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REVIEW

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Neurosteroids, $GABA_A$ receptors, and ethanol dependence

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Abstract *Rationale:* Changes in the expression of type A receptors for γ-aminobutyric acid (GABA) represent one of the mechanisms implicated in the development of tolerance to and dependence on ethanol. The impact of such changes on the function and pharmacological sensitivity of $GABA_A$ receptors ($GABA_A$Rs) has remained unclear, however. Certain behavioral and electrophysiological actions of ethanol are mediated by an increase in the concentration of neuroactive steroids in the brain that results from stimulation of the hypothalamic–pituitary–adrenal (HPA) axis. Such steroids include potent modulators of $GABA_A$R function. *Objectives:* We have investigated the effect of ethanol exposure and withdrawal on subunit expression and receptor function evaluated by subunit selective compounds, as well as the effects of short-term exposure to ethanol on both neurosteroid synthesis and $GABA_A$R function, in isolated neurons and brain tissue. *Results:* Chronic treatment with and subsequent withdrawal from ethanol alter the expression of genes for specific $GABA_A$R subunits in cultured rat neurons, and these changes are associated with alterations in receptor function and pharmacological sensitivity to neurosteroids, zaleplon, and flumazenil. Acute ethanol exposure increases the amount of 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) in hippocampal slices by a local action independent of the activity of the HPA axis. This effect of ethanol was associated with an increased amplitude of $GABA_A$R-mediated miniature inhibitory postsynaptic currents recorded from CA1 pyramidal neurons in such slices. *Conclusions:* Chronic ethanol exposure elicits changes in the subunit composition of $GABA_A$Rs, which, in turn, likely contribute to changes in receptor function associated with the altered pharmacological and behavioral sensitivity characteristic of ethanol tolerance and dependence. Ethanol may also modulate $GABA_A$R function by increasing the de novo synthesis of neurosteroids in the brain in a manner independent of the HPA axis. This latter mechanism may play an important role in the central effects of ethanol.

Keywords $GABA_A$ receptor · Ethanol · Neurosteroids · Tolerance · Dependence · Gene expression · Patch clamp · Hippocampal neurons · Cerebellar granule cells · Benzodiazepines

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Introduction

Type A receptors for γ-aminobutyric acid (GABA) are ligand-gated Cl^- channels and mediate fast inhibitory synaptic transmission in the mammalian central nervous system (CNS) (Barnard et al. 1998; Mehta and Ticku 1999; Vicini 1999). These receptors are heteromeric complexes formed by the assembly of five subunits of various classes, including α_1 to α_6, β_1 to β_4, γ_1 to γ_3, δ, ε, π, θ, and ρ_1 to ρ_3 (Barnard et al. 1998; Sieghart and Sperk 2002; Whiting et al. 1999). The brain region-specific distribution and ontogeny-dependent expression of these various subunits give rise to a relatively large number of $GABA_A$ receptor ($GABA_A$R) subtypes, which differ in their subunit composition as well as in their physiological and pharmacological properties (Sieghart 1995; Sieghart and Sperk 2002; Whiting et al. 1999).

$GABA_A$Rs are the targets both of various classes of clinically relevant drugs, including benzodiazepines, barbiturates, and general anesthetics, as well as of endogenous

compounds such as neuroactive steroids, all of which allosterically modulate receptor function (Barnard et al. 1998; Frye et al. 1981; Mohler et al. 2002; Sieghart 1995). Neurochemical, electrophysiological, and behavioral evidence accumulated over the past two decades suggests that $GABA_A$Rs also mediate certain acute and chronic actions of ethanol (Deitrich et al. 1989; Faingold et al. 1998; Grobin et al. 1998; Harris 1999; Ueno et al. 2001). Similar to the $GABA_A$R modulators mentioned above, ethanol exhibits an array of central depressant actions, including anxiolytic, anticonvulsant, sedative-hypnotic, muscle relaxant, and general anesthetic effects, in a dose-dependent manner (Deitrich et al. 1989; Frye et al. 1981).

A role for $GABA_A$R-mediated neurotransmission in the effects of ethanol was suggested by the early observation that low concentrations (20–60 mM) of ethanol enhanced agonist-stimulated Cl^- flux in brain synaptoneurosomes (Allan and Harris 1986; Morrow et al. 1988; Suzdak et al. 1988) and cultured neurons (Ticku and Burch 1980). In addition, certain behavioral effects of ethanol were shown to be enhanced by $GABA_A$R agonists or positive modulators and to be attenuated or blocked by $GABA_A$R antagonists or negative modulators (Martz et al. 1983). Neurophysiological evidence that ethanol acutely modulates $GABA_A$R function has been somewhat more elusive and controversial; whereas some studies have shown that ethanol potentiates $GABA_A$R function, others have failed to detect a significant effect (Faingold et al. 1998; Grobin et al. 1998). Potentiation of $GABA_A$R function by ethanol has been suggested to be region specific, with the hippocampus generally regarded as a relatively ethanol-insensitive region of the brain. However, ethanol potentiation of $GABA_A$R function has been demonstrated even in the hippocampus under particular experimental conditions, including, for example, blockade of presynaptic $GABA_B$ receptors (Wan et al. 1996), proximal rather than distal stimulation (Weiner et al. 1997), and activation of β-adrenergic signaling mediated by cyclic AMP and protein kinase A (Freund and Palmer 1997; Lin et al. 1991).

More recent studies have provided insight into additional mechanisms by which ethanol might influence the activity of GABAergic synapses. In addition to affecting postsynaptic $GABA_A$Rs, ethanol appears to exert a presynaptic action that results in an increased probability of GABA release. Ethanol has thus been shown to increase the frequency of both potential-dependent and -independent $GABA_A$R-mediated inhibitory postsynaptic currents (IPSCs) in hippocampal CA1 pyramidal neurons (Ariwodola and Weiner 2004; Carta et al. 2003; Sanna et al. 2004), the amygdala (Nie et al. 2004; Roberto et al. 2003), cerebellar granule cells (Carta et al. 2004), and spinal motoneurons (Ziskind-Conhaim et al. 2003). Such an action of ethanol might also be expected to result in an increase in the extracellular concentration of GABA to a level sufficient to activate presynaptic $GABA_B$ receptors, which negatively regulate GABA release from presynaptic terminals (Ariwodola and Weiner 2004). Consistent with this notion, blockade of presynaptic $GABA_B$ receptors with the specific antagonist SCH 50911 greatly increased the modulatory effect of ethanol on $GABA_A$R-mediated IPSCs in the CA1 region (Ariwodola and Weiner 2004). The modulatory activity of ethanol at GABAergic synapses might thus be self-limiting as a result of indirect activation of presynaptic $GABA_B$ receptors.

Expression of mammalian $GABA_A$ receptor subunits in *Xenopus* oocytes revealed that ethanol, at concentrations as low as 3–30 mM, selectively potentiates the function of recombinant receptors containing α_4 or α_6 and δ subunits; ethanol had no effect at these concentrations on receptors that contained the γ_2 subunit in place of δ (Sundstrom-Poromaa et al. 2002; Wallner et al. 2003). $GABA_A$Rs containing α_4 or α_6 and δ subunits (unlike γ_2 subunit-containing receptors) are located exclusively at extrasynaptic sites in the brain and are thought to mediate tonic inhibitory activity (Mody et al. 1994; Semyanov et al. 2004). Extrasynaptic $GABA_A$Rs in the dentate gyrus and thalamus ($\alpha_4\beta\delta$) or cerebellar granule cells ($\alpha_6\beta\delta$) are characterized by a higher affinity for GABA (~0.5 μM), a slower desensitization rate, and a higher sensitivity to neuroactive steroids (Semyanov et al. 2004). A low concentration of ethanol (30 mM) was shown to increase $GABA_A$R-mediated tonic activity in dentate gyrus granule cells (Wei et al. 2004). Given the important role of tonic inhibitory activity in the fine tuning of neuronal excitability, these observations suggest that extrasynaptic $GABA_A$Rs may be an important target of ethanol at pharmacologically relevant concentrations.

