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Invited review

Activation-induced regulation of $GABA_A$ receptors: Is there a link with the molecular basis of benzodiazepine tolerance?

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ABSTRACT

Benzodiazepines have been used clinically for more than 50 years to treat disorders such as insomnia, anxiety, and epilepsy, as well as to aid muscle relaxation and anesthesia. The therapeutic index for benzodiazepines if very high and the toxicity is low. However, their usefulness is limited by the development of either or both tolerance to most of their pharmacological actions and dependence. Tolerance develops at different rates depending on the pharmacological action, suggesting the existence of distinct mechanisms for each behavioral parameter. Alternatively, multiple mechanisms could coexist depending on the subtype of $GABA_A$ receptor expressed and the brain region involved. Because most of the pharmacological actions of benzodiazepines are mediated through $GABA_A$ receptor binding, adaptive alterations in the number, structure, and/or functions of these receptors may play an important role in the development of tolerance. This review is focused on the regulation of $GABA_A$ receptors induced by long-term benzodiazepine exposure and its relationship with the development of tolerance. Understanding the mechanisms behind benzodiazepine tolerance is critical for designing drugs that could maintain their efficacy during long-term treatments.

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Contents

Abbreviations: GABA, γ-aminobutyric acid; BDNF, brain derived neurotrophic factor; CaMKII, calcium/calmodulin-dependent kinase II; PKC, calcium/phospholipid-dependent protein kinase; PKA, cAMP-dependent protein kinase; NGFI-A, nerve growth factor induced factor gene-A; PKG, cGMP-dependent protein kinase; PRIP1, phospholipase C-related catalytically inactive protein 1; PKB, protein kinase B.

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1. Introduction

GABA, the main inhibitory neurotransmitter in the central nervous system, is present in at least 30% of synapses. The actions of GABA result from binding to two classes of receptors, $GABA_A$ and $GABA_B$. Ionotropic $GABA_A$ receptors mediate fast inhibitory actions, whereas metabotropic $GABA_B$ receptors are coupled to G proteins and mediate slow and prolonged inhibitory responses. $GABA_A$ receptors are heteropentameric and belong to the cys-loop ligand-gated ion channel superfamily. The nineteen $GABA_A$ receptor subunits identified in mammals are classified in 8 subunit classes based on sequence homology: α (1–6), β (1–3), γ (1–3), δ, ε, θ, π, and ρ (1–3) (Fig. 1A) [1]. Additional subunit diversity is generated by alternative splicing and alternative promoter usage, thereby increasing the number of possible pentameric combinations. However, a limited number of $GABA_A$ receptor subtypes exist in the central nervous system. Most $GABA_A$ receptors are composed of 2 α, 2 β, and 1γ subunits, with the most abundant receptor subtype composed of 2 α1, 2 β2, and 1 γ2 subunits [2].

$GABA_A$ receptors are the target of many different clinically relevant drugs, including benzodiazepines, barbiturates and anesthetics. Benzodiazepines display multiple actions such as anxiolysis, sedation, muscle relaxation, and sleep induction, and they can act as anticonvulsants. Benzodiazepines bind to the extracellular interface between the α and γ subunits (Fig. 1B). $GABA_A$ receptor subtypes containing α1, α2, α3, or α5 subunits in combination with β and γ subunits are sensitive to benzodiazepines, whereas receptors composed of α4 or α6 subunits in combination with β and γ or δ subunits are insensitive to benzodiazepines [3]. The structural elements linking benzodiazepine binding with a modulation of the $GABA_A$ receptor channel function are incompletely understood. Using the substituted cysteine accessibility method, Hanson et al. [4] have investigated the mechanisms by which different benzodiazepine site ligands can potentiate or inhibit GABA responses. They showed that the Loop F region of the $GABA_A$ receptor γ2 subunit plays a role in controlling the efficacy of benzodiazepine ligands and is part of the allosteric pathway by which the binding of a positive benzodiazepine modulator is transmitted to the channel gate.

It was initially proposed that benzodiazepines act only by increasing the affinity of GABA agonists for their receptors. However, subsequent studies have suggested that benzodiazepines also influence $GABA_A$ receptor gating. Indeed, benzodiazepine agonists increase the efficacy of partial $GABA_A$ receptor agonists and act as direct agonists of spontaneously active mutated $GABA_A$ receptors [5,6]. Moreover, another report has indicated that benzodiazepines modulate receptor function by affecting the pre-activation step preceding channel opening [7]. On the other hand, a more recent study demonstrated that benzodiazepine ligands modulate $GABA_A$ receptor-mediated neurotransmission by altering the diffusion and clustering of the receptor at inhibitory synapses [8].

2. Tolerance to benzodiazepines

The prolonged activation of $GABA_A$ receptors induces adaptive changes in the function of the receptors, which constitutes an example of neuronal plasticity. Chronic exposure to different exogenous drugs that potentiate GABA responses, such as benzodiazepines, barbiturates, and ethanol, induces tolerance by a process considered to be homeostatic.

