Regulation of GABA\(_A\) Receptor Structure and Function by Chronic Drug Treatments In Vivo and with Stably Transfected Cells

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ABSTRACT—In this article, we review the use of stably transfected cells to study the regulation of receptor structure and function by chronic drug treatments and compare results from these cells to results obtained from other systems, including neuronal cultures and intact animals. We focus on the \(\gamma\)-aminobutyric acid type A (GABA\(_A\)) receptor complex. Sedative/hypnotic drugs such as benzodiazepines, barbiturates and alcohol that potentiate GABA\(_A\) receptor function produce tolerance and dependence. Chronic treatment of GABA\(_A\) receptor preparations from brain and neuronal cultures with GABA\(_A\) agonists, as well as these other three classes of drugs, results in regulation of several properties of the receptor. Drug treatments may regulate levels of binding sites, allosteric binding interactions, receptor function, levels of receptor subunit mRNA and levels of receptor subunit protein. Some or all of these effects may comprise the molecular mechanisms of tolerance to these GABA\(_A\)-modulatory drugs. The use of cells stably transfected with neurotransmitter receptors provides a homogeneous population that can be cultured under controlled conditions. As most preparations contain mixed populations of GABA\(_A\) receptor subunits, stably transfected cells offer the advantage of the expression of receptors with a defined subunit composition. We conclude that chronic drug treatments regulate allosteric coupling and function of GABA\(_A\) receptors in stably transfected cells. This regulation does not appear to be due to decreases in the expression of \(\alpha_1\)- or \(\beta_1\)-receptor subunits or to expression of subunits other than \(\alpha_1\), \(\beta_1\), \(\gamma_2\), \(\delta\). Therefore, it is unlikely to be due to changes in receptor subunit composition and probably represents post-translational changes. The rapid regulation of allosteric coupling and function by drug treatment of the stably transfected cells should provide insights to the mechanisms of coupling between GABA\(_A\) and benzodiazepine receptors as well as tolerance and dependence of benzodiazepines and ethanol.

Keywords: \(\gamma\)-Aminobutyric acid type A (GABA\(_A\)) receptor, Benzodiazepine, Barbiturate, Alcohol, Chloride channel, Receptor expression, Dependence, Transfection

I. Neurotransmitter receptor down-regulation and tolerance and dependence to drugs of abuse

Chronic exposure of neurotransmitter receptors to their agonist generally results in down-regulation of receptor levels and function. Chronic exposure to drugs of abuse alters specific neurotransmitter receptor pathways and can produce agonist-induced down-regulation of the receptors they affect. These changes may underlie the behavioral changes associated with tolerance and dependence, as both the development of tolerance and neurotransmitter receptor regulation depend on concentrations of drug and times of exposure that are in agreement. Tolerance and dependence to drugs of abuse can lead to addiction, a compulsive use of a drug despite adverse consequences (1). The molecular and behavioral changes that make up drug addiction are examples of plasticity of the nervous system, as are learning and memory. The acute effects of drugs of abuse are rewarding, or positively reinforcing, and this property leads to repeated use. The rewarding properties of drugs are presumably based on an individual's genetic make-up and environmental factors, like stress (1). If tolerance, or a subsensitivity, to a given concentration of drug develops, greater amounts of drug are required for the original effect. Dependence develops when continued drug exposure is required to avoid withdrawal symptoms, which are typically opposite to the acute and rewarding drug effects. In addition to the
pain and potential dangers associated with withdrawal, drug abuse may cause toxicity to tissues unrelated to the rewarding actions.

The phenomenon of down-regulation after exposure to agonist holds true for γ-aminobutyric acid type A (GABA_A) receptors. GABA_A receptors are sites of action for three classes of compounds which potentiate GABA_A receptor function, produce sedative/hypnotic effects and are drugs of abuse: benzodiazepines, barbiturates and ethanol. Researchers have investigated effects of chronic treatments with drugs that stimulate or potentiate GABA_A receptor function on GABA_A receptor binding density, levels of subunit mRNAs and protein, receptor function and allosteric binding interactions.

This paper discusses the use of cells that have been stably transfected with neurotransmitter receptor cDNA for the study of receptor regulation after drug treatments. The use of stably transfected cells has provided much information about mechanisms involved in neurotransmitter receptor regulation. Recent studies from our lab show that some of the same forms of GABA_A receptor regulation occur after drug treatments of stably transfected cells compared to what occurs in vivo.

**GABA_A/benzodiazepine receptor chloride channel complex**

Ligand-gated and voltage-gated ion channels, gap junction proteins, as well as other neuronal membrane proteins like synaptophysin, comprise the family of ion channel proteins. GABA_A receptors are members of the family of ligand-gated ion channels which includes glutamate, nicotinic cholinergic, glycine, and serotonin receptors. GABA_A receptors are oligomeric combinations of protein subunits which are assembled and inserted into the membrane to form Cl^- channels. GABA_A receptor expression is widespread in the brain and nervous system and GABA_A receptor mRNA is also expressed in glia (2). Stimulation of GABA_A receptors with GABA in neurons typically results in a hyperpolarizing influx of Cl^-, although depolarizing responses are seen in some cells (3). Due to the ubiquity of cells that synthesize GABA and cells that express GABA_A receptors, GABA_A-mediated synaptic transmission is the major inhibitory pathway in brain.

**II. GABA_A/benzodiazepine receptor chloride channel complex GABA_A receptor structure**

GABA was identified as a neurotransmitter in the 1960s (4). During the 1970s and 1980s, GABA_A receptor binding sites and GABA-stimulated chloride flux of GABA_A receptors were characterized (5). These studies revealed a receptor complex with an array of distinct pharma-
colocally active domains. GABA<sub>A</sub> receptors contain seven or more such domains including: 1) the GABA<sub>A</sub> agonist/antagonist site, 2) the benzodiazepine site, 3) the barbiturate site, 4) the neurosteroid site, 5) the picrotoxin or channel site, 6) Cl<sup>-</sup> ion site, 7) Zn<sup>2+</sup> site (5–7). These sites are allosterically linked to each other; e.g., GABA, barbiturates and neurosteroids potentiate benzodiazepine binding; benzodiazepines and barbiturates potentiate low affinity GABA binding; and GABA, neurosteroids and benzodiazepines decrease binding of the channel blocker TBPS (5–8).

