processes will be relevant for therapeutic benefits.

1. Introduction

Brain function depends on a balance between excitation and inhibition. Although most studies on neuronal plasticity have been focused on excitatory transmission, in recent decades, numerous examples of activity-dependent alterations in inhibitory synapses have emerged (for a review, see (Castillo et al., 2011; Flores and Mendez, 2014; Mendez and Bacci, 2011)). Regulation of the expression, subcellular distribution and function of $GABA_A$ receptors, which mediate most of the fast inhibition in the central nervous system, influences neural excitability under physiological, pharmacological and pathological situations. $GABA_A$ receptors are ion channels permeable to chloride and bicarbonate ions (Kaila, 1994). Although $GABA_A$ receptor-mediated responses are predominantly hyperpolarizing and inhibitory in mature neurons, they are depolarizing and excitatory in immature neurons (Ben-Ari et al., 1989). This developmental switch in GABA action appears to be attributable to the differential expression of a set of membrane ion transporters that produces changes in the chloride equilibrium potential during postnatal maturation (Ben-Ari, 2014; Farrant and Kaila, 2007; Rivera et al., 2005). In addition, there are numerous examples of excitatory effects of GABA on the mature brain (Marty and Llano, 2005).

$GABA_A$ receptors are heteropentameric complexes that can be constructed in mammals from 19 different subunits classified in 8 subunit classes according to sequence similarity: α (1-6), β (1-3), γ (1-3), δ, ε, θ, π and ρ (1-3) (Olsen and Sieghart, 2008) (Fig. 1). Alternative splicing generates additional subunit diversity. Although an enormous number of different subunit combinations can theoretically exist, a limited number of receptor subtypes has been detected on the cell surface. Less than 25% of synthesized subunits are part of assembled receptors, suggesting the existence of a mechanism to control receptor oligomerization (Gorrie et al., 1997). The majority of $GABA_A$ receptors comprise 2 α, 2 β and 1 γ subunits, and the most common receptor subtype is formed by 2 α1, 2 β2 and 1 γ2 subunits, comprising 40–50% of brain receptors (Olsen and Sieghart, 2008; Whiting, 2003). GABA has two binding sites on αβγ $GABA_A$ receptors, located at the interface between α and β subunits (Sieghart, 2015). The ρ subunits form homo- and heterooligomeric receptors that are mainly located in the retina (Bormann and Feigenspan, 1995). Different $GABA_A$ receptor subtypes exhibit distinct regional, cellular and subcellular distributions. $GABA_A$ receptors formed by α(1-3) in combination with β and γ subunits are primarily localized at synaptic sites, whereas α5βγ and α(4 or 6)βδ

Abbreviations: GABA, γ-aminobutyric acid; BDNF, brain-derived neurotrophic factor; ERK, extracellular signal-regulated kinase; CaMKII, calcium/calmodulin-dependent protein kinase II; GAD, glutamate decarboxylase; *Lcn2*, lipocalin 2; MAP, mitogen-activated protein; NGFI-A, nerve growth induce gene-A; PKA, protein kinase A; PKC, protein kinase C; TrkB, tropomyosin receptor kinase B; VGCC, voltage-gated calcium channel

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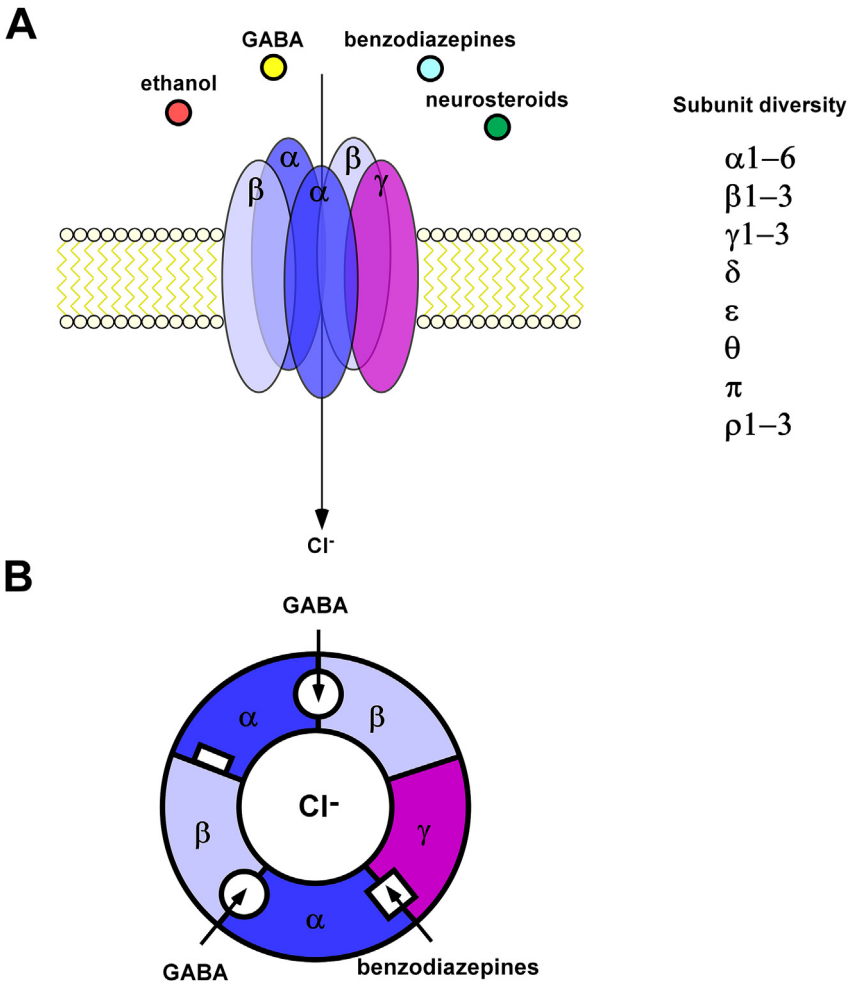