Effects of chronic ethanol exposure on $GABA_A$Rs

Long-term exposure to ethanol can result in adaptive changes in $GABA_A$R-mediated neurotransmission (Chandler et al. 1998; Faingold et al. 1998; Grobin et al. 1998). Altered $GABA_A$R function, characterized by a decreased responsiveness to GABA, a decreased sensitivity to ethanol, cross-tolerance to benzodiazepines and barbiturates, as well as an increased sensitivity to neurosteroids and inverse agonists, is thought to be important in the development of tolerance to and dependence on ethanol (Allan and Harris 1987; Devaud et al. 1996; Morrow et al. 1988; Sanna et al. 1993; Ticku and Burch 1980). Although the molecular mechanisms responsible for the changes in $GABA_A$R function induced by persistent ethanol exposure remain unclear, they have been proposed to involve effects on receptor density (Ticku and Burch 1980), posttranslational modification (Kumar et al. 2002), receptor trafficking (Grobin et al. 1998), and subunit expression (Devaud et al. 1995, 1997; Follesa et al. 2003; Mhatre et al. 1993; Sanna et al. 2003).

Chronic ethanol exposure is associated with marked changes in $GABA_A$R subunit expression at both the mRNA and protein levels in various brain regions (Grobin et al. 2000). Such treatment has thus been shown to result in a decrease in the expression of α_1 and α_2 subunits and a parallel increase in that of α_4, α_6, β_1, β_2, β_3, γ_1, and γ_2 subunits in the cerebral cortex and cerebellum. In addition, chronic intermittent ethanol treatment resulted in down-

regulation of α_1 and δ subunit expression and up-regulation of α_4, γ_1, and γ_2 subunit expression in the hippocampus (Cagetti et al. 2003; Mahmoudi et al. 1997). Thus, in the hippocampus, the α_1 subunit was altered only after multiple withdrawals (Cagetti et al. 2003) but not by chronic ethanol treatment (Matthews et al. 1998). Together, these results suggest that long-term ethanol exposure triggers cellular adaptive mechanisms that involve alterations in the subunit composition of $GABA_A$Rs with regional differences, which, in turn, might affect receptor function and underlie the altered pharmacological and behavioral sensitivity characteristic of ethanol tolerance and dependence. Multiple mechanisms could contribute to the regulation of GABA(A) receptor expression. These mechanisms may include the involvement of other neurotransmitter systems, endogenous steroids, and second or third messenger cross talk (Grobin et al. 1998, 2000).

Role of neurosteroids in the effects of ethanol on $GABA_A$Rs

Neurosteroids are steroid derivatives that are synthesized de novo in the CNS from cholesterol (Hu et al. 1987; Mathur et al. 1993). They are generally distinguished from neuroactive steroids, which are steroids produced by peripheral sources (adrenals and gonads) that exert their effects within the CNS. Certain neuroactive steroids, rather than exhibiting the classical genomic action of steroid hormones, directly modulate the function of $GABA_A$Rs with potencies and efficacies that are similar to or greater than those of benzodiazepines or barbiturates (Harrison and Simmonds 1984; Majewska et al. 1986). These molecules have thus been implicated as endogenous modulators of $GABA_A$R-mediated neurotransmission.

The progesterone metabolite 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) potentiates the GABA-induced opening of the $GABA_A$R-associated Cl^- channel at nanomolar concentrations in vitro, and its systemic administration induces pharmacological and behavioral effects similar to those elicited by anxiolytic, anticonvulsant, and hypnotic drugs that modulate $GABA_A$R function (Lambert et al. 2001; Majewska 1992). The anxiolytic and anticonvulsant properties of progesterone are mostly attributable to its conversion to allopregnanolone (Bitran et al. 1995; Kokate et al. 1994). Fluctuations in plasma or brain concentrations of allopregnanolone have been associated with stress, pregnancy, the menstrual cycle, menopause, regulation of neuronal excitability, and a variety of neurological or psychiatric disorders (Barbaccia et al. 1996; Bicikova et al. 1998; Biggio and Purdy 2001; Concas et al. 1998; Genazzani et al. 1998). In addition, prolonged physiological or pharmacologically induced changes in allopregnanolone concentrations are implicated in regulation of $GABA_A$R plasticity in addition to that of receptor function (Brussaard et al. 1997; Concas et al. 1998; Follesa et al. 2000; Smith et al. 1998b) as well as in development of the GABAergic system in the prefrontal cortex (Grobin et al. 2003).

Certain acute effects of ethanol on $GABA_A$Rs have been proposed to be mediated by the peripheral secretion of neuroactive steroids (Morrow et al. 1999). Acute ethanol administration indeed increases the concentrations of allopregnanolone in the plasma, cerebral cortex, and hippocampus (Barbaccia et al. 1999; Morrow et al. 2001; VanDoren et al. 2000). Furthermore, pretreatment of animals with the 5α-reductase inhibitor finasteride, which inhibits the biosynthesis of allopregnanolone, reduced the extent of the ethanol-induced increase in the cerebrocortical level of allopregnanolone and prevented certain neurochemical, electrophysiological, and behavioral effects of ethanol (Khisti et al. 2002b; VanDoren et al. 2000). The ability of ethanol to increase allopregnanolone biosynthesis is thought to be dependent on its stimulatory effect on the hypothalamic–pituitary–adrenal (HPA) axis (Ellis 1966; Khisti et al. 2003a; Ogilvie et al. 1997; Rivier 1996; Rivier et al. 1984). Indeed, ethanol fails to increase the plasma or brain levels of allopregnanolone or to induce certain of its pharmacological effects in adrenalectomized rats (Khisti et al. 2002a,b). In addition, these investigators showed that in adrenalectomized rats, the administration of the immediate precursor of allopregnanolone restored the effect of ethanol, suggesting that both peripheral precursors and brain synthesis contribute to the effect of this drug (Khisti et al. 2003b).

These various observations suggest that neuroactive steroids produced by peripheral organs in response to activation of the HPA axis mediate certain effects of ethanol on $GABA_A$Rs. However, given that neurosteroids are produced in the brain in a manner independent of peripheral organs (Hu et al. 1987; Khisti et al. 2003b; Mathur et al. 1993; Purdy et al. 1991), it is important to clarify further the pharmacology of ethanol as well as whether this addictive drug is able to stimulate neurosteroidogenesis directly in the brain.

Ethanol and $GABA_A$R plasticity in cultured cerebellar granule cells and hippocampal neurons

Long-term administration and subsequent withdrawal of ethanol elicit neurochemical and molecular effects similar to those induced by drugs that positively modulate $GABA_A$R function (Biggio et al. 2003; Devaud et al. 1997; Mhatre et al. 1993; Morrow et al. 1990). We here summarize the results of several studies from our laboratory in which primary neuronal cultures were subjected to long-term treatment with and withdrawal of ethanol and in which the effects of diazepam, γ-hydroxybutyrate (GHB), and baclofen on $GABA_A$R expression and function were evaluated during ethanol withdrawal in order to obtain insight into the role of individual receptor subunits in the actions of ethanol.

Effects of chronic exposure to and withdrawal of ethanol on GABA$_A$R gene expression

We incubated rat cerebellar granule cells for 5 days in the absence or presence of 100 mM ethanol and then determined the abundance of mRNA or protein for α_1 to α_6, δ, γ_2L, and γ_2S subunits of the GABA$_A$R. Ethanol induced a decrease in the amounts of the mRNAs for the γ_2L and γ_2S splice variants as well as an increase in the amount of the α_3 subunit mRNA, but it had no effect on the abundance of the other subunit mRNAs or proteins examined (Table 1). In contrast, in rat hippocampal neurons, chronic ethanol treatment resulted in a decrease in the amounts of α_1, α_3, γ_2L, and γ_2S subunit mRNAs and a marked increase in those of the δ subunit mRNA and protein (Table 1).