In 1987, two GABA<sub>A</sub> receptor subunit cDNA sequences were reported. This led to an explosion of information regarding receptor primary, secondary, tertiary and quaternary structure and receptor binding and function in expression systems (9–12). GABA<sub>A</sub> receptor subunits are classified into 5 families by sequence homology. The intrasubunit family homology is 70–80%, the intersubunit family homology is 30–40% and a 20–30% homology exists between GABA<sub>A</sub>-receptor subunits and non-GABA<sub>A</sub>-receptor ligand-gated ion channels. There are 6 αs, 3 βs, 3 γs, 1 δ and 2 ρ cDNA(s) cloned, and γ2 (13) and β4 (14) mRNAs can be expressed in alternatively spliced forms.

The sequence and hydropathy data revealed a receptor structure which follows the model derived from nicotinic receptors. GABA<sub>A</sub> receptor polypeptides are believed to fold and insert into membrane with a putative extracellular N-terminus, 4 hydrophobic membrane spanning α-helices, a large hydrophilic intracellular loop between membrane spanning domains 3 and 4, and an extracellular C-terminus. The intracellular loop is the most variable region between subunits. There are consensus phosphorylation sequences for cAMP-dependent protein kinase (PKA) on the intracellular loop on α4-, α6- and β1–4-subunits; Ca<sup>2+</sup>/phospholipid-dependent protein kinase (PKC) on the intracellular loop of α6-, β1–4-, γ2- and ρ1–2-subunits; Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaM kinase II) on γ2-subunits; and protein tyrosine kinase on the intracellular loop of γ1–3-subunits (6). Extracellular N-glycosylation sites are present on all GABA<sub>A</sub> receptor subunit N-terminal sequences (5, 6). GABA<sub>A</sub> receptors are believed to be pentameric structures where the second membrane spanning domains of the 5 subunits make up the Cl<sup>-</sup> ion pore (15). Recent studies by Nayeem et al. (16) have determined a pentameric structure for GABA<sub>A</sub> receptors by electron microscopy of purified receptors and harmonic analysis of rotational symmetry. Therefore, there is a conserved pentameric structure for members of the superfamily of ligand-gated ion channels, including nicotinic-, glycine- and GABA-gated ion channels (16).

With the possibility of homomeric receptors or heteromeric receptors randomly assembled from 2–5 different subunits, the potential for GABA<sub>A</sub> receptor heterogeneity is large, and the precise structure of GABA<sub>A</sub> receptors is not known. Specific subunit mRNAs are expressed in certain brain regions as shown by in situ hybridization (17); e.g., α6 in cerebellum, γ1 in limbic structures and glial cells, β1 in hippocampus, and ρ in retina (6). By purifying benzodiazepine receptors from cortex and immunoprecipitating with subunit specific antibodies, it was determined that virtually all GABA<sub>A</sub>/benzodiazepine receptors contain β-subunits, 75% contain γ2-subunits (18) and 20–30% contain δ-subunits (6). The localization of subunit expression is presumably important for specific brain functions as well as disease states.

Other studies demonstrate that GABA<sub>A</sub> receptor assembly is nonrandom (19). When mouse L929 cells were transfected with α1-, β1- and γ2-subunits, a single population of α1β1γ2 receptors were preferentially expressed, as determined by single channel parameters and benzodiazepine and Zn<sup>2+</sup> sensitivity (20). Considering the data to date, it is safe to say that the majority of GABA<sub>A</sub> receptors in vivo are αδγ trimers and in cortex, most are α1β2γ2, although subunit stoichiometry is uncertain.

Benzodiazepines, such as diazepam and flunitrazepam, allosterically potentiate GABA action by increasing the frequency of channel opening (21). Barbiturates, such as pentobarbital and phenobarbital, allosterically potentiate GABA action in a manner distinct from benzodiazepines. Neurosteroids, such as 5α-pregnan-3α-ol-20-one (5α3α), are metabolites of progesterone produced in neurons. Several of these pregnanolone derivatives are capable of potentiating GABA actions (22) in a similar but not identical manner as barbiturates.

Behavioral effects of alcohol are related to GABA<sub>A</sub> receptors as GABA<sub>A</sub> agonists enhance acute effects of ethanol and reduce withdrawal symptoms, and GABA<sub>A</sub> antagonists shorten ethanol narcosis and potentiate withdrawal (7, 23). The acute actions of ethanol on GABA<sub>A</sub> receptors are generally characterized by a potentiation of GABA<sub>A</sub> receptor function (24–26). The action of ethanol on GABA<sub>A</sub> receptors may underlie the anxiolytic and sedative/hypnotic effects properties of ethanol because physiologically relevant concentrations of ethanol (10–60 mM) produce potentiation. Ethanol potentiation of GABA<sub>A</sub> receptor function is highly complex, however, as ethanol action depends on preparation (27), brain region (28), subunit composition (29), and genetic strain (23, 24). This complexity may explain why many authors do not observe effects of physiologically relevant concentrations of ethanol (<80 mM) on GABA<sub>A</sub> receptor function (30, 31).
III. Effects of drug treatments on receptor binding, function and expression of GABAA receptors

GABAA agonists

Desensitization (fast and slow phases) of GABAA receptors occurs within msec-sec (32) of agonist exposure and, after removal of agonist, reverses within minutes (33). Chronic treatment of neuronal primary cultures with GABAA agonists produces down-regulation of GABAA receptors. Maloteaux et al. (34) were the first to demonstrate this phenomenon, which has subsequently been observed by others (35–37). The magnitude of agonist-induced down-regulation of GABAA receptors from rat, chick and mouse neurons is a 30–40% decrease in benzodiazepine binding sites, which occurs after 2–3 days of treatment (34, 36, 37). EC50 concentrations for GABA-induced down-regulation (36, 37), as well as desensitization (32), are about 100 μM.