Fig. 1. $GABA_A$ receptor structure. A Schematic representation of $GABA_A$ receptors. $GABA_A$ receptors are pentameric complexes that can be formed from a repertoire of 19 different subunit subtypes. The majority of receptors are composed of two α, two β and one γ subunits. They are targets of numerous drugs, such as benzodiazepines, neurosteroids and ethanol. **B** Top view of $GABA_A$ receptors. GABA has two binding sites on αβγ receptors, at the interfaces between α and β subunits. The benzodiazepine binding site is located at the interface between α and γ subunits.

receptors are predominantly localized at extrasynaptic sites (Brunig et al., 2002; Nusser et al., 1998). In addition, the presence of αβ receptors at extrasynaptic sites has been demonstrated in hippocampal neurons (Mortensen and Smart, 2006).

$GABA_A$ receptors are the site of action of numerous compounds, such as benzodiazepine-site ligands, barbiturates, neurosteroids, anesthetics and ethanol (Sieghart, 1995). Adaptive alterations in $GABA_A$ receptors induced by prolonged exposure to these drugs contribute to the development of tolerance and dependence. This review is focused on the effect of sustained exposure of $GABA_A$ receptors to GABA, benzodiazepines, neurosteroids and ethanol under physiological and pharmacological conditions.

2. GABA agonists

Use-dependent regulation of $GABA_A$ receptors is relevant to diverse physiological and pathological conditions. After a single action potential, GABA reaches high concentrations (mM) in the synaptic cleft and is then rapidly cleared (Clements, 1996; Cherubini and Conti, 2001). However, the high basal firing rates of GABAergic neurons (5–50 Hz, meaning one action potential every 200-20 ms) (Czurko et al., 1999) in addition to the long-lasting activation of the $GABA_A$ receptor (decay constants of 50 and 171 ms) (Bianchi and Macdonald, 2001; Jones and Westbrook, 1995) support the conclusion that at least a fraction of postsynaptic receptors (approximately 30%) are persistently activated by millimolar neurotransmitter concentrations under physiological conditions. This conclusion suggests the existence of regulatory mechanisms to control GABA responses. Increases in extracellular GABA concentration are more evident under pathological conditions. For example, numerous studies have demonstrated a significant increase in GABA levels during epileptic seizures (Thomas et al., 2003; Wilson et al., 1996) and ischemic episodes (Kett-White et al., 2005; Li and Yan, 2010). These changes may result in enhanced stimulation of $GABA_A$ receptors and subsequent downregulation of the receptor function. In fact, an increase in $GABA_A$ receptor endocytosis which leads to a decrease in synaptic receptors has been demonstrated in animal models of *status epilepticus* (Goodkin et al., 2005; Terunuma et al., 2008). In addition, selective alterations in the mRNA levels of $GABA_A$ receptor subunits have been detected in rat models of temporal lobe epilepsy (Brooks-Kayal et al., 1998).

Insights into the mechanism of agonist-induced $GABA_A$ receptor regulation have been provided by cell culture experiments. The exposure of chick brain cultures to GABA for 48 h induced a decrease in the number of $GABA_A$ receptors and the uncoupling of GABA/benzodiazepine site interactions with a half-time of 24–25 h, detected by means of binding assays (Roca et al., 1990a). Downregulation of the receptor number was associated with a reduction of mRNA levels of the α1, β2S and γ1 $GABA_A$ receptor subunits, probably due to the transcriptional repression of the subunit genes (Lyons et al., 2000; Russek et al., 2000). This regulatory process was dependent on the calcium

influx through L-type voltage-gated calcium channels (VGCCs) (Lyons et al., 2001). The decrease in $GABA_A$ receptor number observed in mouse cortical neuronal cultures treated with GABA for 5 days was accompanied by a reduction in GABA-gated chloride influx (Mehta and Ticku, 1992). In contrast, GABA-induced uncoupling was independent of L-type VGCC activation (Lyons et al., 2001), suggesting that downregulation and uncoupling are induced by the activation of distinct signal transduction pathways.

A brief treatment of rat cortical neurons with GABA (half-time of 3 min) has been demonstrated to be sufficient to induce uncoupling of GABA/benzodiazepine interactions hours later (half-time of 12 h), suggesting that uncoupling is relevant to physiological conditions involving the persistent activation of $GABA_A$ receptors for several minutes. This rapidly induced uncoupling occurs in the absence of changes in $GABA_A$ receptor number (Gravielle et al., 2005), further suggesting that the two regulatory processes, uncoupling and downregulation of receptor number, are mediated by independent mechanisms. The mechanism of GABA-induced uncoupling is still unknown, but it has been suggested to be mediated by increased $GABA_A$ receptor internalization (Gutiérrez et al., 2014b). The agonist-induced internalization of $GABA_A$ receptors has also been demonstrated in rat hippocampal cultures exposed to muscimol for 30 min and in cell lines expressing recombinant $GABA_A$ receptors treated for 30 min with GABA (Chaumont et al., 2013). Studies from Barnes'group performed in chick brain cultures suggested that GABA induced an increase in the sequestration of $GABA_A$ receptors, followed by degradation, after several hours of exposure (Calkin and Barnes, 1994). Longer exposures to the neurotransmitter resulted in downregulation of the mRNA levels of the α1, β2, β4, γ1 and γ2 $GABA_A$ receptor subunits (Baumgartner et al., 1994). These authors have hypothesized that internalized receptors may provide the signal for subsequent regulation of receptor subunit gene expression (Barnes, 2000). Thus, uncoupling might represent an early regulatory event that is followed by downregulation of receptor number after longer GABA exposures.