In addition, tolerance can develop under certain physiological conditions, such as during the menstrual cycle and pregnancy, in which $GABA_A$ receptors are continuously exposed to the endogenous ligand allopregnanolone. This tolerance was associated with selective alterations in $GABA_A$ receptor subunit expression [9].

Benzodiazepines have been used in the clinic for over 50 years due to their high therapeutic index and low toxicity. However, their long-term use is limited by the development of tolerance to most of their pharmacological actions and by dependence.

The abrupt discontinuation of long-term benzodiazepine treatments produces a withdrawal syndrome, considered to be a sign of dependence, which is characterized by increased anxiety, insomnia, and sensory disturbances [10,11]. Because the acute effects of benzodiazepines are opposite to those of the withdrawal syndrome, it is tempting to speculate that tolerance and withdrawal result from the same compensatory mechanisms. However, dependence can occur in the absence of tolerance and tolerance can develop without any manifestation of dependence [12]. The symptoms occur for 1 or 2 weeks after discontinuation of the drug [11] and are thought to be the consequence of neuronal adaptations produced over the course of chronic treatments [10]. The dependence liability of benzodiazepines is affected by different factors, such as the dose administered, duration of the treatment, drug-specific pharmacokinetic properties, and individual differences between patients [10]. Discontinuation of treatment, but not the long-term exposure of cerebellar granule cells to diazepam or imidazenil (a benzodiazepine partial agonist), produces a selective increase in the $GABA_A$ receptor α4 subunit mRNA and protein levels, suggesting a change in the receptor subunit composition [13]. A similar alteration was observed after withdrawal of other drugs that positively modulate $GABA_A$ receptor functions [14]. The abuse liability of a drug is related to its propensity to produce dependence [10]. Benzodiazepine abuse often occurs in conjunction with the abuse of other drugs, such as opiates and alcohol [12,15,16].

Benzodiazepine tolerance seems to develop at different rates depending on the pharmacological action. Studies using animal models have indicated that tolerance to the sedative and hypnotic effects of benzodiazepines develops rapidly, followed by tolerance to their anticonvulsant actions. In several preclinical studies, tolerance to the anxiolytic effects has been demonstrated to occur as a consequence of a longer exposure to benzodiazepines, but has been difficult to demonstrate in humans. The different timescale of each tolerance manifestation suggests the existence of distinct mechanisms underlying each of the behavioral parameters. Moreover, multiple adaptive changes can coexist depending on the $GABA_A$ receptor subtype and the brain region involved [17–19].

Tolerance to benzodiazepines may result from alterations in the absorption, distribution, metabolism, or excretion of the drug, which, in turn, lead to a reduction of the benzodiazepine concentrations at the sites of action. However, several studies in humans and animals have shown that the plasma and cortical concentrations of benzodiazepines do not decrease over time during chronic treatment. The plasma diazepam concentrations after acute administration were similar in both patients chronically treated with alprazolam and in untreated patients. However, chronic diazepam treatments induce tolerance to the acute amnesic and sedative

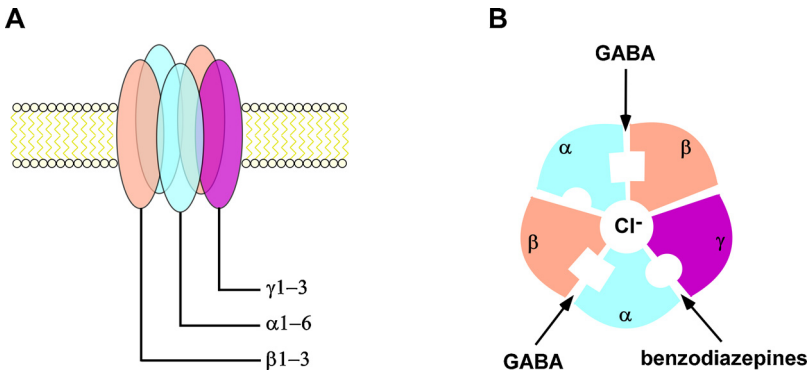

Fig. 1. $GABA_A$ receptor structure. (A) The majority of $GABA_A$ receptors are composed of five subunits belonging to the α, β, and γ subfamilies. (B) A transverse view of the $GABA_A$ receptor. The five subunits assemble to form a chloride-permeable channel. The GABA binding sites are located at the interface between the α and β subunits, while the benzodiazepine binding site is located at the interface between the α and γ subunits.

effects of the drug [20]. Moreover, some reports have indicated that, in rats, the tolerance to the sedative and anxiolytic actions of diazepam induced by chronic treatment was not accompanied by a reduction of the serum and brain concentrations [21–23]. Altogether, these results indicate that benzodiazepine tolerance is not produced by a change in the pharmacokinetic properties of the drugs.

Because most pharmacological effects of benzodiazepines are mediated through binding to the $GABA_A$ receptor, the mechanisms underlying tolerance may involve compensating changes in the number, structure, and/or function of this receptor. In addition, changes in other neurotransmitter systems may also be involved in the development of tolerance. In fact, prolonged benzodiazepine exposure produces alterations in the glutamatergic system [10]. This review is focused on the regulation of the $GABA_A$ receptor in association with benzodiazepine tolerance. From a therapeutic point of view, it is critical to understand the mechanism of benzodiazepine tolerance to achieve the design of drugs that will maintain their efficacy during long-term treatments. Unfortunately, biochemical studies performed in combination with behavioral tests are lacking in the investigation of the molecular mechanisms behind tolerance.