As well as decreases in binding sites, 40–60% decreases in the peak amplitude of GABA-induced currents in chick neurons (38) and GABA-induced 36Cl− flux in mouse neurons (37) result from chronic agonist treatments. Chronic agonist treatments result in 50–60% decreases in GABAA receptor α1-subunit mRNA levels (39, 40) and subunit protein levels (41) in chick neurons. In addition, α2 and α3 mRNA and protein levels are reduced by GABA treatment in mouse neurons (42). GABAA receptor activation is required for GABA-induced down-regulation of mRNAs, as these effects are prevented by cotreatment with the GABAA receptor antagonists SR 95531 (39) or R 5135 (42).

Chronic agonist-induced decreases in receptor binding sites, mRNA, and protein are accompanied by GABAA receptor internalization and association with clatharin-coated vesicles. Studies by Tehrani and Barnes demonstrate intracellular pools of benzodiazepine binding with the use of the membrane-impermeant ligand SPTC-1012S. Intracellular binding represented about 7% of the total benzodiazepine binding sites in chick neurons, and chronic GABA treatment increased the intracellular sites by 60–70% (35). Furthermore, the authors demonstrated specific benzodiazepine binding in fractions enriched for clatharin coated vesicles (43). The authors hypothesize that GABAA receptor trafficking from the membrane involves endocytosis to clatharin coated vesicles, and this process is increased by chronic agonist treatment.

One consistent feature of chronic agonist treatment is uncoupling of GABA and benzodiazepine binding sites on the receptor complex. In chick (36) and mouse neurons (37), the enhancement of benzodiazepine binding by GABA is decreased by 30–40%, which occurs within 1–5 days of agonist treatment. Concentrations of GABA and muscimol in these experiments are 0.1–1 mM. In mouse neurons (37), the uncoupling represents a decrease in the Emax of the GABA enhancement of benzodiazepine binding.

Benzodiazepines

Chronic exposure to benzodiazepine agonists also causes uncoupling of GABAA and benzodiazepine binding sites of GABAA receptors of brain and cultured neurons. Gallager et al. (44) were the first to demonstrate this effect by administering diazepam to rats for 2 weeks and measuring benzodiazepine binding in cortex. The authors observed a 50% decrease in the GABA enhancement of benzodiazepine binding in diazepam-treated rats and no changes in the affinity or density of receptors. This effect has been reproduced with primary chick and mouse neuronal cultures. In these studies, benzodiazepine (flurazepam) treatment produces 40–80% decreases in coupling with t1/2 values for uncoupling of 1–3 days of treatment. For both of these studies, flurazepam EC50 values for uncoupling are 1–2 μM. These effects are blocked by the benzodiazepine antagonist Ro 15-1788 but not the GABAA antagonist SR 95531 or the Cl− channel blocker picrotoxin (45).

Although receptor levels are unaffected, Gallager et al. (44) demonstrated that diazepam treatment produces a postsynaptic subsensitivity to GABA, as well as allosteric uncoupling. Studies of chronic benzodiazepine treatment of animals demonstrate decreases (47) and no change (44) in the levels of benzodiazepine binding sites after chronic exposure. With prolonged continuous administration of benzodiazepines using minipumps or silastic pellets, decreases in binding sites are observed (48, 49). These changes are accompanied by decreases in the GABA-mediated Cl− flux (48, 49) as well as the benzodiazepine potentiation of GABA-mediated Cl− flux (48). With chick neurons, 10-day clonazepam treatment produced a 40% decrease in benzodiazepine binding sites and a 60% decrease in GABA-induced 36Cl− uptake (49). Results from mouse neuronal cultures are qualitatively different as a 10-day flurazepam treatment caused a 50% decrease in the benzodiazepine potentiation of GABA-induced 36Cl− uptake, although GABA-induced 36Cl− uptake and levels of binding sites were unaffected by benzodiazepine treatment. Therefore, embryonic neuronal primary culture models may express the necessary factors for some but not other forms of GABAA receptor regulation.

Prolonged benzodiazepine exposure (2–4 weeks) decreases levels of α1-subunit mRNA in rat cortex by 25% (50) and by 50% in mouse cortex (51) and γ2-subunit mRNA by 50% in mouse cortex (51) and by 30–40% in rat cortex and hippocampus (52). Two studies suggest that α5-subunit mRNA is more rapidly regulated. Decreases in α5 mRNA levels by 20–35% can be detected.
in 2–4 hr in rat cortex and hippocampus (52) and rat whole brain (53). The latter two studies failed to detect changes in \( a1 \) mRNA levels, and O'Donovan et al. (53) observed 40% increases in \( a3 \) and \( a6 \) mRNA levels after 8–16 days of flurazepam treatment. With respect to the \( \beta \)-subunits, Heninger et al. (50) reported no change in \( \beta1 \) mRNA levels in rat cortex, hippocampus and cerebellum, and in \( \beta3 \) mRNA in hippocampus and cerebellum after 4 weeks of flurazepam treatment. Chronic treatment of rats with the benzodiazepine inverse agonist FG 7142 for 8 days by minipump produced increases in \( a1 \) mRNA levels (15%) and \( \gamma2 \) mRNA levels (25%) and no change in \( \beta1 \) mRNA levels in cortex (54).

One explanation for reductions in GABA\( _{A} \) receptor allostERIC coupling and benzodiazepine function is the idea of \( \alpha \)-subunit replacement. Because different \( \alpha \)-subunits confer different benzodiazepine sensitivity (10, 11), a logical hypothesis for changes in GABA\( _{A} \) receptor coupling and function could be explained by an altered expression of subunits after drug exposure (7). As the levels of GABA\( _{A} \) receptor \( \alpha5 \), \( \beta2 \), \( \beta3 \) and \( \gamma2 \)-subunit mRNAs are decreased by benzodiazepine treatment (51, 52), a hypothesis of receptor regulation by replacement of specific subunits is most attractive.