Uncoupling induced by a short-time (10 min) exposure of rat cortical cultures to GABA was associated with a decrease in the percentage of receptors containing α3 subunits (Gutiérrez et al., 2014b). Therefore, a change in the receptor subunit composition may produce receptors with a lower degree of coupling between GABA and benzodiazepines. In addition, the uncoupling induced by this brief GABA exposure was contingent on the phosphorylation of the $GABA_A$ receptor γ2 subunit by protein kinase C (PKC) (Gutiérrez et al., 2014a). Collectively, these results may indicate that GABA-induced uncoupling is initiated by a $GABA_A$ receptor internalization step that activates multiple signal transductional pathways leading to several adaptive changes in the structure and function of $GABA_A$ receptors.

Some reports have demonstrated that $GABA_A$ receptor activity induces novel mechanisms of synaptic plasticity that involve changes in receptor localization. The exposure of hippocampal cultures to muscimol for 30–120 min reduced $GABA_A$ receptor and gephyrin levels at synapses and accelerated lateral diffusion of the receptors. The opposite effects were produced by a treatment with the $GABA_A$ receptor antagonist gabazine (Gouzer et al., 2014). The following results from experiments performed in rat cortical cultures have suggested the occurrence of agonist-induced structural changes that decrease the strength of the GABAergic synapse (Brady et al., 2018). A 30-min treatment with muscimol produced a reduction in the synaptic localization of γ2-containing $GABA_A$ receptors and the gephyrin postsynaptic scaffold concomitantly with a decrease in synaptic currents and an increase in tonic currents mediated by this receptor population. In addition, this treatment produced a decrease in presynaptic glutamate decarboxylase 65 (GAD65) levels. These alterations resulted from the activation of different signaling pathways. Extracellular signal-regulated kinase (ERK) and brain-derived neurotrophic factor (BDNF) signaling mediated the dispersal of γ2-containing $GABA_A$ receptors from synapses, whereas the activation of ERK alone was responsible for gephyrin declustering. The reduction in presynaptic GAD65 was the result of BDNF/tropomyosin receptor kinase B (TrkB) signaling activation. The muscimol-induced depolarization stimulated calcium release from intracellular stores which in turn activated ERK and stimulated the release of BDNF. Altogether, these results indicate that activation of $GABA_A$ receptors can contribute to alterations in synaptic and circuit function on a timescale of minutes (Brady et al., 2018).

3. Benzodiazepines

Benzodiazepines have anxiolytic, sedative, muscle relaxant, hypnotic and anticonvulsant actions, and they facilitate anesthesia (Haefely, 1989). These pharmacological effects are mediated by binding to $GABA_A$ receptors (Haefely, 1984). The benzodiazepine binding site is located at the interface between α and γ $GABA_A$ receptor subunits (Sieghart, 2015; Smith and Olsen, 1995). The presence of particular α subunit isoforms in a receptor subtype is the main determinant of the sensitivity of the $GABA_A$ receptor to benzodiazepines. Thus, the presence of α1, α2, α3 or α5 subunits in $GABA_A$ receptors confers benzodiazepine sensitivity, whereas receptors containing α4 or α6 subunits are insensitive to benzodiazepines (Duncalfe et al., 1996; Wieland et al., 1992). Benzodiazepines increase the opening frequency of receptors without altering the mean open time or conductance (Vicini et al., 1987). By enhancing the action of endogenous GABA instead of directly activating the receptor, benzodiazepines exhibit a large therapeutic index and low toxicity. In addition, evidence suggests that benzodiazepines influence the gating of $GABA_A$ receptors. In fact, some reports support a two-state allosteric model in which benzodiazepine binding stabilizes the active state of the receptor relative to the inactive state (Campo-Soria et al., 2006; Downing et al., 2005). Moreover, the results from another study suggest that benzodiazepines allosterically modulate $GABA_A$ receptors by shifting the equilibrium between the ligand-bound resting and preactivated steps before channel opening (Gielen et al., 2012). In addition to modulating $GABA_A$ receptor gating and/or GABA binding, a more recent study demonstrated that benzodiazepine site ligands can also alter the amplitude of synaptic currents by rapidly influencing receptor diffusion and synaptic aggregation (Levi et al., 2015).