We next investigated the effects of ethanol withdrawal by incubating the cultured neurons first with 100 mM ethanol for 5 days and then in the absence of ethanol for 3–24 h. The effects of withdrawal, which peaked between 3 and 6 h after discontinuation of ethanol treatment, included a decrease in the abundance of the α_1 and α_6 subunit mRNAs as well as in that of the δ subunit mRNA and protein, with the amounts of the γ_2L and γ_2S subunit mRNAs remaining decreased, in cerebellar granule cells (Table 1). In addition, ethanol withdrawal increased the expression of the α_2, α_4, and α_5 subunits in these cells. In hippocampal neurons, the abundance of the α_1, γ_2L, and γ_2S subunit mRNAs remained decreased, and the expression of the δ subunit remained increased after ethanol withdrawal (Table 1). In addition, ethanol withdrawal increased the expression of α_2, α_3, and α_4 subunits in these neurons.

These changes in the expression of specific GABA$_A$R subunits might contribute to the molecular mechanisms responsible for development of tolerance to and dependence on ethanol as well as for the central hyperexcitability induced by abrupt discontinuation of prolonged exposure to this drug (Faingold et al. 1998; Grobin et al. 1998). This hypothesis is supported by the fact that changes in the subunit composition of GABA$_A$Rs have pronounced effects on their physiological and pharmacological properties (Barnard et al. 1998; Hevers and Luddens 1998; Sieghart 1995) and thus might underlie the reduced receptor function and altered pharmacological and behavioral sensitivity characteristic of ethanol tolerance and dependence. Given the diversity and heterogeneity of GABA$_A$Rs expressed in different neuronal cell types, the ethanol-induced changes in the expression of each subunit might differ among neuronal populations.

Table 1 Effects of long-term treatment with and withdrawal of ethanol on GABA$_A$R gene expression in rat cerebellar granule cells and hippocampal neurons in culture

GABA$_A$R subunit	Cerebellar granule cells		Hippocampal neurons	
	Chronic ethanol	Ethanol withdrawal	Chronic ethanol	Ethanol withdrawal
α_1	↔	↓	↓	↓
α_2*	↔	↑↑↑	↔	↑↑
α_3	↑	↔	↓	↑↑
α_4*	↔	↑↑	↔	↑↑
α_5	↔	↑	↔	↔
α_6	↔	↓	Not expressed	Not expressed
δ*	↔	↓	↑↑↑	↑↑
γ_2L	↓↓	↓↓↓	↓↓	↓↓
γ_2S	↓	↓↓	↓	↓

Cells were incubated in the presence of 100 mM ethanol for 5 days (chronic ethanol) and then in the absence of ethanol for an additional 3–6 h (ethanol withdrawal). Changes in the amounts of GABA$_A$R subunit mRNAs relative to those in control cultures incubated continuously in the absence of ethanol were determined by RNase protection assay. Changes in the abundance of those subunits indicated with an asterisk were also determined by immunoblot or fluorescence analysis; similar qualitative results were obtained for the mRNA and protein of a given subunit. The arrows indicate an increase, decrease, or no change. The number of arrows indicates: one, from 20 to 30%; two, from 30 to 50%; three, more than 50%. Data for α_3 and α_5 subunit mRNAs in cerebellar granule cells are original; all other data are published (Follesa et al. 2003, 2004, 2005; Sanna et al. 2003)

Effects of chronic exposure to and withdrawal of ethanol on GABA$_A$R function

The pharmacology of benzodiazepine receptor ligands depends on the subunit composition of GABA$_A$Rs, especially with regard to the specific α and γ subunit isoforms present (Barnard et al. 1998; Pritchett et al. 1989). We therefore next examined the impact of the changes in GABA$_A$R subunit gene expression induced by chronic exposure to and withdrawal of ethanol on benzodiazepine pharmacology in both cerebellar granule cells and hippocampal neurons. With the use of patch-clamp electrophysiological recording from single neurons in culture, we evaluated the effects of the benzodiazepine receptor agonist zaleplon, which is selective for GABA$_A$Rs containing the α_1 subunit (Damgen 1999), as well as those of flumazenil, which acts as a competitive antagonist at the benzodiazepine site of most GABA$_A$Rs but as an agonist at that of GABA$_A$Rs containing the α_4 subunit (Wafford et al. 1996; Whittemore et al. 1996).

Consistent with its predominant pharmacological profile of a pure antagonist devoid of intrinsic activity, flumazenil (3 μM) exhibited only small effects on GABA-evoked Cl^- currents in either cerebellar granule cells or hippocampal neurons cultured in the absence of ethanol (Table 2). Treatment of either cell type with ethanol for 5 days had no effect on the action of flumazenil. In contrast, in granule cells or hippocampal neurons subjected to ethanol withdrawal for 6 h, flumazenil induced marked potentiation (+53±5 and +61±8%, respectively) of GABA-evoked Cl^- currents. Given that the presence of the α_4 subunit in recombinant GABA$_A$Rs confers positive (rather than no) allosteric modulation by flumazenil, our electrophysiological data showing positive modulation of GABA$_A$R function by flumazenil in cultured neurons subjected to ethanol withdrawal are consistent with our observations that, in both cerebellar granule cells and hippocampal neurons,

Table 2 Modulation of $GABA_A$ receptor function by flumazenil or zaleplon after long-term treatment with or withdrawal of ethanol in rat cerebellar granule cells and hippocampal neurons in culture

Drug	Potentiation of GABA-evoked Cl^- current (%)					
	Cerebellar granule cells			Hippocampal neurons		
	Control	Chronic ethanol	Ethanol withdrawal	Control	Chronic ethanol	Ethanol withdrawal
Flumazenil	20±3	18±2	53±5*	9±1	11±1	61±8*
Zaleplon	42±3	43±5	24±5*	41±5	23±5*	24±4*

Cells were untreated (control), treated with 100 mM ethanol for 5 days (chronic ethanol) or subjected to ethanol withdrawal for 6 h (ethanol withdrawal). They were then subjected to whole-cell patch-clamp recording. GABA was first applied to the cells at a concentration that induced a Cl^- current with an amplitude of 5–10% of the maximal response. It was then applied together with 3 μM flumazenil or 0.1–0.5 μM zaleplon. Some data are derived from published studies (Sanna et al. 2003, copyright of the Society for Neuroscience, and Follesa et al. 2003, with permission of ASPET)

*$P<0.05$ vs corresponding value for control cells

ethanol withdrawal also induced up-regulation of α_4 subunit expression (Table 1).

Chronic treatment of rats with ethanol was found to increase $GABA_AR$ α_4 subunit gene expression in both the cerebral cortex and hippocampus (Cagetti et al. 2003; Devaud et al. 1995, 1997; Mahmoudi et al. 1997; Matthews et al. 1998). Our data show that ethanol withdrawal results in a marked increase in expression of the α_4 subunit gene in cultured neurons. Up-regulation of α_4 subunit expression is also induced by withdrawal of benzodiazepine receptor ligands (Follesa et al. 2001, 2002) or of neurosteroids (Follesa et al. 2000; Smith et al. 1998b), suggesting that it might play an important role in the cellular hyperexcitability and anxiety-like behavior apparent in both animals and humans during withdrawal from these positive allosteric modulators of the $GABA_AR$. Increased expression of the α_4 subunit was also observed in the hippocampus of rats subjected to electrical kindling, a condition associated with reduced GABAergic function, a lower threshold for convulsions, and conflict behavior (Kamphuis et al. 1995). Furthermore, depletion of the α_4 subunit with the use of antisense RNA prevented the development of withdrawal symptoms in a progesterone withdrawal paradigm (Smith et al. 1998a). Flumazenil ameliorates ethanol withdrawal symptoms such as anxiety and hyperexcitability in animals and human alcoholics (Buck et al. 1991; File et al. 1989; Gerra et al. 1991; Moy et al. 1997; Nutt et al. 1993), an effect that has been proposed to result from blockade of the action of a putative endogenous benzodiazepine receptor ligand endowed with inverse agonist activity. Our data suggest that this effect of flumazenil might also be attributable to an increase in the number of $GABA_ARs$ containing the α_4 subunit induced by ethanol withdrawal, given that flumazenil acts as an agonist at these receptors.