Recent work by Tehrani and Barnes (55), with mice exposed to lorazepam for 7 days by minipump, demonstrated a 21% decrease in benzodiazepine binding sites of membranes compared to the control. Benzodiazepine binding to fractions of clathrin-coated vesicles was increased by 83% after benzodiazepine treatment, suggesting receptor down-regulation occurs by receptor endocytosis and degradation via clathrin coated vesicles.

Barbiturates

Chronic barbiturate treatments do not produce a consistent effect on levels of GABA\( _{A} \)-receptor binding sites (7, 56, 57). In primary chick neurons, 2-day barbiturate treatment does not affect the levels of benzodiazepine binding sites but reduces the allostERIC coupling between GABA and benzodiazepine sites by 25% (45). Pentobarbital administration to rats for 6 weeks reduces GABA\( _{A} \)-mediated Cl\(^{-} \) uptake by 27% and the pentobarbital potentiation by 43% (7). With a 7-day phenobarbital treatment of mice, there is no change in GABA\( _{A} \)-mediated Cl\(^{-} \) flux, but the potentiating effects of both phenobarbital and flunitrazepam are reduced by 41% (58). Chronic pentobarbital has no effects on \( a1 \)-subunit mRNA levels in rat cortex (7). Withdrawal of pentobarbital in rats made tolerant to the drug results in increases in \( a1 \)-, \( \beta3 \)- and \( \gamma2 \)-subunit mRNA levels by 25–50% in several specific brain regions (57, 59).

Neurosteroids

The in vitro binding interactions of neurosteroids and ligands for GABA\( _{A} \) receptors are well understood (60, 61), but only two studies address chronic treatments with neurosteroids. With primary chick neuronal cultures, neurosteroid treatment completely uncouples neurosteroid and benzodiazepine binding sites in chick neurons in 2 days (62). In cultures from mouse, neurosteroid treatment for 5 days uncouples the GABA and neurosteroid enhancement of benzodiazepine sites (63).

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Alcohol

Chronic exposure to ethanol in mice and rats generally results in increases or decreases of specific GABA\( _{A} \) receptor subunit mRNA and protein levels, alterations of GABA\( _{A} \) receptor binding. Chronic ethanol does not affect the binding of benzodiazepine agonists to the GABA\( _{A} \) receptor complex (64), but chronic ethanol does increase the binding levels of a benzodiazepine inverse agonist by 40–60% in rat cortex and cerebellum and mouse spinal cord neurons (65, 66). Studies of the allostERIC coupling of GABA and benzodiazepine binding sites suggest that chronic ethanol treatments can produce decreases (67), increases (68) or no effects (69, 70).

Although levels of GABA\( _{A} \) receptor binding sites are unchanged by chronic ethanol, decreases in levels of subunit mRNAs for \( a1 \) (40–60%), \( a2 \) (30–60%) and \( a5 \) (50%) in rat cortex; increases in \( a6 \) (76%) in rat cerebellum; and increases in \( \beta1 \) (29%), \( \beta2 \) (55%) and \( \beta3 \) (72%) in rat cortex occur (66, 71, 72). However, one study reported a 50% increase in \( a1 \) mRNA in mouse whole brain after chronic ethanol (73). At the protein level, chronic ethanol exposure decreases \( a1 \)-, \( a2 \)- and \( a3 \)-subunit protein levels by 30–60% and increases \( \beta2 \)- and \( \beta3 \)-subunit protein by 23% in rat cortex (72, 74). Chronic ethanol exposure decreases GABA-mediated Cl\(^{-} \) flux in some (74, 75) but not other studies (70, 76). Chronic ethanol treatment results in an attenuation in the effects of ethanol, pentobarbital and flunitrazepam potentiation of GABA\( _{A} \) receptor function and an augmentation of the effects of benzodiazepine inverse agonists (70, 75, 77).
IV. The use of stably transfected cells to study neurotransmitter receptor regulation by chronic drug treatments

Advantages of stably transfected cell lines

The expression of neurotransmitter receptors in cultured cells is a useful tool for investigations of neurotransmitter receptor function and regulation. The advantages of cultured cells include a relatively homogeneous cell population that can be well-characterized with respect to receptor subunit composition, structure and function. Culture conditions can be tightly controlled which facilitates the study of the mechanisms of neurotransmitter receptor regulation.

As a result of the developmental regulation, it is difficult to completely define the subunit composition of receptors expressed in primary cultures. These receptors may be different from the adult, which is another potential disadvantage of the primary cultures. In cells which have been stably transfected with neurotransmitter receptor cDNAs, the expression of receptors with a defined subunit composition in a clonal cell line facilitates the investigation of neurotransmitter receptor regulation after chronic drug treatments.

The majority of these studies involve G-protein coupled receptors and they include measurements of receptor binding, coupling to cAMP accumulation and receptor mRNA levels. For most neurotransmitter receptors in vivo, chronic agonist exposure produces down-regulation and chronic antagonist produces up-regulation. As discussed below, this axiom holds for some, but not other, receptors with chronic treatments of transfected cells. Results from such studies are producing novel hypotheses about the mechanisms of neurotransmitter receptor regulation. In fact, studies of stably expressed β2-adrenergic receptors provide the hallmark model of neurotransmitter receptor regulation.

Recent studies of stably expressed nicotinic receptors and GABA_A receptors involve chronic treatments of cells stably expressing ligand-gated ion channels.

Neurotransmitter receptor systems

Adrenergic receptors: Studies of adrenergic receptors stably expressed in cells provide the most detailed information regarding receptor regulation in response to agonist exposure. For expressed β2-adrenergic receptors, agonists cause desensitization, receptor sequestration, and down-regulation of receptor binding sites (78). Desensitization represents an uncoupling of receptors from G proteins and cAMP accumulation. Sequestration is defined by an increase in the receptor pool that binds hydrophobic but not hydrophilic ligands. The receptor sequence requirements of these events have been elegantly characterized. In seconds to minutes of agonist exposure, receptors are desensitized and sequestered. Removal of ligand results in a reversal of desensitization and sequestration in minutes. Down-regulation of receptor levels occurs more slowly, in times greater than 1 hr (78).