Long-term treatments with benzodiazepines are limited by the development of tolerance to the majority of their pharmacological actions and by the development of dependence (von Moltke and Greenblatt, 2003; Westra and Stewart, 2002). The development of tolerance to each pharmacological effect follows different temporal courses, suggesting the existence of multiple mechanisms. Tolerance to the sedative and hypnotic effects develops first, followed by tolerance to the anticonvulsant effects. Tolerance to the anxiolytic effects is observed after longer exposures in animal models, but it is not clear if this occurs in patients (File, 1985; Hutchinson et al., 1996). Benzodiazepine dependence manifests as a withdrawal syndrome, which is observed after discontinuation of prolonged treatments; this withdrawal is characterized by effects opposite to those produced by acute benzodiazepine administration (Khong et al., 2004; Licata and Rowlett, 2008). Because the overt effects of benzodiazepine are mediated by $GABA_A$ receptors, tolerance and dependence may be the result of adaptive changes in these receptors; however, additional alterations in other neurotransmitter systems may also be involved (Allison and Pratt, 2003).

Although the molecular bases of tolerance and dependence are still unknown, multiple alterations in $GABA_A$ receptor structure, localization and function have been reported as a consequence of long-term benzodiazepine treatments (Bateson, 2002; Gravielle, 2016; Vinkers and Olivier, 2012; Wafford, 2005). Most reports have indicated that prolonged *in vivo* or *in vitro* benzodiazepine treatments do not produce alterations in $GABA_A$ receptor number (Ferreri et al., 2015; Gallager et al., 1984; Heninger and Gallager, 1988; Impagnatiello et al., 1996; Lewin et al., 1989; Ramsey-Williams et al., 1994; Stephens and Schneider, 1985); however, few studies have reported changes in

receptor density. For example, a 4-week administration of flurazepam in rats has been demonstrated to produce a reduction in the maximal binding of flunitrazepam in the cerebral cortex (Rosenberg and Chiu, 1981). In contrast, the exposure of HEK293 cells expressing recombinant $GABA_A$ receptors to diazepam for 48–72 h induced an increase in the maximal binding of flunitrazepam and muscimol (Pericic et al., 2007).

Persistent benzodiazepine treatments induced selective changes in $GABA_A$ receptor subunit levels. The results are mixed due to differences in the treatment paradigm, species and bran region investigated (Bateson, 2002; Gravielle, 2016; Uusi-Oukari and Korpi, 2010; Vinkers and Olivier, 2012). Different reports suggest a switch in receptor subunits, although, unfortunately, most of the studies did not directly analyze the subunit composition of receptors. For example, a decrease in the maximal binding capacity of zolpidem, a ligand selective for α1-containing $GABA_A$ receptors, was observed in the cerebellum of rats chronically treated with flurazepam in the absence of changes in flunitrazepam binding (Wu et al., 1994). These results suggest a change in receptor subunit composition. On the other hand, a 24-h treatment of hippocampal neurons with flurazepam produced a decrease in the number of $GABA_A$ receptors containing α2 subunits due to increased degradation, without changes in α1 subunit levels (Jacob et al., 2012), suggesting an alteration in the combination of receptor subunits.

Numerous studies have demonstrated that prolonged *in vivo* or *in vitro* exposure to benzodiazepines produces uncoupling of GABA/benzodiazepine site interactions, estimated by means of electrophysiological (Prasad and Reynolds, 1992; Tietz et al., 1999), chloride uptake (Hu and Ticku, 1994a; b; Marley and Gallager, 1989) and binding assays (Ali and Olsen, 2001; Ferreri et al., 2015; Gallager et al., 1984; Hu and Ticku, 1994a; Klein et al., 1994; Pericic et al., 2007; Primus et al., 1996; Roca et al., 1990b; Tietz et al., 1989; Wong et al., 1994). A report from Holt et al. demonstrated that a single diazepam dose in rats produced uncoupling in the cerebral cortex hours later (Holt et al., 1999). These results may indicate that uncoupling is the initial step of an adaptive process that leads to benzodiazepine tolerance. The link between uncoupling and tolerance has not yet been established.

The mechanism of benzodiazepine-induced uncoupling, similar to that of GABA-induced uncoupling, seems to involve a step of increased $GABA_A$ receptor endocytosis. The Barnes group demonstrated that a 7-day lorazepam treatment of mice induced the sequestration of $GABA_A$ receptors on clathrin-coated vesicles, suggesting an increase in receptor internalization (Tehrani and Barnes, 1997). Results from the same group also indicated that clathrin-coated vesicles isolated from bovine brain contained uncoupled $GABA_A$ receptors (Tehrani et al., 1997). In agreement with the results from these *in vivo* experiments, Ali and Olsen (2001) reported that the uncoupling induced by a 60-h diazepam treatment of a cell line expressing recombinant $GABA_A$ receptors was produced by the acidic environment inside intracellular vesicles as a consequence of an increase in receptor internalization.

Several reports indicate that the adaptive changes in $GABA_A$ receptors produced by prolonged benzodiazepine treatments are mediated by phosphorylation/dephosphorylation mechanisms. The decline in GABA response in CA1 pyramidal neurons induced by 1-week flurazepam administration was associated with changes in PKA activity (Lilly et al., 2003). In addition, a more recent study demonstrated that a 7-day diazepam treatment of rats resulted in an increase in the phosphorylation of $GABA_A$ receptor γ2 subunits at serine 327 in the cerebral cortex (Ferreri et al., 2015). Similar results were derived from *in vitro* treatments suggesting that sustained exposure of cell cultures to benzodiazepines involves changes in the activity of protein kinases. The downregulation of the $GABA_A$ receptor α1 subunit produced by a 48-h treatment of rat cerebellar granule cells with flunitrazepam was prevented by the protein kinase inhibitor staurosporine (Brown and Bristow, 1996). On the other hand, the uncoupling produced by the exposure of $GABA_A$ receptors expressed in a cell line to diazepam for 60 h was blocked by PKA activation (Ali and Olsen, 2001).