To evaluate the functional effects of the changes in α_1 subunit gene expression induced by chronic ethanol treatment or withdrawal, we examined the modulatory action of the pyrazolopyrimidine zaleplon. Zaleplon at low concentrations in vitro is selective for $GABA_ARs$ that contain the α_1 subunit (Sanna et al. 2002). It also shows a lower receptor affinity and modulatory potency compared with other α_1 subunit-selective drugs (Sanna et al. 2002). We therefore examined the effects of zaleplon at a concentration at which it is selective for α_1 subunit-containing receptors in order to discriminate between receptors containing α_1 and those containing other α subunits.

The potentiating effect of a low concentration of zaleplon (0.5 μM) in control cerebellar granule cells (+42±3%) was similar to that observed in those subjected to chronic ethanol treatment (+43±5%). In contrast, this effect of zaleplon was reduced by about half in cerebellar granule cells subjected to ethanol withdrawal (Table 2). In hippocampal neurons, the potentiating effect of zaleplon observed under control conditions (+41±5%) was reduced by about half in neurons subjected either to chronic ethanol treatment or to ethanol withdrawal (Table 2).

The reduced efficacies of zaleplon in cerebellar granule cells subjected to ethanol withdrawal and in hippocampal neurons after chronic ethanol treatment or withdrawal (Table 2) are thus consistent with the down-regulation of α_1 subunit gene expression apparent under these conditions (Table 1). Given that $GABA_AR$ subtypes that contain α_1 or α_2 subunits mediate the sedative and anxiolytic effects of benzodiazepines, respectively (Mohler et al. 2002), our results are also consistent with the reduced sedative efficacy of benzodiazepines in human alcoholics as well as with the ability of these drugs to reduce the anxiogenic effect of ethanol withdrawal (Lejoyeux et al. 1998; Sellers et al. 1983).

With the same cell culture system, we have also shown that the changes in $GABA_AR$ gene expression induced by ethanol withdrawal are similar to those induced by withdrawal of benzodiazepines (Follesa et al. 2001), of imidazopyridines or pyrazolopyrimidines (Follesa et al. 2002), or of neurosteroids (Follesa et al. 2000), suggesting that these various modulators elicit changes in $GABA_AR$ function by a common molecular mechanism and that these changes underlie the development of withdrawal symptoms.

We also examined whether the differential changes in expression of the $GABA_AR$ δ subunit gene induced by chronic exposure to and withdrawal of ethanol in cerebellar granule and hippocampal neurons were accompanied by parallel changes in $GABA_AR$ function. Given that receptors containing the δ subunit ($\alpha_4\beta_3\delta$ receptors) manifest a greater sensitivity to the partial agonist 4,5,6,7-tetrahydroisoxazolo-pyridin-3-ol (THIP or gaboxadol) than do those containing the γ_2 subunit ($\alpha_4\beta_3\gamma_2$ receptors) (Adkins et al. 2001; Brown et al. 2002), we used this compound to

Table 3 Effects of chronic exposure to and subsequent withdrawal of ethanol on THIP-evoked Cl^- current and $GABA_AR$ sensitivity to allopregnanolone in cerebellar granule and hippocampal neurons

	Cerebellar granule cells THIP-evoked Cl^- current			Hippocampal neurons THIP-evoked Cl^- current		
	Control	Chronic ethanol	Ethanol withdrawal	Control	Chronic ethanol	Ethanol withdrawal
THIP (EC_{50}, μM)	18±1	16±1	51±2*	72±3	18±3*	31±2*
Allopregnanolone (% potentiation)	492±95%	499±76%	164±14%*	326±41%	895±179%**	652±103%*

Cells were incubated for 5 days in the absence (control) or presence of 100 mM ethanol (chronic ethanol) or were subjected to ethanol withdrawal for 6 h (ethanol withdrawal). The median effective concentration (EC_{50}) for THIP-evoked Cl^- current was determined from the dose–response relation, and the potentiation by allopregnanolone (0.3–1 μM) of THIP-evoked Cl^- current was determined at a THIP concentration that yielded 5–10% of the maximal response. Data are means±SEM of values from 10 to 22 neurons
*P<0.05, **P<0.01 vs corresponding value for control cells

evoke $GABA_AR$-mediated Cl^- currents in both types of neuron.

In cerebellar granule cells, the potency of THIP apparent under control conditions (EC_{50}, 18±1 μM) was not significantly affected by chronic ethanol treatment (Table 3). However, withdrawal of ethanol resulted in a significant reduction in THIP potency (EC_{50}, 51±2 μM). In hippocampal neurons, the potency of THIP was significantly increased after chronic exposure to ethanol (EC_{50}, 18±3 μM), compared with that apparent in control cells (EC_{50}, 72±3 μM), and it remained significantly increased after ethanol withdrawal (EC_{50}, 31±2 μM) (Table 3).

The modulatory effect of several neuroactive steroids on $GABA_AR$ function is markedly enhanced by the presence of the δ subunit (Adkins et al. 2001; Brown et al. 2002; Wohlfarth et al. 2002). This modulatory action is thus impaired in mice that lack the δ subunit (Mihalek et al. 1999; Spigelman et al. 2003). We therefore examined whether the different effects of δ subunit expression elicited by chronic ethanol exposure and ethanol withdrawal in cerebellar granule cells and hippocampal neurons were associated with parallel changes in the effect of allopregnanolone on THIP-evoked Cl^- currents. In cerebellar granule neurons, the modulatory action of allopregnanolone on THIP-evoked Cl^- current apparent under control conditions (+492±95%) was not significantly affected by chronic ethanol treatment (Table 3). However, withdrawal of ethanol resulted in a significant decrease in allopregnanolone efficacy (+164±14%). In hippocampal neurons, the modulatory effect of allopregnanolone on THIP-evoked Cl^- current was increased by a factor of 2.7 by chronic ethanol treatment and remained significantly increased after ethanol withdrawal (Table 3).

The δ subunit confers on $GABA_ARs$ a greater sensitivity to GABA, to the partial agonist THIP (Adkins et al. 2001; Brown et al. 2002), and to neurosteroids (Mihalek et al. 1999; Spigelman et al. 2003; Wohlfarth et al. 2002) than does the γ_2 subunit. Moreover, coassembly of the δ subunit with the α_4 and β_3 subunits yields $GABA_ARs$ whose function is enhanced by ethanol at low concentrations (Sundstrom-Poromaa et al. 2002; Wallner et al. 2003). The changes in the expression of the δ subunit induced by chronic ethanol treatment and withdrawal in cerebellar granule and hippocampal neurons (Table 1) were thus consistent with the corresponding changes in THIP potency and allopregnanolone efficacy (Table 3).