Phosphorylation sites for protein kinase A and β-adrenergic receptor kinase are required for desensitization as determined by expressing mutant receptors which are not phosphorylated at these sites (79). The cytosolic protein β-arrestin is also involved in desensitization. Over-expression of β-arrestin augmented desensitization in cells with high levels of β2-receptors (80). This effect did not occur in cells which express low levels of β2-receptors, however. Mutagenesis and stable expression determined several tyrosine residues critical for β2-receptor regulation. Tyrosine 326, a residue at the proposed junction of the seventh membrane spanning domain and the proximal part of the carboxyl terminus, is highly conserved among G protein-coupled receptors. Mutation of this residue resulted in receptors that are not sequestered by agonist treatment (81). Mutating tyrosine 350 and 354 of the carboxyl tail results in expressing receptors that are sequestered similar to wild-type but are not down-regulated (82). Tyrosine residues are conserved in the carboxyl terminus of membrane bound receptors, and mutations of these residues of the low density lipoprotein, polyimmunoglobulin and mannose-6-phosphate receptors, disrupt agonist-induced internalization (82).

This wealth of information about β2-receptor regulation supports a model whereby brief agonist exposure induces receptor phosphorylation and desensitization, followed by sequestration to phosphatase-enriched vesicle compartments and resensitization and reinsertion into the membrane (78). In contrast to brief exposure, prolonged exposure to agonist inducers receptor down-regulation. This alternate pathway likely involves endocytosis of receptors to clathrin-coated vesicles and protein degradation (82).

Studies of expressed α2A-receptors show that they are phosphorylated by β-adrenergic receptor kinase, at sites on the third intracellular loop, which are required for agonist-induced desensitization (83). Studies of β1-receptors stably transfected in cells show that agonist treatments desensitize receptor function when expressed in HEK 293 cells, but not when expressed in CHO cells (84).

Dopamine receptors: Regulation of stably expressed dopamine D2-receptors occurs in response to chronic agonist and antagonist treatments (85, 86). D2L-receptors stably expressed in HEK 293 cells are coupled to inhibition of cAMP accumulation, and agonists cause a 3–5 fold increase in receptor density which occurs rapidly (t1/2 = 6 hr). Interestingly, D2L-receptor levels were also
increased by antagonists, while neither agonist nor antagonist treatment affected receptor mRNA levels.

Mechanisms of up-regulation of D2L-receptors by agonists may be different than the mechanisms of up-regulation by antagonists, which occurs less rapidly ($t_{1/2}$ = 8 hr), and to a lesser extent (2–3 fold). Effects of agonists on receptor levels are synergistic with cAMP analogs but effects of antagonists are not. By using cycloheximide, a protein synthesis inhibitor, and a receptor alkylating agent, investigators provided evidence that both agonists and antagonists decrease receptor protein degradation, increase receptor synthesis and increase receptor insertion into membrane (87). Whether all of these mechanisms occur in vivo is not known, but these studies provide strong support for translational and post-translational mechanisms for controlling levels of receptors.

Other authors (86) have studied D2L-receptors expressed in CHO cells and concluded that agonist-induced up-regulation of receptor levels is due to increased receptor mRNA levels by increased transcription of receptor genes. Discrepancies in interpretation for transfected D2L as well as adrenergic receptors could be due to several factors, including: parent cell line, species (or sequence) of receptor clone expressed, promoter used, amount of intact 5' receptor promoter sequence and level of expression.

Serotonin receptors: Groteweil and Sanders-Bush (88) demonstrated that the parent cell line background used for neurotransmitter receptor expression in heterologous systems is a critical determinant for receptor regulation. 5-HT2A receptors were stably expressed in NIH 3T3 cells, Madin-Darby canine kidney cells and AtT-20 cells. In transfected AtT-20 cells, agonists produce down-regulation and antagonists produce up-regulation. The converse situation occurs in transfected Madin-Darby canine cells, while agonists or agonists do not affect receptor levels in NIH 3T3 cells. This dependence on cellular environment may complicate and shroud the determinations of mechanisms important in vivo. On the other hand, these findings could suggest that neurotransmitter receptor genes expressed in many cells in vivo are differentially regulated depending on other genes specifically expressed in the different cells.

Muscarinic receptors: M1- and M2-receptor regulation has been studied in stably transfected mouse fibroblast B82 cells (89). Both M1- and M2-receptors are down-regulated by chronic agonist exposure by 50–70% within 6 hr. M2-receptor down-regulation is accompanied by receptor internalization but M1-receptor down-regulation is not, presumably due to differences in receptor subtype sequence, since the parent cell line background is identical.

M3-receptors stably expressed in CHO cells also exhibit agonist-induced down-regulation of receptor levels. Yang et al. (90) elegantly demonstrated that 3 carboxyl-terminal threonines of the M3-receptors are required for this effect using site-directed mutagenesis, stably expressing wild-type and mutant receptors, treating the cells with agonist and measuring receptor levels.

Opioid receptors: A study of δ-opioid receptors stably expressed in CHO cells (91) brings to light another complication of interpretations of results from these studies. The authors generated 3 cell lines which express low, intermediate and high levels of opioid binding. Chronic exposure to agonist causes down-regulation in these cells. The magnitude of down-regulation was lower by 22–29% in cells expressing low levels of receptors compared to the other 2 cell lines. Receptor desensitization, as measured by cAMP accumulation, was maximal in cells expressing low levels of receptors, and minimal in the other 2 cell lines. Receptor mRNA was unaffected by agonist treatment in all 3 cell lines. Instead of elucidating mechanisms of receptor regulation, this study may reveal an even greater complexity of this process.