Insights into the signaling pathways activated by benzodiazepines that are responsible for the adaptive alterations in the central nervous system have been provided by several lines of evidence. Some reports have investigated the benzodiazepine-induced changes in transcript levels by means of microarray experiments. One study (Huopaniemi et al., 2004) reported that acute diazepam administration produced a decrease in the mRNA levels of the α subunit of the calcium/calmodulin-dependent protein kinase II (CaMKIIα), BDNF, mitogen-activated protein (MAP) kinase phosphatase, transcription factor GIF, c-fos and nerve growth factor-induced gene-A in the mouse cerebral cortex by the selective activation of α1-containing $GABA_A$ receptors. Notably, the decrease in the CaMKIIα transcript level persisted 40 h after benzodiazepine administration, suggesting a role in the development of tolerance and dependence. Another microarray study (Furukawa et al., 2017) demonstrated that lipocalin 2 (*Lcn2*) mRNA was the most upregulated gene in the mouse cerebral cortex, hippocampus and amygdala after a 10-day diazepam treatment, suggesting a correlation with iron homeostasis. On the other hand, some reports have suggested a role of VGCC activation mediating the effects of long-term benzodiazepine treatments. A one-week treatment of rats with flurazepam stimulated L-type VGCC-mediated calcium currents (Xiang et al., 2008) and induced a reduction in $GABA_A$ receptor-mediated synaptic currents that was dependent on L-type VGCC activation in hippocampal CA1 neurons (Xiang and Tietz, 2008). In addition, a 3-day exposure of mouse cortical neurons to different benzodiazepines produced the upregulation of L-type VGCCs (Katsura et al., 2007).

4. Neurosteroids

Neurosteroids are defined as steroids synthesized *de novo* in the brain by neurons and glia from cholesterol independently of peripheral steroidogenic endocrine glands (Baulieu, 1981). In addition, the term neuroactive steroid refers to natural or synthetic steroids that rapidly alter neuronal excitability by binding to cell surface receptors (Paul and Purdy, 1992). Numerous neurosteroids potentiate $GABA_A$ receptor function and have anxiolytic, sedative, hypnotic, anticonvulsant and anesthetic effects (Belelli and Lambert, 2005; Paul and Purdy, 1992). At low concentrations (nanomolar range), these neurosteroids act as positive allosteric modulators of $GABA_A$ receptors and, at higher concentrations, they can directly activate the receptor in the absence of GABA (for a review, see (Carver and Reddy, 2013)). Positive neurosteroid modulation of $GABA_A$ receptors occurs by increasing both the frequency and duration of channel opening (Hosie et al., 2007; Lambert et al., 2009). Two neurosteroid binding sites within the transmembrane domains of the α and β subunits for allosteric modulation or direct activation of the $GABA_A$ receptor, respectively, were initially identified based on homology modeling and mutagenesis studies (Hosie et al., 2006, 2007; Lambert et al., 2009; Mitchell et al., 2008; Sieghart, 2015). However, in a recent report, the crystal structure of the transmembrane domain of the $GABA_A$ receptor α1 subunit was analyzed using a receptor chimera (Laverty et al., 2017). The results from this study indicated that the binding site for potentiating neurosteroids was located at the subunit interface and that a single site was responsible for both activation and potentiation. In contrast to potentiating neurosteroids, some sulfated neurosteroids inhibit $GABA_A$ receptor function (Belelli and Lambert, 2005; MacKenzie and Maguire, 2013). These negative modulators act as noncompetitive antagonists of $GABA_A$ receptors, reducing the frequency of channel opening (Reddy, 2010). Analysis of the crystal structure of the $GABA_A$ receptor α1 chimera suggested that inhibitory neurosteroids bind to a discrete intrasubunit site within the transmembrane domain (Laverty et al., 2017). Unlike benzodiazepines, neurosteroids modulate most $GABA_A$ receptor subtypes; however, $GABA_A$ receptors containing δ subunits are more sensitive to neurosteroids (Lambert et al., 2003).

The allosteric modulation of $GABA_A$ receptors by neurosteroids is

influenced by the activity of protein kinases (Comenencia-Ortiz et al., 2014; Nakamura et al., 2015). The activation of protein kinases enhances the positive neurosteroid modulation of $GABA_A$ receptors. Inhibition of PKA or PKC reduced the sensitivity of the $GABA_A$ receptor to neurosteroids in rat CA1 neurons (Harney et al., 2003) and the hypothalamus (Fancsik et al., 2000), whereas in rat dentate gyrus neurons (Harney et al., 2003) and *Xenopus* oocytes expressing recombinant $GABA_A$ receptors (Leidenheimer and Chapell, 1997), the stimulation of PKC enhanced neurosteroid effects. In addition, the phosphorylation of the β3 and α4 subunits by PKC in extrasynaptic $GABA_A$ receptors expressed in HEK293 cells was necessary for the potentiating effects of neurosteroids (Adams et al., 2015). The reduction in neurosteroid sensitivity observed in $GABA_A$ receptor δ-deficient mice is restored by PKC activation (Vicini et al., 2002). In contrast, kindling (a model of temporal lobe epilepsy) stimulated the phosphorylation of $GABA_A$ receptors, resulting in a reduction in the modulatory effect of neurosteroidsof the pyramidal neurons in the rat piriform cortex (Kia et al., 2011). These controversial results may be explained by differences in the subunit composition of $GABA_A$ receptors. On the other hand, through a reciprocal regulatory mechanism, neurosteroids stimulate the phosphorylation of $GABA_A$ receptors. Neurosteroids enhanced the phosphorylation of $GABA_A$ receptor α4 subunits, leading to increased insertion of α4-containing receptors into the plasma membrane and a consequent stimulation of tonic inhibition in the mouse hippocampus (Abramian et al., 2014). Additionally, an increase in the phosphorylation of $GABA_A$ receptor β3 subunits by neurosteroids was reported in HEK293 cells expressing recombinant receptors (Adams et al., 2015).