2.1. Role of different $GABA_A$ receptor subtypes

The $GABA_A$ receptor subtype-specificity of benzodiazepine actions has been extensively studied using different strategies, including gene-knockout, gene knock-in (generating point mutations that abolish the binding of classical benzodiazepines), and subtype-selective drugs [16,24–30]. Analyzing the differential contribution of distinct $GABA_A$ receptor subtypes to the adverse effects of benzodiazepines, such as tolerance and dependence, may be important for the development of selective drugs with therapeutic potential.

The role of α1-containing $GABA_A$ receptors in the development of benzodiazepine dependence is controversial [12,31]. Both experiments performed with mice and epidemiological data obtained from patients have suggested that zolpidem, which is selective for α1-containing receptors, has a reduced dependence liability compared to classical benzodiazepines [12]. However, studies performed in non-human primates suggest that withdrawal effects are produced after chronic zolpidem treatment [32,33]. A study from Kovacevic et al. [34] showed that the anxiety-like behavior induced by diazepam withdrawal in rats was not affected by co-administration of the α1-selective neutral modulator βCCt. However, βCCt prevented the decrease in the pentylenetetrazole-induced seizures produced by diazepam withdrawal [34]. These results suggest that the role of the α1-containing $GABA_A$ receptors in the development of dependence varies depending on the behavioral effect analyzed. Mirza and Nielsen [35] reported that, in mice, physical dependence failed to occur after chronic treatments with several subtype-selective compounds. These results may indicate that the manifestation of physical dependence requires the activation of all $GABA_A$ receptor subtypes [12].

Collectively, the results of different groups suggest that benzodiazepine tolerance depends on the activation of specific $GABA_A$ receptor subtypes. In one study, the development of tolerance was analyzed in knock-in mice harboring a mutation in the $GABA_A$ receptor α1, α2, α3, or α5 subunit that results in diazepam-insensitive receptors [36]. In this model, chronic diazepam administration resulted in a decreased sedative effect of the benzodiazepine in wild-type and mutant mice carrying the mutation in the α2 or α3 subunit. Diazepam did not produce a sedative effect in mice with a mutation in the α1 subunit, whereas mice with a mutated α5 subunit did not develop tolerance to this pharmacological effect. These studies suggest that tolerance to diazepam-induced sedation requires the concomitant activation of the α1- and α5-containing $GABA_A$ receptors. These results are in agreement with another study in which tolerance to the anxiolytic, hypothermic, and sedative effects of diazepam was investigated in mice chronically treated with different positive allosteric modulators that bind to the benzodiazepine site [37]. Chronic treatment of mice with diazepam, a non-selective benzodiazepine, produced tolerance to its anxiolytic, hypothermic and sedative effects. Mice chronically treated with bretazenil, a partial non-selective positive allosteric modulator, developed tolerance to the anxiolytic and hypothermic effects of diazepam, but not to its sedative effects. The chronic administration of zolpidem, which is selective for α1-contaning $GABA_A$ receptors and has no efficacy at α5-containing receptors, induced tolerance to the hypothermic effect, partial tolerance to the anxiolytic actions, and no tolerance to the sedative effects of diazepam. Finally, chronic treatment with TPA023, a selective ligand for the α2/3-contaning receptors, failed to produce tolerance to the anxiolytic, hypothermic and sedative actions of diazepam. The lack of tolerance to the sedative actions of zolpidem has also been reported by other research groups [38,39]. Therefore, the chronic activation of $GABA_A$ receptors containing the α1 and α5 subunits may be crucial for the development of tolerance to the sedative effect of diazepam. On the other hand, Auta et al. [40] demonstrated that long-term treatment with zolpidem or diazepam but not with imidazenil, which has low intrinsic efficacy at α1-containing $GABA_A$ receptors and full intrinsic action at α5-containing $GABA_A$ receptors, resulted in anticonvulsant tolerance in rats. Besides, imidazenil did not induce anticonvulsant cross tolerance to diazepam or zolpidem. These results suggest that the prolonged activation of the α1- but not of the α5-containing $GABA_A$

receptors is crucial for the development of tolerance to the anticonvulsant effect of benzodiazepines. In agreement with this, Vlainic et al. [41] showed that repeated diazepam and zolpidem treatments in mice result in tolerance to the anticonvulsant effect, suggesting that the α5-containing receptors are not essential for the development of anticonvulsant tolerance. In contrast to Vinkers et al. [37], these authors [41] also demonstrated that prolonged diazepam and zolpidem administration results in tolerance to the sedative effects, suggesting that activation of the α5 subunit-containing receptors is not necessary for the development of sedative tolerance. These discrepancies may be explained by differences in the treatment paradigms used.