Nicotinic receptors: An exception to the axiom of agonist-induced down-regulation is the nicotinic receptor, a member of the family of ligand-gated ion channels. The levels of this receptor are increased in brain with chronic nicotine exposure (92). M10 cells are a mouse Ltk-cell line that stably express nicotinic α4β2-subunits. Receptor cDNAs were transfected in the pMSGneo vector. Receptor expression is controlled by a dexamethasone-sensitive promoter in these cells; therefore, M10 cells are highly analogous to the GABA_A-receptor-expressing PA3 cells (93).

Nicotine treatment (3 days) produces twofold up-regulation of expressed receptors. A channel blocking antagonist also produces up-regulation, a phenomenon that also occurs in brain. Receptor mRNA levels are unaffected by agonist treatments. With the use of cycloheximide, the authors demonstrated that agonist-induced up-regulation is a result of a decreased protein degradation of agonist-bound receptor. This study represents another example of post-translational regulation of stably expressed neurotransmitter receptors.

Potential limitations of these studies

The studies described above demonstrate that stable expression of neurotransmitter receptors in mammalian cells provides a means of observing the phenomenon of down-regulation and determining the receptor amino acids sequences and the mechanisms involved in down-regulation. Although these systems are a useful tool, interpretations about the mechanisms of receptor regulation must be conservative, as many factors can affect
results from chronic drug treatments, including parent cell line, receptor species or subtype, promoter system and level of receptor expression.

V. Results from chronic drug treatments of cells that stably express GABA<sub>A</sub> receptors

Mammalian cell expression system

As discussed above, chronic drug treatments can alter GABA<sub>A</sub> receptor binding, allosteric coupling, function and levels of subunit mRNAs and protein in brain and neurons. Performing similar experiments with stably transfected cells can address the cellular requirements of GABA<sub>A</sub> receptor regulation. It is possible that mechanisms which regulate some forms of GABA<sub>A</sub> receptor down-regulation may be present in stably transfected cells while others are missing. For example, the regulation of allosteric coupling by chronic drug treatments is shared by distinct classes of drugs. Regulation of subunit mRNA levels is also common to chronic treatments with GABA or benzodiazepines. However, it is not clear if decreases in allosteric coupling are connected to decreases in subunit mRNAs. Comparing results from transfected cells and neurons implicates the importance of characteristics of the neuronal phenotype. Furthermore, the study of mechanisms of receptor regulation may be facilitated by using a homogeneous population of cells (see Fig. 1).

In cells which stably express GABA<sub>A</sub> receptor α1-, β1- and γ2L-subunits (PA3 cells) (93), expression of receptor subunit mRNA is controlled by an exogenous dexamethasone-sensitive promoter; the 5’ upstream sequences for GABA<sub>A</sub> receptor genes are missing. This could be considered a disadvantage of this expression system. However, under these conditions, it is unlikely that chronic treatments would affect receptor gene transcription. In this manner, one can assess whether changes in receptor transcription are a requirement for receptor regulation. It is hypothesized that receptor regulation observed with PA3 represents post-transcriptional regulation and probably post-translational regulation.

If no changes in the various forms of GABA<sub>A</sub> receptor down-regulation occur after chronic treatment of PA3 cells, the importance of intact promoter regions and control of transcription would be indirectly implicated. However, the PA3 cells may also be missing factors present in neurons that are involved in the translational or post-translational regulation of GABA<sub>A</sub> receptors in response to drug treatments.

Considering the enormous heterogeneity of GABA<sub>A</sub> receptors, it is possible that chronic drug treatments regulate such receptors through the expression of specific subunits. We know that chronic treatment with GABA or benzodiazepines affects levels of GABA<sub>A</sub> receptor mRNAs (39, 51), although uncoupling can occur without changes in receptor density (44–46). The predominant hypothesis for such changes in neuronal systems is the “subunit replacement” mechanism (7). Because different α-subunits can confer different degrees of allosteric coupling; e.g., GABA enhancement of benzodiazepine binding is greater in receptors expressing α3-versus α1- or α2-subunits (11), an attractive mechanism for the regulation of uncoupling would be replacement of subunits, leading to expression of receptors with reduced coupling. For example, if chronic exposure to agonists affects the expression of receptor genes, leading to lower levels of GABA<sub>A</sub> receptors containing α3-subunit in neurons, a decrease in allosteric coupling would be postulated.

Because the receptor composition of PA3 cells is known to be α1β1δ1γ2L, regulation of allosteric coupling could not be attributed to any variant in α-subunit or to expression of non-α1β1δ1γ2L-subunits. Therefore, PA3 cells can be used to address the importance of subunit replacement as a mechanism of GABA<sub>A</sub> receptor regulation.

Results from drug treatments

Uncoupling of GABA and benzodiazepine binding sites occurred in PA3 cells after treatments with GABA<sub>A</sub> agonists (94) and benzodiazepines (95). For GABA<sub>A</sub> agonists, the effective length of treatment (2–4 days) as well as the extent of uncoupling (40–50%) are in agreement with agonist-induced uncoupling of chick neurons (36). However, benzodiazepine-induced uncoupling occurred more rapidly, at lower concentrations, and to a greater extent (80–90%) in the stably transfected cells compared to mouse and chick neurons (46, 47). Chronic benzodiazepine treatments also decreased benzodiazepine potentiation of receptor function by 70–80%. The levels of receptor subunit mRNAs and α1-subunit protein were not affected for GABA<sub>A</sub> agonist and benzodiazepine treatments in PA3 cells.