Chronic exposure of $GABA_A$ receptors to endogenous neurosteroids under different physiological conditions, such as the estrous cycle and pregnancy, induces tolerance associated with alterations in receptor function (Maguire and Mody, 2007, 2009; Turkmen et al., 2010). The effect of long-term neurosteroid treatments on the expression of $GABA_A$ receptor subunits has been extensively studied by means of *in vivo* and *in vitro* experiments. Prolonged *in vivo* treatments with progesterone or allopregnanolone produced an increase in the $GABA_A$ receptor α4 (Gulinello et al., 2001a; Shen et al., 2005; Uusi-Oukari and Korpi, 2010) and δ subunits (Maguire and Mody, 2007; Shen et al., 2005; Smith et al., 2007; Uusi-Oukari and Korpi, 2010) and a decrease in the α1 and γ2 subunits (Shen et al., 2005; Uusi-Oukari and Korpi, 2010) in the rodent hippocampus. These changes may result in an increase in tonic inhibition. Neurosteroid-induced upregulation of the α4 subunit was detected in association with an increase in anxiety in the elevated plus maze (Gulinello et al., 2001b). The results of *in vitro* experiments have indicated that continuous progesterone exposure in rodent cerebellar cultures induced a decrease in the mRNA levels of the $GABA_A$ receptor α1, α3, α5 and γ2 subunits (Biggio et al., 2003; Follesa et al., 2000, 2004), whereas the persistent progesterone treatment of rodent cerebrocortical cultures produced a decrease in the mRNA levels of the α2, α3, β2 and β3 subunits (Follesa et al., 2004). The investigation of the mechanism of the regulation of $GABA_A$ receptor subunit expression has been focused on the α4 subunit. The results from Roberts et al., 2005, 2006 indicated that the increase in α4 subunit gene expression in rat hippocampal neurons produced by *status epilepticus* was mediated by BDNF, which induced the expression of the early growth response factor 3 (Egr3) via a PKC/MAPK-dependent pathway. Likewise, Gangisetty et al. (Gangisetty and Reddy, 2010) reported that the α4 upregulation induced in the rat hippocampus by progesterone withdrawal was mediated by Egr3 via a progesterone receptor-independent mechanism.

Chronic neurosteroid treatments produced, similar to GABA and benzodiazepines, the uncoupling of GABA/benzodiazepine site interactions. Thus, prolonged *in vivo* progesterone administration produced a reduction in the potentiation of GABA currents by benzodiazepines in the rat hippocampus (Gulinello and Smith, 2003). In a similar fashion, the persistent exposure of chick brain cultures to pregnenolone induced a decrease in the stimulation of benzodiazepine binding by GABA without changes in the total receptor number (Friedman et al., 1993, 1996).

5. Ethanol

Ethanol acts in the central nervous system by modulating different ion channels, such as $GABA_A$, glycine and NMDA receptors (Crews et al., 1996). The regulation of GABAergic transmission is responsible for many of the behavioral effects of ethanol, including anxiolytic, anticonvulsant, sedative/hypnotic, cognition-impairing and motor-incoordination-inducing actions (Kumar et al., 2009). These effects are similar to those produced by benzodiazepines. The ethanol binding site is still unknown, but two regions of the $GABA_A$ receptor have been demonstrated to be critical for the ethanol modulation occurring at the α/β subunit interface and in transmembrane regions 2 and 3 of the α or β subunits (Forstera et al., 2016; Mihic et al., 1997; Ueno et al., 2000).

The mechanisms underlying the acute actions of ethanol on the GABAergic system are complex because they include direct and indirect effects on $GABA_A$ receptors. Acute ethanol directly stimulates the function of the typical heteromeric $GABA_A$ receptors formed by the 2α, 2β and 1γ or δ subunits (Kumar et al., 2009). The acute indirect actions of ethanol on $GABA_A$ receptors include the phosphorylation of $GABA_A$ receptors, elevation of neurosteroid levels and regulation of presynaptic GABA release (Kumar et al., 2009). It has been demonstrated that changes in the phosphorylation state of the $GABA_A$ receptor by PKA, PKC and fyn kinase are involved in some of the ethanol-induced alterations in receptor function (Kumar et al., 2009; Trudell et al., 2014). On the other hand, ethanol administration produces an increase in the brain and plasma levels of neurosteroids, reaching physiologically relevant concentrations that can stimulate GABA responses (Khisti et al., 2005). Finally, ethanol-stimulated presynaptic GABA release is responsible for many of the effects of ethanol on GABAergic neurotransmission (Kumar et al., 2009; Trudell et al., 2014).