Downstream signaling cascades have been analyzed to understand the mechanism of neuronal adaptation induced by the selective activation of the α1-containing $GABA_A$ receptors by benzodiazepines. Using microarray analyses on cerebral cortex samples, the transcription profile changes induced by acute diazepam administration were compared between wild-type and knock-in mice carrying a mutation in the $GABA_A$ receptor α1 subunits that renders the receptors insensitive to diazepam [42]. It was hypothesized that the transcripts showing expression level changes in wild-type but not in knock-in mice could be involved in the signaling pathways activated selectively by diazepam via the α1-containing receptors and may be associated with the sedative actions of benzodiazepines. In wild-type mice, diazepam treatment induced a decrease in the mRNA levels of the α subunit of calcium/calmodulin-dependent protein kinase II (CaMKIIα), brain-derived neurotrophic factor (BDNF), MAP kinase phosphatase, transcription factor GIF, c-fos and nerve growth induced gene-A (NGFI-A). None of these alterations were induced by diazepam in knock-in mice. Unlike most transcript level changes, the decrease in CaMKIIα mRNA levels persisted for 40 h after the diazepam treatment, suggesting that it can contribute to the development of tolerance and dependence produced by chronic treatments.

3. Effects of long-term benzodiazepine exposure

3.1. $GABA_A$ receptor number

GABA reaches high concentrations (1–3 mM) in the synaptic cleft immediately after an action potential [43], suggesting that saturation of the post-synaptic $GABA_A$ receptors occurs during a synaptic event. Therefore, a change in receptor number would be an effective mechanism to regulate GABAergic transmission. Moreover, the development of tolerance to benzodiazepines could result from a decrease in the number of $GABA_A$ receptors.

It was reported that the oral administration of flurazepam for 4 weeks in rats induces a tolerance to the locomotor impairment caused by flurazepam. Tolerance is associated with a decrease in the maximal binding capacity (B_{max}) of [^{3}H]flunitrazepam (a non-selective benzodiazepine) in the cerebral cortex [44]. The same treatment results in a reduction of the B_{max} of [^{3}H]RY-80 (selective for α5-containing $GABA_A$ receptors) in rat hippocampus, suggesting that changes in a selected population of receptors may contribute to the mechanism of benzodiazepine tolerance [45]. These results suggest that tolerance is mediated by a decrease in the $GABA_A$ receptor number, although very high benzodiazepine concentrations were administered (100–150 mg/kg daily). In contrast, most reports have demonstrated that a prolonged diazepam treatment in rats induces tolerance without altering the maximal binding of benzodiazepines, suggesting a lack of change in the $GABA_A$ receptor number [23,46–51].

The molecular basis of the usage-dependent regulation of $GABA_A$ receptors has also been investigated in cell culture. The prolonged exposure of cultured chick primary neurons to 1 mM of GABA for 48 h resulted in a down-regulation of the receptor number that was mediated by the transcriptional repression of receptor subunit genes [52–54]. Conversely, exposure of similar neuronal cultures to 10 μM of flurazepam for 48 h did not induce changes in receptor density [55]. These results suggest that prolonged activation of $GABA_A$ receptors by the neurotransmitter or allosteric modulators triggers distinct regulatory mechanisms.

The prolonged exposure (48 and 72 h) of HEK293 cells stably transfected with $GABA_A$ receptor α1β1γ2S subunits to 1 μM of diazepam failed to induce alterations in the maximum number or affinity of the benzodiazepine binding sites [56]. Similarly, exposure of Sf9 cells expressing α1β1γ2 $GABA_A$ receptors to 1 μM of diazepam for 60 h did not produce changes in benzodiazepine sites number or affinity [57]. In contrast, exposure of HEK293 cells expressing α1β1γ2 $GABA_A$ receptors to a higher concentration of diazepam (50 μM) for 48 and 72 h resulted in the up-regulation of the receptor number, measured as an increase in the B_{max} of [^{3}H]flunitrazepam and [^{3}H]muscimol [58]. This suggests that the different adaptive alterations observed depend on the benzodiazepine concentration administered.

3.2. $GABA_A$ receptor subunit composition

Numerous studies demonstrated that the tolerance induced by chronic benzodiazepine administration was associated with selective changes in the mRNA and protein levels of $GABA_A$ receptor subunits (reviewed in [14]). Therefore, a transcriptional switch between $GABA_A$ receptor subunits expression represents a potential mechanism for benzodiazepine tolerance. However, very few studies have directly analyzed the effect of chronic benzodiazepine administration on the subunit composition of the $GABA_A$ receptors.

Because most $GABA_A$ receptors contain α, β and γ2 subunits, the regulation of these subunits by chronic benzodiazepine treatment has been extensively investigated. The results have been mixed due to differences in the species, treatment paradigms and brain regions analyzed. For example, mixed results have been reported regarding the regulation of the α1 subunit, the most abundant and ubiquitous α subunit variant. Indeed, chronic diazepam treatment in rats has been reported either to produce a decrease [51,59–61], an increase [23,62], or no change [63–65] in the α1 mRNA and peptide levels in the cerebral cortex.