Chronic ethanol treatments (96) decreased the benzodiazepine potentiation of receptor function in a manner consistent with results from mice and mouse brain mRNA-expressing oocytes (71, 97). Chronic ethanol treatments did not affect levels of receptor subunit mRNAs or α1- or β1-proteins in PA3 cells. Treatments with barbiturate or neurosteroid (94) did not affect coupling of GABA and benzodiazepine binding sites, which has been observed in primary neuronal cultures (36, 64). Therefore, in comparison to neurons, some forms of receptor regulation (uncoupling of binding sites and decreases in benzodiazepine action) are observed in the stably transfected cells while others (changes in levels of receptor binding, mRNA and protein) are not.
Conclusions from the stably transfected cells

The regulation of allosteric coupling and benzodiazepine function by chronic drug treatments that occur in neurons can be observed in stably transfected cells. Down-regulation of receptor density, α1-subunit mRNA or protein was not observed. The 5' upstream regions of the receptor cDNAs are not intact, and the presence of intact promoters and transcriptional regulation of receptor genes may be a requirement for the down-regulation seen in neurons. Another possibility for these results are that factors involved in the post-transcriptional regulation of receptors in neurons are missing in the transfected cells. Other possible mechanisms of drug regulation of receptor mRNA levels must address mRNA processing, transport to the cytoplasm, targeting to dendrites, assembly into polysomes, stability and rate of translation (1). It is possible that regulation of one of these steps between transcription and translation of receptor mRNAs is the target of regulation of receptor mRNA levels in neurons.

Another feature of neurons missing in PA3 cells is GABA_A-mediated synaptic neurotransmission. This pathway is presumably important for receptor regulation as distinct classes of drugs which increase GABA_A receptor function produce some of the same chronic effects. Finally, these cells are exposed to dexamethasone, a potent glucocorticoid. Effects of dexamethasone on gene expression could potentially be synergistic or inhibitory on the regulatory effects of chronic drug treatments.

The transfected cells express receptors with defined subunit composition. Several subtypes of each subunit family can be expressed in neuronal cultures, and the regulation of specific subunits may be targeted in the regulation by chronic treatments. Only the transfected subunit cDNAs are expressed in PA3 cells. Regulation in PA3 cells does not support a requirement of changes in the expression of specific subunits or subunit replacement. Because some studies with chronic ethanol (70) and chronic benzodiazepines (45, 46) have shown changes in receptor functional phenotype without changes in levels of receptor binding sites, exchanging specific subunits is a highly logical explanation. This process is known to occur in the development of the neuromuscular junction. For example, acetylcholine receptor subunit expression and channel conductance and gating are different in embryonic cultures compared to adult cells (98). Replacing γ-subunits with ε-subunits during development explains these differences, as receptors containing γ display the embryonic phenotype and receptors containing ε display the adult phenotype (98). GABA_A receptor mRNA expression in cerebellar granule cells also changes with time in culture; e.g., increases in α6-subunits, and diazepam-insensitive [3H]Ro 15-4513 binding over time (99). GABA_A receptor phenotype changes with time in culture in mouse cortical neuron cultures, resulting in a shift from benzodiazepine type 2 to benzodiazepine type 1 receptors (100).

PA3 cells that are not induced with dexamethasone do not express [3H]muscimol or [3H]flunitrazepam binding and do not display muscimol-stimulated 36Cl uptake. Thus, it is a safe assumption that no other GABA_A receptor subunit subtypes are expressed in these cells other than α1, β1 and γ2L. Therefore, a subunit replacement mechanism would not be possible in this system and cannot account for observed changes in receptor properties.

VI. Post-translational regulation of GABA_A receptors which may occur during chronic drug treatment

The intracellular loop PKA and PKC consensus phosphorylation sequences and in vitro phosphorylation of β- and γ2-subunits have been well-characterized (101-103). Both PKA and PKC phosphorylation affect measurements of GABA_A receptor function, although there is not a consensus of findings in this area. Many studies demonstrate decreases in GABA-mediated responses associated with increased cAMP levels or introduction of the catalytic peptide of PKA. In brain microsacs, introduction of PKA reduced muscimol-stimulated 36Cl uptake (104). When this response was measured in transfected 293 cells, it was eliminated by site-directed mutagenesis of serine 409 of β1-subunits (105). However, in L929 cells stably transfected with α1β1γ2-subunits, cotransfection of the catalytic subunit of PKA enhanced GABA-mediated currents in comparison to cells not expressing the catalytic subunit. Site-directed mutagenesis of the PKA phosphorylation on the β-subunit abolished the observed increases in function (19).

Phorbol esters activate PKC. In oocytes injected with receptor mRNAs, phorbol esters reduce GABA_A receptor function (106, 107). Phosphorylation of serine 410 (108) of the β2-subunit and serines 327 (108) and 343 (109) of the γ2-subunit contributes to this effect as determined by mutagenesis of these residues. In contrast, in transiently transfected L929 cells, the catalytic peptide from PKC enhances GABA_A single channel responses by increasing opening frequency and duration (110). Differences in the stimulation of phosphorylation of these critical residues; e.g., by phorbol esters or kinase catalytic domain, as well as differences in expression system background may explain some of the discrepancies of the various studies of effects of phosphorylation on GABA_A receptor function.

Other known and unknown kinases may also affect GABA_A receptor function, including tyrosine kinases, camK II and cGMP-dependent protein kinase (PKG). The latter two kinases phosphorylate serine 409 of β1-
subunits (111). Therefore, the phosphorylation state of this serine is likely a major target for the regulation of receptor function. In electrophysiological studies, cGMP prevents rundown of GABA_A responses in frog dorsal root ganglion cells (112) and introduction of cGMP-dependent kinase II increases maximal GABA_A receptor currents in transiently transfected L929 cells (113). Tyrosine kinases can phosphorylate both the γ- and β-subunits of the GABA_A receptor and tyrosine phosphorylation appears to increase the function of the receptor (114).

A candidate mechanism for GABA_A receptor regulation after chronic drug exposure is phosphorylation of receptor protein. Conformational changes induced by GABA_A agonists and benzodiazepines may unveil receptor serine or tyrosine residues that are inviting to PKA, PKC, camK II, PKG, PTK or other kinases. It is possible that the observed decreases in GABA_A receptor function after chronic agonist treatment (37, 38) are mediated by PKA or PKC phosphorylation of GABA_A receptor protein or associated proteins. Unfortunately, most of the phosphorylation studies did not look at allosteric interactions of GABA_A receptors. In one study, phorbol ester treatment of oocytes expressing GABA_A receptors enhanced both benzodiazepine and barbiturate potentiation of GABA-induced currents (107). If PKC phosphorylation does enhance benzodiazepine and barbiturate functional coupling to GABA_A sites in neurons, the reductions in allosteric binding interactions after drug treatments in neurons (37, 46, 63) may occur through dephosphorylation of PKC sites on GABA_A receptor protein or associated proteins. Therefore, chronic drug treatments may affect specific phosphatases.