Chronic ethanol administration induces tolerance to most of its effects on the GABAergic system, such as sedation, motor incoordination and cognitive impairment, and produces cross-tolerance to the actions of benzodiazepines and barbiturates. Tolerance to ethanol was accompanied by a loss of the ethanol-induced elevation in neurosteroid levels (Morrow et al., 2001). Tolerance is associated with different adaptive alterations of the GABAergic system (Grobin et al., 1998; Kumar et al., 2009). Persistent ethanol exposure produces, similar to GABA, benzodiazepines and neurosteroids, the uncoupling of GABA/benzodiazepine site interactions. For instance, chronic *in vivo* intermittent ethanol exposure induced a decrease in the benzodiazepine-mediated potentiation of miniature inhibitory postsynaptic currents in the rat hippocampus (Cagetti et al., 2003). Furthermore, a decrease in the ability of benzodiazepines to enhance muscimol-induced chloride uptake in the mouse cerebral cortex was produced by prolonged *in vivo* ethanol administration (Buck and Harris, 1990).

Prolonged ethanol treatments result in the regulation of $GABA_A$ receptor subunit expression, which varies according to the brain region. For example, chronic *in vivo* treatments with ethanol induce a decrease in α1, α2, and α3 and an increase in α4, β1, β2, β3, γ1 and γ2 subunit mRNA and peptide levels in the rodent cerebral cortex (Uusi-Oukari and Korpi, 2010). The *in vitro* exposure of cortical cultures to ethanol produces a decrease in α1, α2 and γ2 and an increase in α4 subunit mRNA and peptide levels (Uusi-Oukari and Korpi, 2010).

Alterations in the synaptic localization of $GABA_A$ receptors have been reported after chronic ethanol treatments. Thus, the pharmacological changes in receptors, detected by means of electrophysiological experiments, suggest that the expression of α4-containing receptors increased at synapses and decreased at extrasynaptic sites in the rat hippocampus following a chronic *in vivo* intermittent ethanol treatment (Liang et al., 2004). The translocation of α4-$GABA_A$ receptors from extrasynaptic to synaptic sites was further supported by electron microscopy, which showed an increase in the synaptic localization of α4

Table 1
Principal alterations in $GABA_A$ receptors induced by chronic exposure to GABA and positive allosteric modulators.

Alteration	Treatment			
	GABA	Benzodiazepines	Neurosteroids	Ethanol
Downregulation of receptor number	+ (Mehta and Ticku, 1992; Roca et al., 1990a)	– (Ferreri et al., 2015; Gallager et al., 1984; Impagnatiello et al., 1996; Ramsey-Williams et al., 1994)	– (Friedman et al., 1993, 1996)	– (Uusi-Oukari and Korpi, 2010)
Uncoupling	+ (Gravielle et al., 2005; Lyons et al., 2000; Roca et al., 1990a)	+ (Bateson, 2002; Gravielle, 2016; Vinkers and Olivier, 2012)	+ (Friedman et al., 1993, 1996; Gulinello and Smith, 2003)	+ (Buck and Harris, 1990; Cagetti et al., 2003)
Internalization	+ (Calkin and Barnes, 1994; Chaumont et al., 2013; Gravielle et al., 2005)	+ (Ali and Olsen, 2001; Tehrani and Barnes, 1997)	**ND**	+ (Kumar et al., 2003)
Changes in subunit levels	+ (Gravielle et al., 2005; Lyons et al., 2000)	+ (Bateson, 2002; Ferreri et al., 2015; Vinkers and Olivier, 2012)	+ (Uusi-Oukari and Korpi, 2010)	+ (Uusi-Oukari and Korpi, 2010)
Posttranslational modifications (phosphorylation)	+ (Gravielle et al., 2005)	+ (Ferreri et al., 2015)	**ND**	+ (Kumar et al., 2002)

+: evidence of alteration; -: no alteration; ND: not determined.