Effects of diazepam, GHB, and baclofen on $GABA_AR$ plasticity during ethanol withdrawal

Benzodiazepines, GHB, and the $GABA_B$ receptor agonist baclofen reduce withdrawal symptoms and the craving for ethanol in both human alcoholics and ethanol-dependent laboratory animals (Addolorato et al. 1996, 2002; Agabio et al. 1998; Colombo et al. 2000; Fadda et al. 1989; Gallimberti et al. 1992; Lejoyeux et al. 1998). We therefore examined the effects of diazepam, GHB, and baclofen on the changes in $GABA_AR$ gene expression and function induced by ethanol withdrawal in cultured cerebellar granule and hippocampal neurons. Exposure of cerebellar granule cells or hippocampal neurons to diazepam or GHB at the time of ethanol withdrawal completely antagonized the withdrawal-induced increase in the abundance of the α_2 and α_4 subunit mRNAs (Table 4). In contrast, baclofen did not antagonize these effects of ethanol withdrawal in either cell type. Neither diazepam, GHB, nor baclofen affected

Table 4 Prevention by diazepam and GHB, but not by baclofen, of the increase in $GABA_AR$ α_2 and α_4 subunit gene expression induced by ethanol withdrawal

$GABA_AR$ subunit mRNA	Drug	Effect on cerebellar granule cells and hippocampal neurons
α_1	Diazepam	Does not antagonize decrease
	GHB	Does not antagonize decrease
	Baclofen	Does not antagonize decrease
α_2	Diazepam	Antagonizes increase
	GHB	Antagonizes increase
	Baclofen	Does not antagonize increase
α_4	Diazepam	Antagonizes increase
	GHB	Antagonizes increase
	Baclofen	Does not antagonize increase
γ_2	Diazepam	Does not antagonize decrease
	GHB	Does not antagonize decrease
	Baclofen	Does not antagonize decrease

Cerebellar granule cells or hippocampal neurons were incubated first for 5 days with 100 mM ethanol and then for 3 h in ethanol-free medium in the presence of 10 μM diazepam, 100 mM GHB, or 100 μM baclofen. The abundance of the indicated $GABA_AR$ subunit mRNAs was then determined by RNase protection assay. Most data are derived from published studies (Follesa et al. 2003, 2004; Sanna et al. 2003)

the decrease in the abundance of the α_1 or γ_2 subunit mRNAs induced by ethanol withdrawal. Furthermore, substitution of diazepam or GHB for ethanol prevented development of the positive modulatory effect of flumazenil on $GABA_AR$ function conferred by ethanol withdrawal in both cerebellar granule cells and hippocampal neurons (Fig. 1). These latter results are thus consistent with the ability of diazepam and GHB to abolish the ethanol withdrawal-induced up-regulation of α_4 subunit expression (Table 4).

Benzodiazepines are among the most effective drugs available for treatment of the life-threatening condition of alcohol withdrawal syndrome in humans (Mayo-Smith 1997). These drugs prevent the more severe clinical manifestations of the syndrome, including seizures and delirium. Our data demonstrate that the ethanol withdrawal-induced

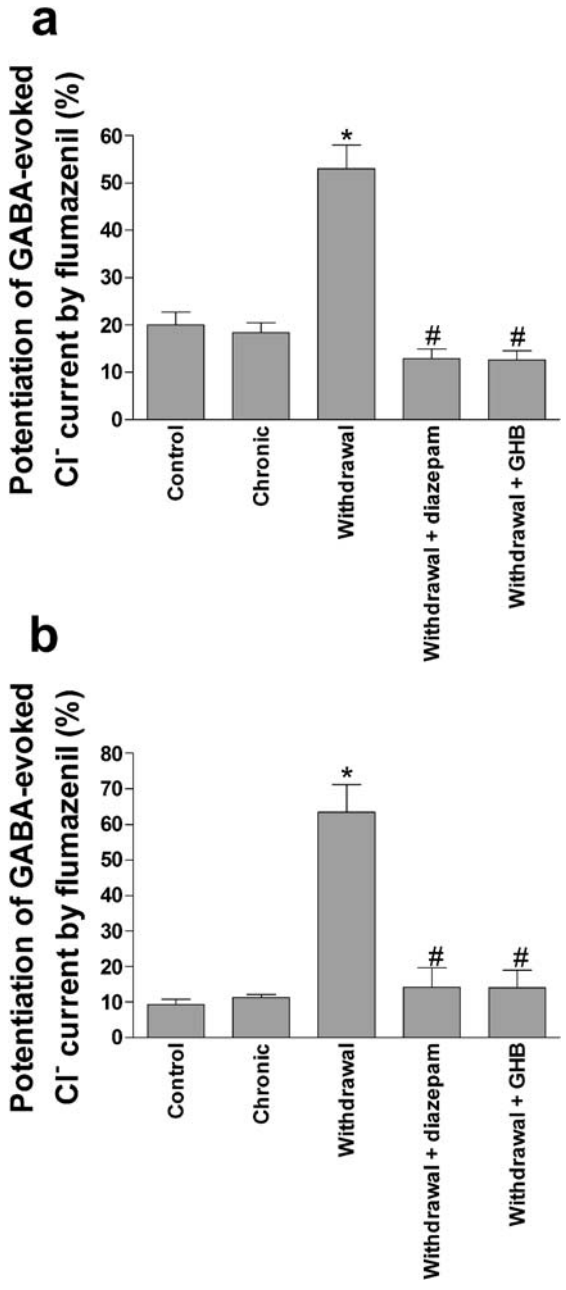

Fig. 1 Potentiation of $GABA_AR$ function by flumazenil in cultured rat cerebellar granule cells (**a**) and hippocampal neurons (**b**) subjected to ethanol withdrawal and its prevention by diazepam or GHB. Cells were untreated (control), treated with 100 mM ethanol for 5 days (chronic), or subjected to ethanol withdrawal for 6 h in the absence (withdrawal) or presence of 10 μM diazepam or 100 mM GHB (withdrawal + diazepam or GHB). They were then subjected to whole-cell patch-clamp recording. GABA was first applied to the cells at a concentration (1–3 μM) that induced a Cl^- current with an amplitude of 5–10% of the maximal response. It was then applied together with 3 μM flumazenil. *P<0.01 vs control; $^{\#}P$<0.01 vs withdrawal. Data are derived from published studies (Sanna et al. 2003, copyright 2003 by the Society of Neuroscience, and Follesa et al. 2003, with permission of ASPET)

Table 5 Effects of ethanol, progesterone, CB34, and GHB on allopregnanolone concentration in rat hippocampal slices

Treatment	Allopregnanolone (ng/g tissue)	Change (%)
Vehicle	0.50±0.04	
Ethanol		
25 mM	0.56±0.03	13±6.1
50 mM	0.85±0.04*	70±8.3*
100 mM	0.96±0.02*	92±5.0*
Progesterone (1 μM)	1.05±0.03*	110±7.1*
CB34 (30 μM)	1.22±0.04*	145±7.8*
GHB (300 μM)	1.37±0.04*	175±8.0*

Freshly isolated hippocampal slices were incubated for 30 min at 34°C in the presence of the indicated agents, after which the amount of allopregnanolone in the tissue and medium was determined and expressed both as nanograms of steroid per gram of tissue and as percentage change relative to vehicle-treated slices. Data are derived from Sanna et al. (2004), copyright 2004 by the Society for Neuroscience

*P<0.01 vs vehicle-treated slices

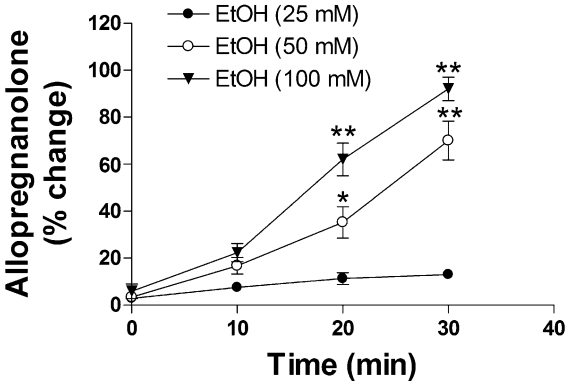

Fig. 2 Time- and concentration-dependent stimulatory effect of ethanol on allopregnanolone production in rat hippocampal slices. Fresh hippocampal slices were incubated for the indicated times at 34°C in the presence of ethanol (25, 50, or 100 mM) or vehicle, after which the total amount of allopregnanolone in the tissue and medium was determined. At time zero (0 min), the incubation was stopped immediately after the addition of ethanol. Data are expressed as percentage change in the abundance of allopregnanolone relative to the corresponding value for vehicle-treated slices. *P<0.05, **P<0.01 vs corresponding value for vehicle. Adapted with permission from Sanna et al. (2004), copyright 2004 by the Society of Neuroscience

increase in expression of the $GABA_AR$ α_2 and α_4 subunit genes and the associated changes in receptor function in cultured neurons are prevented by both diazepam and GHB. A rapid and marked increase in the abundance of the α_2 and α_4 subunits may thus contribute to the development of alcohol withdrawal symptoms that are ameliorated by diazepam or GHB.