Regarding ethanol enhancement of GABA_A receptor function, electrophysiological recordings from CA1 hippocampal neurons show that activation of G proteins facilitates ethanol potentiation (115) and that the ethanol potentiation is blocked by PKC inhibitors (116). Furthermore, ethanol potentiation of GABA_A action in this system is enhanced by using hippocampal slices placed in cold incubation buffer which is then allowed to equilibrate to room temperature. Preliminary data suggest that in samples from the cold slices where ethanol potentiation is elevated, the basal in vitro phosphorylation is greater compared to normal preparations (J.L. Weiner, personal communication). Therefore, the findings of Allan and Harris (77) and Morrow et al. (75), which show that chronic ethanol treatment causes a reduction in ethanol potentiation of GABA-mediated 36Cl^- flux, might be explained by dephosphorylation of PKC sites.

For the down-regulation of GABA_A (117, 118) as well as NMDA (119) receptor levels by chronic agonist treatments, it appears that regulating receptor mRNA levels is not critical, and the regulation of receptor protein by post-translational modifications may occur on several levels. Rates of insertion into membrane, rates of degradation, subcellular trafficking, and association with specific organelles and macromolecular complexes may be affected by chronic drug treatments (1). There is evidence for GABA_A agonist-induced receptor internalization (43), and [3H]flunitrazepam binding sites are present on clathrin-coated vesicles (44). Furthermore, chronic benzodiazepine treatment increases the receptor density of this fraction by about 80%, while simultaneously decreasing receptor density of membranes by about 20% (120). These studies provide a compelling argument for the mechanism of down-regulation of GABA_A receptor levels in neurons: chronic GABA_A or benzodiazepine agonist treatments increase receptor endocytosis and degradation via clathrin coated vesicles.

Further investigation using biochemical and electrophysiological methods are needed to determine the importance of GABA_A receptor phosphorylation in the regulation of receptors by drug treatments. Conformational changes induced by GABA_A agonists, benzodiazepines and ethanol could potentially induce lasting interactions with other molecules including associated proteins (such as kinases) or lipids. The result of these associations may energetically favor a receptor conformation with diminished allosteric coupling. Using brain preparations, neuronal cultures, as well as stably transfected cells, will help in the understanding of the interactions that cause receptor regulation, as well as the tolerance and dependence properties of benzodiazepines and ethanol.

VII. Conclusions

It is clear that chronic exposure of neuronal GABA_A receptors to GABA agonists or allosteric modulators such as benzodiazepines and ethanol alters the function of these receptors. These observations raise several questions. One is the mechanism(s) responsible for these changes. Transfected cells will likely be useful for dissecting and elucidating at least some of these mechanisms. As discussed in detail in this review, three of the most likely mechanisms for regulation of GABA_A receptor function are subunit substitution, receptor internalization/recycling, and receptor phosphorylation. Subunit substitution cannot occur in many transfected cells and thus can be ruled out as a mechanism for effects of chronic drug treatments on these cells. Subunit substitution would be expected to provide rather slow changes in GABA_A receptor function as changes in gene expression must first occur in the nucleus, then protein must be synthesized and transported to the cell membrane, which,
in the case of dendrites or axons, may be some distance from the nucleus. Lastly, the new receptors must be inserted into the membrane, and the receptor pool will slowly be changed as "old" receptors are internalized and the "new" receptors become dominant in numbers. In contrast, post-translational processes such as receptor internalization or phosphorylation would be much more rapid. In fact, chronic drug treatments with stably transfected cells can produce changes in receptor function within hours (95).

The differences in these processes raises a second question: Which of these various receptor changes and mechanisms are relevant to tolerance and dependence in vivo? A prevalent idea is that benzodiazepine or ethanol tolerance and dependence develop slowly; most animal studies (reviewed in section III) use treatment times in the range of 4 days to 4 weeks. This would suggest regulation of gene expression, rather than post-translational modification, as mechanisms of tolerance and dependence. However, this may not be correct. In fact, there are a number of studies showing tolerance and dependence after only brief treatments with benzodiazepines or ethanol (121). For example, Lister and Nutt (122) studied mice and rats for lorazepam tolerance and dependence. The shortest interval they used was six hours (after a single injection of lorazepam). However, this was sufficient to produce both tolerance and dependence. Crabbe et al. (123) found that a single injection of diazepam, ethanol, pentobarbital, t-butanol or acetaldehyde produced signs of acute dependence within a few hours. Haigh and Feely (121) noted that 12 hr after a single dose of lorazepam, the tolerance in mice is as great as after 10 days of treatment. Careful clinical studies of this question are impossible, but Crawford et al. (124) attempted to address the issue of rapid tolerance to lorazepam in treatment of childhood status epilepticus and suggested that tolerance develops over a period of hours. Thus, it is clear that brief treatment with benzodiazepines or ethanol can produce tolerance and dependence in vivo and rapid post-translational modifications of GABA<sub>A</sub> receptors may be responsible for these behavioral responses.

A key, unanswered, question is what signaling system can be responsible for transcriptional and post-translational responses to chronic drug treatment. In some cases (e.g., GABA agonists) activation of the channel is required. In this instance, the signal could be a change in chloride levels or in membrane potential, but how such changes could affect gene expression or kinase activities is completely unknown. In other cases (e.g., benzodiazepine agonists), channel activation is not required; receptor occupation is sufficient by itself. Study of the processes responsible for these drug actions may lead to a new understanding of intracellular signaling and receptor regulation.

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