A 4-week flurazepam treatment in rats resulted in a reduced [^{3}H]zolpidem maximal binding capacity in the cerebral cortex (22%), cerebellum (32%), and hippocampus (25%). However, the [^{3}H]flunitrazepam maximal binding capacity decreased in the cerebral cortex (13%) and hippocampus (14%), while no change was observed in the cerebellum [63]. These differences may indicate a shift in the $GABA_A$ receptor subtype composition with a decrease in the percentage of receptors containing the α1 subunit, especially in the cerebellum.

A recent report indicated that the development of tolerance to the diazepam-induced anxiolytic effects in rats over a 14-day treatment period occurred concomitantly with an increase in the α1 mRNA and protein levels in the cerebral cortex, which is also associated with an increased number of $GABA_A$ receptors composed of α1 subunits [23].

The decreased $GABA_A$ receptor number in neuronal cultures after a prolonged activation of the receptors by GABA seems to be mediated by the transcriptional repression of specific subunit genes [53,54] via the activation of L-type voltage-gated calcium channels (L-VGCCs) [66]. However, the signaling pathway that links long-term benzodiazepine exposure with the regulation of $GABA_A$ receptor subunit levels remains unknown. A long-term treatment with $GABA_A$ receptor positive allosteric modulators, such as benzodiazepines, ethanol, or other abused drugs, modulated the VGCCs [67,68]. In particular, chronic flurazepam administration potenti-

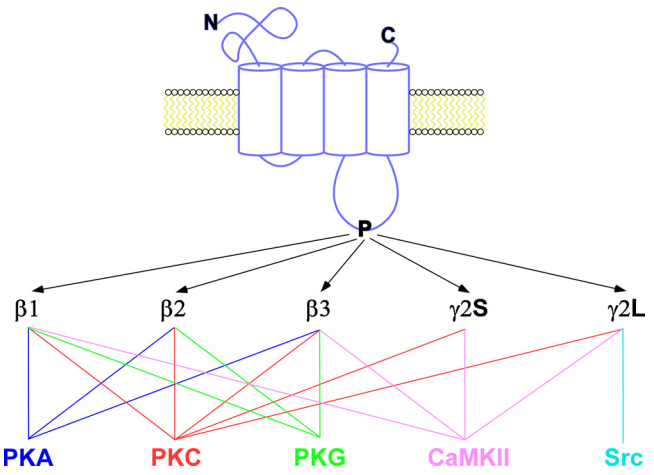

Fig. 2. Phosphorylation of the $GABA_A$ receptors by different protein kinases. Each $GABA_A$ receptor subunit contains 4 hydrophobic transmembrane domains. The large intracellular loop between transmembrane domains 3 and 4 is phosphorylated by multiple kinases.

ated calcium currents through L-VGCCs in rat CA1 neurons [69]. In addition, L-VGCC inhibitors prevented the reduction of the GABA currents induced by chronic flurazepam in the rat hippocampus [70]. Therefore, it is possible to hypothesize that prolonged benzodiazepine exposure stimulates the calcium influx through the L-VGCCs, which, in turn, activate a signaling cascade leading to the transcriptional regulation of specific $GABA_A$ receptor subunit genes.

Experiments performed in primary cultures of rat hippocampal neurons showed that a 24-h flurazepam treatment reduced the amplitude of the miniature inhibitory post-synaptic currents and decreased the surface and total levels of α2-containing $GABA_A$ receptors. This effect seems to be selective for this receptor subtype because flurazepam exposure failed to induce changes in α1-containing receptors [71]. Collectively, these results suggest that prolonged benzodiazepine treatment induces a change in the subunit composition of the $GABA_A$ receptor pool at the plasma membrane, which would in turn lead to a reduction in the efficacy of inhibitory neurotransmission. The mechanism regulating the down-regulation of α2 subunit-containing $GABA_A$ receptors seems to involve an increase in the degradation of this receptor population after endocytosis.

3.3. Uncoupling

A decrease in the allosteric interaction between the GABA and benzodiazepine sites, referred to as uncoupling, may be responsible for the reduced benzodiazepine activity observed after chronic exposure. Different reports have indicated that the development of tolerance after prolonged benzodiazepine administration in rodents was accompanied by an uncoupling of the GABA/benzodiazepine site interactions. Uncoupling has been detected as a decrease in the ability of benzodiazepines to potentiate the GABA currents [72] or the GABA-induced chloride influx [73], as well as a reduction in the stimulation of benzodiazepine binding by GABA [23,46,74].

Uncoupling has been observed not only after *in vivo* benzodiazepine treatments [23,46,74] but also as the consequence of the *in vitro* exposure of neuronal cultures and cell lines expressing recombinant $GABA_A$ receptors to benzodiazepines [55,57,58,75–78]. Therefore, although the timescale of benzodiazepine-induced uncoupling *in vitro* is faster than *in vivo*, the cell culture systems seem to represent a good model to study the molecular mechanisms of tolerance.