Whereas the effects of diazepam during ethanol withdrawal are consistent with its mechanism of action at the $GABA_AR$, the mechanism by which GHB elicits its effects is not clear. Despite its similarities to GABA and GABAergic drugs in terms of chemical structure and pharmacological profile, GHB does not possess activity at $GABA_ARs$ (Feigenbaum and Howard 1996; Follesa et al. 2003; Serra et al. 1991). GHB has been suggested to exert its central depressant effects by increasing the synthesis and extracellular concentration of GABA in specific brain regions (Gobaille et al. 1999). Furthermore, administration of GHB, like that of ethanol (Morrow et al. 2001), increases the formation of neuroactive steroids in rats (Barbaccia et al. 2002), an effect mediated by $GABA_B$ receptors. The accumulation of neuroactive steroids in the brain would be expected to result in an increased GABAergic tone, mediated by $GABA_A$ receptors. However, the $GABA_B$ receptor antagonist SCH 50911 failed to inhibit the actions of GHB, which were also not mimicked by the $GABA_B$ receptor agonist baclofen, during ethanol withdrawal in cultured cerebellar granule and hippocampal neurons (Follesa et al. 2004; Sanna et al. 2003), suggesting that $GABA_B$ receptors do not contribute to the effects of GHB in our experimental models.

Role of neurosteroids in the acute effects of ethanol on hippocampal $GABA_AR$ function

Effect of ethanol on neurosteroid synthesis in isolated hippocampal tissue

Given that experimental evidence indicated that certain pharmacological actions of ethanol require an increase in the synthesis and secretion of neuroactive steroids from

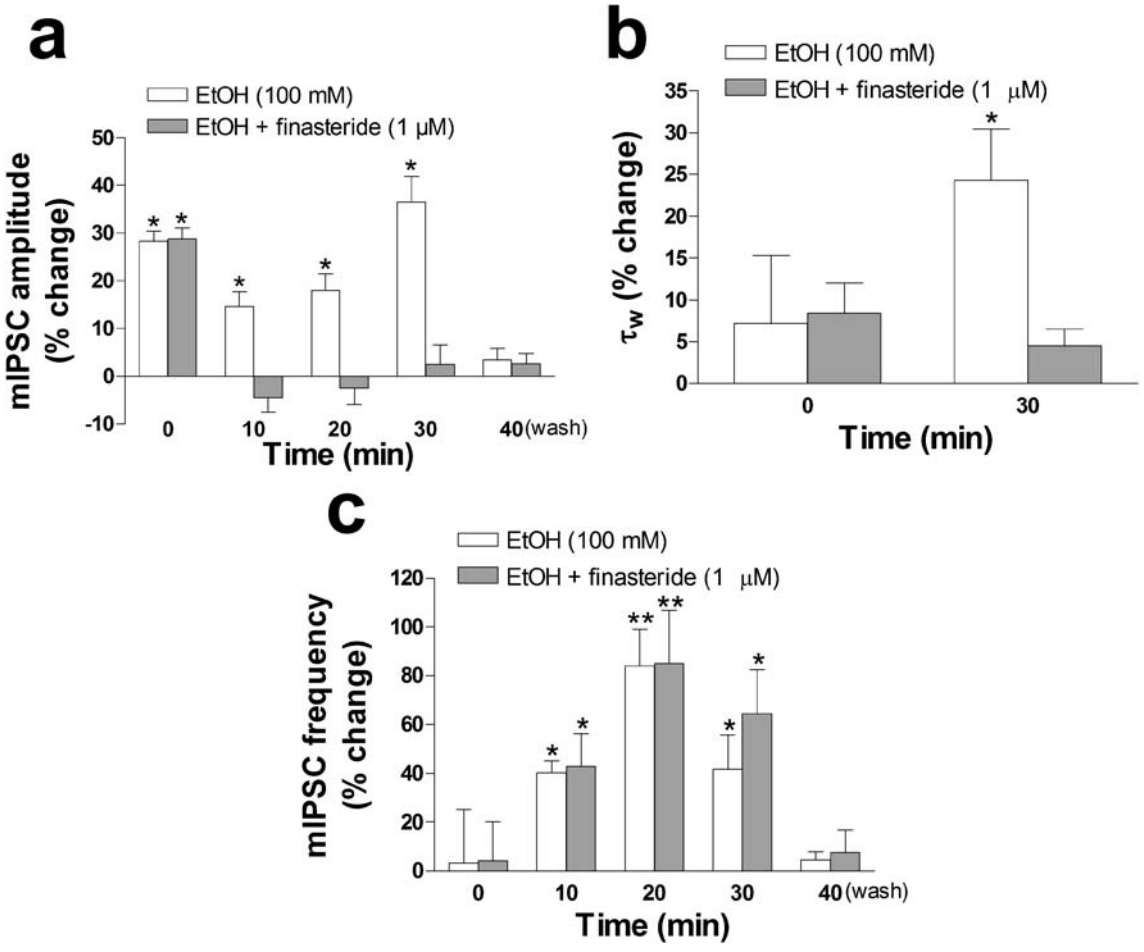

Fig. 3 Effects of ethanol on $GABA_AR$-mediated mIPSCs in CA1 pyramidal cells of rat hippocampal slices. The mean amplitude (**a**), decay time constant (τ_w) (**b**), and frequency (**c**) of mIPSCs were determined at the indicated times during bath application of 100 mM ethanol for 30 min in the absence or presence of 1 μM finasteride. The time zero point (0 min) recording occurred during the initial 3 min (0–3 min) of ethanol application. Data are expressed as percentage change in each parameter induced by ethanol. *P<0.05, **P<0.01 vs control values. Adapted with permission from Sanna et al. (2004), copyright 2004 by the Society of Neuroscience

peripheral organs (Morrow et al. 2001), we investigated whether such a mechanism might also operate in the brain. Indeed, brain cells express steroid synthetic enzymes, and neurosteroid formation has been demonstrated to occur independently of peripheral sources (Hu et al. 1987; Khisti et al. 2003b; Mathur et al. 1993; Purdy et al. 1991). We found that exposure of hippocampal slices freshly isolated from 3-week-old rats to ethanol resulted in a concentration- and time-dependent increase in the amount of allopregnanolone (Table 5, Fig. 2); the effect was apparent at 50 and 100 mM (but not 25 mM) ethanol and at 20 and 30 min. The abundance of allopregnanolone in isolated hippocampal slices was also increased after incubation for 30 min with various agents that stimulate neurosteroid synthesis by different mechanisms, including progesterone, which is converted to allopregnanolone by neurons and glial cells, CB34, a selective agonist of the peripheral benzodiazepine receptor (Serra et al. 1999), whose activation promotes translocation of cholesterol into mitochondria (Gavish et al. 1999), and GHB, which has been suggested to promote steroidogenesis through $GABA_B$ receptor-mediated stimulation of the HPA axis (Barbaccia et al. 2002; Table 5).