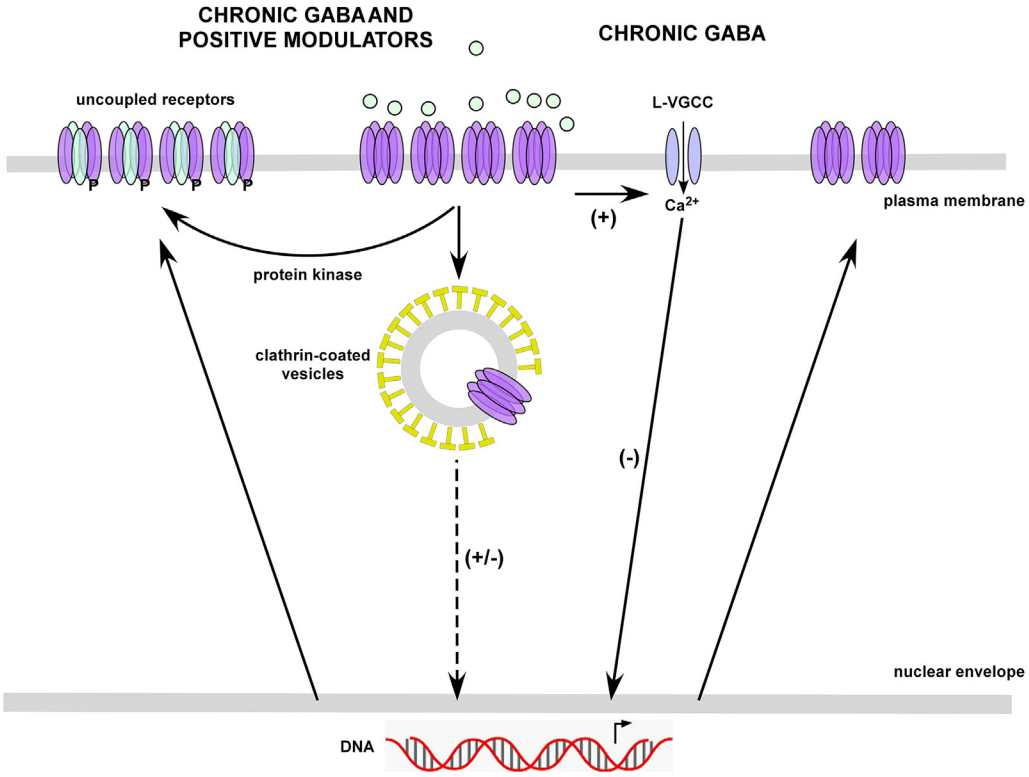

Fig. 2. Effect of prolonged exposure of $GABA_A$ receptors to GABA, benzodiazepines, neurosteroids and ethanol. Chronic GABA produces downregulation of receptor number and uncoupling of GABA/benzodiazepine site interactions. Downregulation is probably the result of transcriptional repression of subunit genes and depends on the activation of L-type VGCCs (L-VGCC). Persistent exposure to positive modulators induces uncoupling without changes in receptor number. The mechanism of uncoupling remains unknown but it seems to be mediated by multiple alterations in $GABA_A$ receptors, such as an increase in receptor internalization, receptor phosphorylation, and changes in receptor composition. It has been hypothesized that internalized receptors provide a signal for subsequent regulation of subunit gene expression.

subunits (Liang et al., 2006).

Adaptive changes in $GABA_A$ receptors induced by chronic ethanol also include regulation of the intracellular trafficking of receptors. Chronic administration of ethanol in rats produced a selective increase in the internalization of α1-containing $GABA_A$ receptors and a consequent decrease in these receptors in the synaptic fraction from the cerebral cortex. In contrast, these treatments failed to induce a change in the internalization of α4-containing receptors (Kumar et al., 2003). This differential regulation of receptor endocytosis may be mediated by a phosphorylation mechanism. The chronic treatment of rats with ethanol also induced a decrease in the association of PKCγ with α1-containing $GABA_A$ receptors and a reduction in the expression of these receptors in the plasma membrane in the cerebral cortex. Conversely, an increase in the association of PKCγ with α4-containing $GABA_A$ receptors and in the expression of these receptors at the plasma membrane was observed in the cerebral cortex. The phosphorylation of α4 subunits has been suggested to prevent the recognition of the $GABA_A$ receptor by adaptor complex 2 (AP2), thus inhibiting receptor internalization (Kumar et al., 2002).

It has been demonstrated in rat cortical cultures that protein kinases play a critical role in the regulation of $GABA_A$ receptors by prolonged ethanol exposure. The activation of PKA and PKC had opposite effects on the regulation of $GABA_A$ receptor α1 and α4 subunits. PKA activity positively regulated (Carlson et al., 2013) whereas PKC activity negatively regulated (Kumar et al., 2010) synaptic α1 subunit abundance and function after persistent ethanol treatment. In contrast, PKA activity negatively regulated whereas PKC activity positively regulated synaptic α4 subunit abundance and function following the ethanol

treatment (Carlson et al., 2016a). Ethanol treatment induced an increase in extrasynaptic α4 subunit abundance and function in a PKA-dependent manner, but ethanol activation of PKC did not affect these subunits (Carlson et al., 2016b). The results from the experiments performed in rat cortical cultures in the absence of ethanol provided some insights into the mechanism of the regulation of $GABA_A$ receptors by these protein kinases. The decreased expression of synaptic α4 subunits induced by PKA activation was associated with an increase in the phosphorylation of β3 receptor subunits at S408/409, whereas the increased expression of synaptic α4 subunits induced by PKC activation was accompanied by an increase in the phosphorylation of γ2 receptor subunits at S327 (Bohnsack et al., 2016).

6. Conclusions

The prolonged exposure of $GABA_A$ receptors to GABA or positive allosteric modulators under diverse physiological, pathological and pharmacological conditions produces different adaptive alterations of receptors (Table 1, Fig. 2). The principal adaptive changes are down-regulation of receptor number and uncoupling of allosteric interactions between GABA and benzodiazepine sites. Chronic GABA treatments induce both, downregulation and uncoupling; however, sustained exposure to positive allosteric modulators only produces uncoupling, suggesting the existence of two independent regulatory mechanisms.

The molecular bases of these $GABA_A$ receptor alterations have been investigated by means of *in vivo* and *in vitro* studies. Experiments performed in cell cultures seem to reproduce quite well the results from *in vivo* treatments. Downregulation of $GABA_A$ receptor number is induced by transcriptional repression of receptor subunit genes and is probably mediated by activation of L-type VGCCs. The mechanism underlying the uncoupling of GABA/benzodiazepine site interactions is unclear but seems to be associated with different alterations in $GABA_A$ receptors, such as an increase in receptor internalization, changes in receptor subunit composition and/or posttranslational modifications.

The comprehension of the mechanisms underlying these adaptive processes is important for therapeutic purposes. For example, the elucidation of the molecular bases of benzodiazepine tolerance will help to design new drugs that can maintain their efficacies during long-term treatments. On the other hand, understanding the ethanol-induced adaptations will be crucial for the treatment of alcoholism.

Conflicts of interest

None.

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