The relevance of coupling alterations of GABA-benzodiazepine sites for the development of tolerance *in vivo* remains controversial. The Gallager group demonstrated that chronic administration of the benzodiazepine antagonist flumazenil in rats failed to induce uncoupling, whereas exposure to other benzodiazepine site ligands produced different degrees of uncoupling that were correlated with their efficacies. Moreover, they showed a relationship between the efficacy of these compounds to induce uncoupling and the magnitude of the anticonvulsant tolerance induced by chronic treatment [79]. These results may indicate that uncoupling contributes to the development of benzodiazepine tolerance. In support of this hypothesis, other reports have demonstrated that uncoupling was induced by chronic but not acute benzodiazepine treatment in rats [23,74]. Moreover, uncoupling was reversed 2 days after the end of the treatment [74], a time-course that is similar to that of the reversal of tolerance to benzodiazepine-induced locomotor impairment [44]. In contrast, Holt et al. reported that a single diazepam dose in rats induced uncoupling 4 h later and that this process was reversed after 24 h [80]. These authors suggested that the uncoupling detected immediately at the end of the chronic benzodiazepine treatment was produced by the acute action of the last benzodiazepine administration.

The uncoupling of allosteric interactions of the binding sites in $GABA_A$ receptors seems to represent a general regulatory process. Indeed, uncoupling is also induced by a persistent stimulation of the $GABA_A$ receptor by different allosteric modulators, such as neurosteroids and barbiturates. Experiments performed on cultured chick brain neurons showed that the exposure to pregnanolone for 48 h resulted in a decreased allosteric interaction of the GABA/benzodiazepine, benzodiazepine/neurosteroid, and benzodiazepine/barbiturate sites [81]. Similarly, a 48-h barbital and pentobarbital treatment of these cultures produced the uncoupling of the GABA/benzodiazepine and benzodiazepine/barbiturate sites [55].

Additionally, an exposure of chick brain neuronal cultures to GABA for 48 h produced a decrease in the allosteric GABA/benzodiazepine and benzodiazepine/barbiturate sites interaction [81]. The uncoupling of the GABA/benzodiazepine sites interaction induced by continuous exposure to GABA developed with a $t_{½}$ of 24 h [52]. However, in rat neocortical neuronal cultures, it was subsequently demonstrated that GABA-induced uncoupling required only a brief activation ($t_{½}$ of 3 min) of the $GABA_A$ receptor with neurotransmitter. Then, an incubation period of 24 h ($t_{½}$ of 12 h) in the absence of GABA was sufficient to allow uncoupling to occur [82]. These results suggest that uncoupling can also be induced under physiological conditions in which the persistent activation of the post-synaptic $GABA_A$ receptors by GABA occurs for only minutes.

The mechanism of uncoupling remains unknown. The ability of benzodiazepines to potentiate the actions of GABA depends on the α subunit subtype present in the $GABA_A$ receptor in the following rank order: α3 > α2 > α1 [83–85]. Therefore, it is possible to speculate that benzodiazepine-induced uncoupling is the result of a change in the subtype of the α subunit, which in turn could result in a lower coupling efficiency. Alternatively, uncoupling may be the consequence of changes in the post-translational modifications of the $GABA_A$ receptor, for example phosphorylation.

3.4. Phosphorylation of the $GABA_A$ receptors

$GABA_A$ receptors are modulated by different post-translational modifications, such as palmitoylation, ubiquitination, and phosphorylation. In particular, $GABA_A$ receptor phosphorylation is an important mechanism that regulates channel activity, sensitivity to different modulators, and trafficking [86–88]. Therefore, a bal-