Neurosteroids and ethanol modulation of hippocampal $GABA_AR$ function

Our results showing that ethanol as well as progesterone, CB34, and GHB increased the concentration of allopregnanolone in isolated hippocampal tissue led us to examine whether this effect results in positive modulation of the function of hippocampal $GABA_A$Rs, which are sensitive targets of neurosteroids (Lambert et al. 2001). Recording of spontaneous $GABA_A$R-mediated miniature IPSCs (mIPSCs) from CA1 pyramidal neurons revealed that the continuous bath application of ethanol induced a time- and concentration-dependent modulation of $GABA_A$R function. At the concentration of 100 mM, ethanol induced an ~30% increase in mIPSC amplitude during the initial 3 min of treatment (Fig. 3a). The extent of this effect was reduced, although still significant, after exposure of the tissue to ethanol for 10 min and had increased again to ~35% at 30 min. Both the decay time constant and frequency of mIPSCs were also increased after exposure to ethanol for 30 min, whereas ethanol had no effect on these parameters during the initial 3 min of treatment (Fig. 3b,c).

To determine whether the increased biosynthesis of allopregnanolone induced by ethanol was responsible for its effects on $GABA_A$R-mediated mIPSCs, we exposed hippocampal slices to the 5α-reductase inhibitor finasteride both before and during treatment with ethanol. Finasteride alone had no effect on mIPSCs and did not modify the early (initial 3 min) effect of ethanol on mIPSC amplitude (Fig. 3a); however, it abolished the effect of ethanol on mIPSC amplitude apparent between 10 and 30 min after the onset of ethanol application. These results thus suggested that the delayed effect of ethanol on mIPSC amplitude was due to increased synthesis of neurosteroids, whereas the finasteride-insensitive immediate increase in mIPSC amplitude was attributable to a direct modulatory action of ethanol at $GABA_A$Rs. Consistent with this conclusion, given the ability of neurosteroids to prolong $GABA_A$R-mediated IPSCs (Harrison et al. 1987; Zhu and

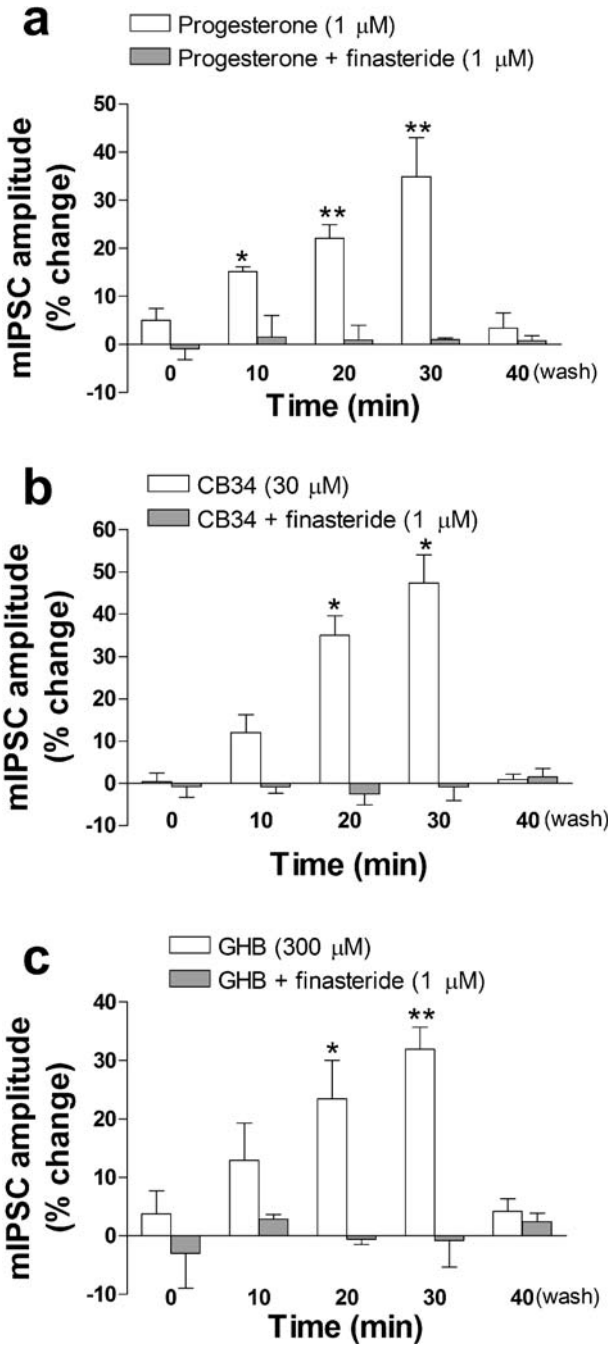

Fig. 4 Effects of progesterone, CB34, and GHB on the mean amplitude of $GABA_A$R-mediated mIPSCs in CA1 pyramidal neurons of rat hippocampal slices. The percentage change in mean mIPSC amplitude was determined at the indicated times during bath application for 30 min of 1 μM progesterone (**a**), 30 μM CB34 (**b**), or 300 μM GHB (**c**), each in the absence or presence of 1 μM finasteride. The time zero point (0 min) recording occurred during the initial 3 min (0–3 min) of ethanol application. **P*<0.05, ***P*<0.01 vs control values. Adapted with permission from Sanna et al. (2004), copyright 2004 by the Society of Neuroscience

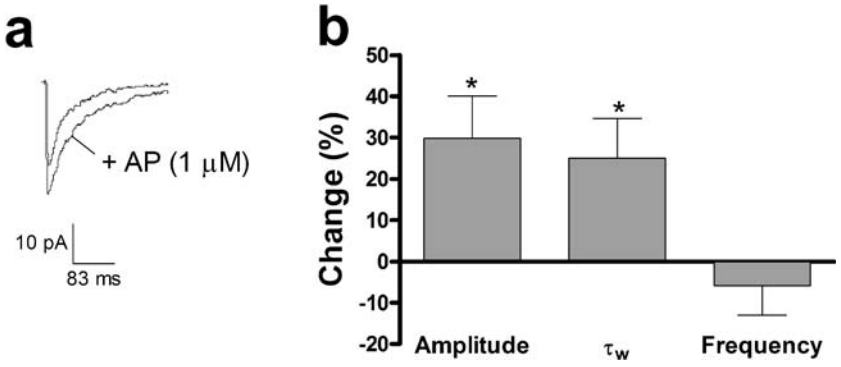

Fig. 5 Effect of allopregnanolone on $GABA_AR$-mediated mIPSCs in CA1 pyramidal neurons of rat hippocampal slices. **a** Averaged mIPSC traces recorded before and 5 min after the onset of bath application of 1 μM allopregnanolone (*AP*). **b** Percentage changes in mean mIPSC amplitude, decay time constant, and frequency determined 5 min after the onset of bath application of 1 μM allopregnanolone. *$P<0.05$ vs control values

Vicini 1997), finasteride also blocked the increase in the decay time constant of mIPSCs induced by ethanol (Fig. 3b). In contrast, finasteride failed to affect the ethanol-induced increase in mIPSC frequency (Fig. 3c), indicating that ethanol increases the probability of GABA release from presynaptic terminals independently of neurosteroids. A presynaptic action of ethanol in the modulation of $GABA_AR$ activity has also been described by other groups (Ariwodola and Weiner 2004; Carta et al. 2004; Roberto et al. 2003; Ziskind-Conhaim et al. 2003) and is consistent with our observation that ethanol also reduced and then reversed the ratio of paired-pulse facilitation in hippocampal slices (Sanna et al. 2004).

$GABA_AR$-mediated mIPSCs in CA1 pyramidal neurons were also modulated by bath application of progesterone, CB34, or GHB (Fig. 4). All three compounds increased in a time-dependent and reversible manner the amplitude of mIPSCs, with the onset of this effect being apparent after 10–20 min. Unlike ethanol, these compounds did not affect mIPSC amplitude during the initial 3 min of application, consistent with their inability to interact directly with the $GABA_AR$ (Lambert et al. 2001; Serra et al. 1991, 1999). Furthermore, progesterone, CB34, and GHB each increased mIPSC decay time but failed to affect mIPSC frequency (Sanna et al. 2004), and pretreatment of hippocampal slices with finasteride prevented the effects of all three compounds on both mIPSC amplitude (Fig. 4) and decay time (Sanna et al. 2004).

To verify further that the delayed effects of ethanol, progesterone, CB34, and GHB on $GABA_AR$-mediated mIPSCs were attributable to the local synthesis of neurosteroids, we examined the effects of bath application of allopregnanolone to hippocampal slices. At the concentration of 1 μM, allopregnanolone induced a rapid increase in both the amplitude and decay time of mIPSCs without an effect on mIPSC frequency (Fig. 5).

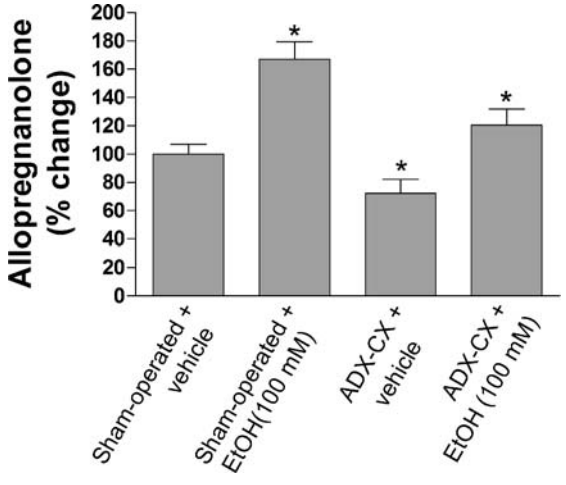

Fig. 6 Ethanol-induced formation of allopregnanolone in hippocampal slices from ADX-CX rats. Freshly isolated hippocampal slices obtained from ADX-CX or sham-operated rats at least 1 week after surgery were incubated in the presence of ethanol (100 mM) or vehicle for 30 min at 34°C, after which the total amount of allopregnanolone in tissue and medium was determined. Data are expressed as a percentage of the allopregnanolone content for vehicle-treated tissue from sham-operated rats (control). *$P<0.05$ vs vehicle-treated group

Effects of ethanol on hippocampal neurosteroidogenesis and $GABA_AR$ function in ADX-CX rats

The molecular mechanism underlying the stimulatory effect of ethanol on allopregnanolone biosynthesis in isolated brain tissue remains unclear. However, to clarify whether ethanol increases the rate of conversion of precursors (such as progesterone) derived from peripheral sources to allopregnanolone by brain cells or whether it promotes the de novo formation of neurosteroids from cholesterol, we studied hippocampal tissue freshly isolated from adrenalectomized-castrated (ADX-CX) rats. The plasma and brain concentrations of progesterone and pregnenolone are markedly reduced in ADX-CX rats 1 week after surgery compared with those in sham-operated animals (Porcu et al. 2004). The basal level of allopregnanolone in hippocampal slices isolated from ADX-CX animals at this time was reduced by only ~40% compared with that in hippocampal tissue from sham-operated animals (Fig. 6), consistent with the notion that brain cells continue to

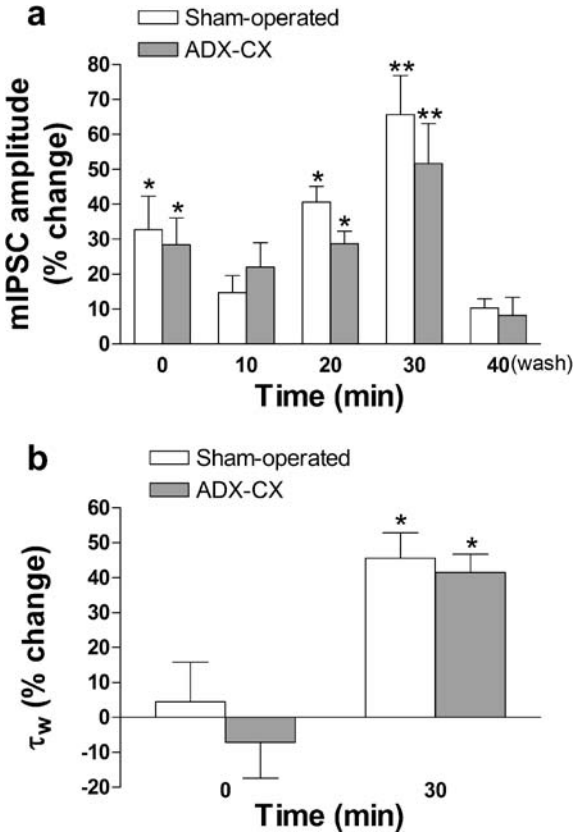

Fig. 7 Effects of ethanol on $GABA_AR$-mediated mIPSCs in CA1 pyramidal cells of hippocampal slices from ADX-CX rats. The mean amplitude (**a**) and decay time constant (**b**) of mIPSCs were determined at the indicated times during exposure of hippocampal tissue from ADX-CX or sham-operated rats to 100 mM ethanol for 30 min. Data are expressed as percentage change in each parameter induced by ethanol. *P<0.05, **P<0.01 vs control values

produce neurosteroids independently of the periphery. Incubation of hippocampal slices from ADX-CX rats with ethanol (100 mM) for 30 min resulted in a significant increase (~65%) in the amount of allopregnanolone, with the size of this effect being similar to that observed with hippocampal tissue from sham-operated animals (Fig. 6). Consistent with these observations, electrophysiological recording of $GABA_AR$ function in CA1 pyramidal neurons revealed that ethanol increased mIPSC amplitude and decay time to similar extents in hippocampal slices from ADX-CX rats and sham-operated animals (Fig. 7).

Together, these results suggest that the abilities of ethanol to both increase the concentration of allopregnanolone and enhance $GABA_AR$ function in hippocampal slices are independent of steroid precursors from the periphery and likely involve the local de novo biosynthesis of neurosteroids from cholesterol. Consistent with our findings, the increase in the brain content of allopregnanolone did not correlate with changes in the plasma concentrations of corticosterone, progesterone, or allopregnanolone in ethanol-treated rats, suggesting that brain and circulating steroid concentrations are regulated differentially (VanDoren et al. 2000).

Conclusions

We have shown that prolonged exposure to and subsequent withdrawal of ethanol are associated with marked, specific, and differential changes in $GABA_AR$ subunit gene expression, as well as with changes in receptor function and pharmacological sensitivity to neurosteroids, zaleplon, and flumazenil, in cultured rat neurons. The changes in receptor function induced by ethanol withdrawal may be an important determinant of alcohol withdrawal syndrome, consistent with the fact that some of these changes are blocked by drugs effective in the treatment of ethanol dependence. It should be kept in mind also that although alterations in subunit expression are correlated with altered receptor function, in the absence of altered subunit composition, selective trafficking of specific subunits could also represent an additional mechanism. Our results also demonstrate that ethanol is able to increase brain steroidogenesis by a local action independent of the activity of the HPA axis. This latter action of ethanol, together with or independent of stimulation of the HPA axis, might thus be important in mediating some of the central effects of this drug of abuse. In addition, this mechanism may be important in mediating the effects of ethanol in such physiological or pathological conditions as the menstrual cycle, pregnancy, menopause, premenstrual syndrome, and a variety of neurological or psychiatric disorders in which steroidogenic activity undergoes pronounced changes.

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