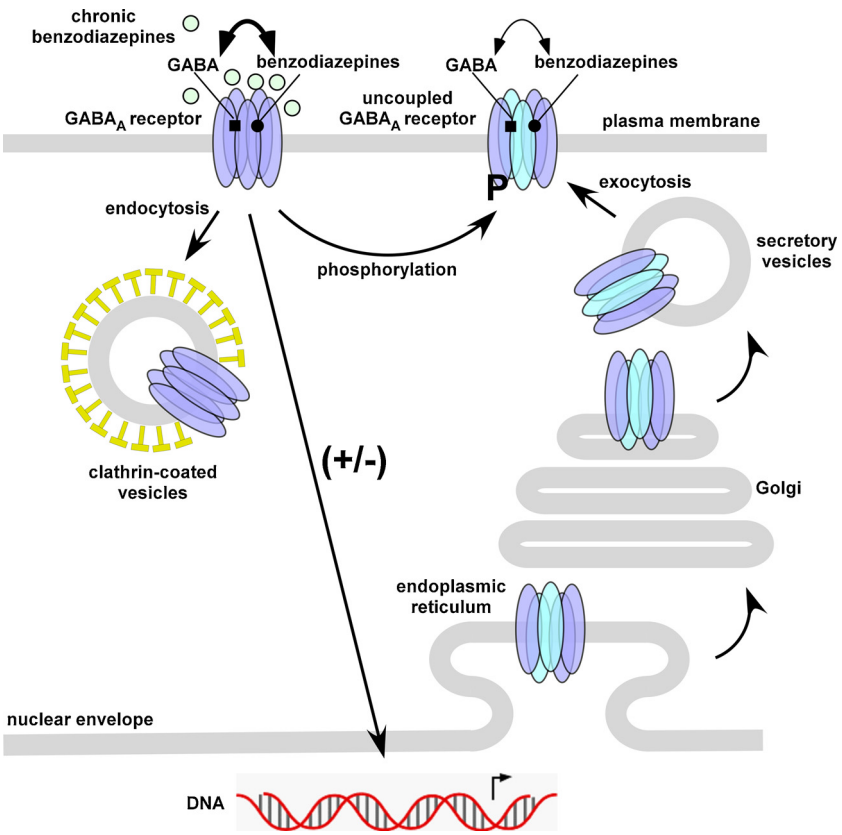

Fig. 3. The effect of chronic benzodiazepine administration on GABA$_A$ receptors. Different reports have suggested that prolonged benzodiazepine exposure induces the following alterations of the GABA$_A$ receptor: a switch of receptor subunits, an increase in receptor internalization, a change in the phosphorylation state of the receptor, and the uncoupling of the GABA/benzodiazepine binding site interactions.

ance between the activity of protein kinases and phosphatases is crucial for the regulation of GABAergic neurotransmission.

GABA$_A$ receptors can be phosphorylated at key residues located within the major intracellular loop of the receptor β1-3 and γ2 subunits by different kinases, including cAMP-dependent kinase (PKA), calcium/phospholipid-dependent protein kinase (PKC), CaMKII, protein kinase B (PKB or AKT), cGMP-dependent protein kinase (PKG), and Src tyrosine kinases. Importantly, different protein kinases can phosphorylate the same residue [86–88] (Fig. 2).

In vitro experiments revealed that the function of GABA$_A$ receptors can be differentially regulated by phosphorylation depending on their subunit composition. For example, modulation of the receptor functions by PKA depends upon the subtype of β subunit present in the GABA$_A$ receptor. PKA activation inhibits GABA currents in HEK-293 cells expressing β1-containing GABA$_A$ receptors, stimulates GABA responses mediated by β3-containing receptors, and has no effect on receptors composed of β2 subunits [89].

The role of PKC in regulating the function of GABA$_A$ receptors is controversial. Activation of PKC by phorbol esters resulted in the down-regulation of GABA responses via the phosphorylation of serine residues in the GABA$_A$ receptor β and γ2 subunits, both in HEK-293 cells [90] and in *Xenopus laevis* oocytes [91]. In contrast, in mouse fibroblasts expressing recombinant receptors, the constitutively active catalytic domain of PKC (PKM) stimulated the GABA$_A$ receptor function via phosphorylation of the β1 and γ2L subunits [92]. In addition, BDNF transiently potentiated GABA$_A$ receptor activity in rat hippocampal neurons, in parallel with the phosphorylation of the GABA$_A$ receptor β3 subunit by PKC and with an increase in the stability of the receptors at the cell surface [93].

Several studies have demonstrated that changes in the phosphorylation state of GABA$_A$ receptors could also regulate the effects of allosteric modulators. However, these results are contradictory. The stimulation of GABA currents by benzodiazepines, barbiturates, and neurosteroids in *Xenopus laevis* oocytes expressing α1β1γ2 GABA$_A$ receptors was potentiated by PKC activation [94,95]. However, in another report using the same expression system, PKC had no effect on the sensitivity of the GABA$_A$ receptors to benzodiazepines and barbiturates [96]. In neuron-like NT2-N cells, PKC activation by different modulators decreased the potency of the benzodiazepine-induced stimulation of GABA$_A$ receptor currents [97], indicating that PKC negatively modulated the action of benzodiazepines. In accordance with these experiments, knockout mice lacking PKCε exhibited an increased sensitivity to the acute behavioral responses to ethanol, benzodiazepines, barbiturates, and neurosteroids, suggesting that this kinase is involved in inhibiting the action of these allosteric modulators [98,99].

The mechanisms underlying benzodiazepine tolerance could be the consequence of changes in the phosphorylation state of GABA$_A$ receptors. Several lines of evidence suggest that the alterations in the function of GABA$_A$ receptors produced by chronic benzodiazepine treatment are mediated by changes in the activity of different protein kinases. In rat cerebellar granule cells, the decreased GABA$_A$ receptor α1 subunit levels induced by a 48-h treatment with flunitrazepam were mediated by PKC activation [100]. Additionally, recent results indicated that the development of tolerance to the sedative effects of diazepam produced by a 7-day benzodiazepine treatment in rats was concurrent with an increase in the phosphorylation of the cortical GABA$_A$ receptor γ2 subunit at serine 327 [23]. Additionally, a one-week flurazepam treatment in rats resulted in a reduced GABA response in CA1 pyramidal cells that was related to changes in endogenous PKA activity [101]. The results from the Olsen group indicated that

the uncoupling induced by prolonged diazepam exposure in Sf9 cells expressing recombinant $GABA_A$ receptors was prevented by PKA activation. However, direct phosphorylation of the receptor subunits was not involved [78]. The phosphorylation of other proteins, such as $GABA_A$ receptor-associated proteins, may participate in the uncoupling mechanism. For example, the activity of gephyrin, a scaffolding protein that regulates the aggregation of $GABA_A$ receptors at the postsynaptic membrane [102], is regulated by a phosphorylation mechanism [103]. Therefore, changes in the phosphorylation state of gephyrin may be part of the adaptive mechanisms induced by chronic benzodiazepine administration. Finally, the decrease in CaMKIIα mRNA levels induced by diazepam may also indicate that tolerance is associated with a phosphorylation process [42].

3.5. Intracellular trafficking

Although most reports have demonstrated that a long-term treatment with benzodiazepines does not decrease the total number of $GABA_A$ receptors, tolerance could be the consequence of a decrease in the number of receptors at the cell surface. The expression and stability of $GABA_A$ receptors at the synapse is an important determinant of the strength of synaptic inhibition. $GABA_A$ receptors continuously cycle between the cell surface and intracellular compartments. This trafficking is controlled by the interaction of different proteins with the subunits of the receptor [87]. Alterations in the activity of $GABA_A$ receptor-associated proteins may mediate the adaptive changes of the receptors that are induced by chronic benzodiazepine exposure. For example, the importance of phospholipase C-related catalytically inactive protein 1 (PRIP1), a protein involved in $GABA_A$ receptor trafficking, for the regulation of the receptor function is evident from electrophysiological studies performed in mouse hippocampal cells. These studies showed that, in PRIP1 knockout mice, the modulation of GABA currents by benzodiazepines and zinc is impaired [104].

The results of several studies suggest that the uncoupling induced by chronic benzodiazepine administration is mediated by an increase in the internalization of $GABA_A$ receptors. The Barnes group has described the presence of uncoupled $GABA_A$ receptors in a fraction of clathrin-coated vesicles isolated from bovine brain [105]. They also observed that chronic lorazepam administration in mice resulted in an increased benzodiazepine binding in the intracellular vesicle fraction of the brain [106]. Experiments performed in cultured Sf9 cells expressing recombinant α1β2γ2 $GABA_A$ receptors [78] indicated that prolonged diazepam exposure for 60 h induced a decrease in the potentiation of benzodiazepine binding by GABA. This uncoupling was prevented by the use of an osmotic shock treatment to lyse the intracellular vesicles in the membrane homogenate used for the binding assays. Because uncoupling was reproduced in untreated cells when the binding assays were performed at a low pH (4–5.2), the authors concluded that uncoupling was produced by the exposure of $GABA_A$ receptors to an acidic environment inside the intracellular vesicles. In addition, immunofluorescence experiments showed that the benzodiazepine treatment produced a loss of surface receptors and an increase of the internalized receptor levels, suggesting that endocytosis of the receptors was stimulated [78]. Furthermore, in a more recent study using rat neocortical cultures [107] GABA-induced uncoupling was prevented by a co-incubation with high concentrations of sucrose, suggesting that uncoupling depends on a $GABA_A$ receptor internalization step. In addition, immunocytochemical studies and biotinylation assays demonstrated that GABA exposure increases receptor endocytosis [107].

The internalization of $GABA_A$ receptors is controlled by receptor phosphorylation. The interaction of phosphorylatable residues within the major intracellular loop of $GABA_A$ receptor β, γ, and δ subunits with the μ2 subunit of the clathrin adaptor protein AP2 regulates $GABA_A$ receptors endocytosis. This interaction is negatively regulated by the phosphorylation of these residues. The phosphorylation of serine 408 and 409 in the receptor β3 subunits by PKA, PKC, CaMKII, and AKT increases the levels of β3-containing $GABA_A$ receptors at the plasma membrane. Conversely, dephosphorylation of serine 408 in β1, serine 410 in β2, and serine 409 in β3 by the PP1α and PP2A phosphatases stimulates $GABA_A$ receptor internalization [108]. Therefore, benzodiazepine tolerance may result from changes in the phosphorylation state of the $GABA_A$ receptors, which alters the rate of receptor internalization.

4. Conclusion

Numerous *in vivo* and *in vitro* experiments have demonstrated that chronic benzodiazepine exposure induces different alterations of the $GABA_A$ receptors (Fig. 3). Although most of these reports have suggested that the occurrence of benzodiazepine tolerance is not associated with a down-regulation of the total $GABA_A$ receptor number, tolerance could be the consequence of changes in the intracellular trafficking of $GABA_A$ receptors, leading, in turn, to a reduction in the number of receptors at the plasma membrane. Multiple studies have indicated that prolonged benzodiazepine treatments induce selective changes in the mRNA and protein levels of $GABA_A$ receptor subunits. Therefore, a change in the $GABA_A$ receptor subunit combination is another possible mechanism underlying the development of tolerance to benzodiazepines. Alternatively, chronic exposure to benzodiazepines results in an uncoupling of the GABA/benzodiazepine site interactions. However, the relevance of this phenomenon for tolerance remains unclear. Different studies have demonstrated that some of the effects of prolonged benzodiazepine treatment on $GABA_A$ receptor functions are mediated by the activity of protein kinases, suggesting that alterations in the phosphorylation state of $GABA_A$ receptors may also contribute to the development of tolerance. In summary, tolerance may be the result of multiple mechanisms that involve different changes in the $GABA_A$ receptors.

Conflict of interest

The authors declare that they have no conflict of interests.

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