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Ethanol and opioid receptor signalling

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Summary. Ethanol may modulate endogenous opioid systems by disrupting opioid receptor signalling. Low concentrations of ethanol slightly potentiate μ-opioid receptor binding by increasing receptor B_{max}, and, in some cases, chronic ethanol exposure decreases the density or affinity of the μ-opioid receptors. By contrast, high concentrations of ethanol acutely decrease δ-opioid receptor binding by decreasing receptor affinity, whereas chronic exposure of animals and neuronal cell lines to lower concentrations of ethanol leads to possibly adaptive increases in the density or affinity of the δ-opioid receptors. In the neuronal cell line NG108-15, ethanol does not up-regulate the δ-opioid receptor by blocking receptor degradation or endocytosis, but protein synthesis is required for this response. Up-regulation of the δ-opioid receptor renders ethanol-treated NG108-15 cells 3.5-fold more sensitive to opioid inhibition of adenylyl cyclase. Long-term treatment with ethanol also increases maximal opioid inhibition in NG108-15 cells, possibly by decreasing levels of $G\alpha_s$ and its mRNA. Ethanol differentially modulates signal transduction proteins in three additional neuronal cell lines, N18TG2, N4TG1, and N1E-115. Ethanol-treated N18TG2 cells show the least up-regulation of the δ-opioid receptor, little heterologous desensitization of adenylyl cyclase, and no changes in $G\alpha_s$ or $G\alpha_i$. By contrast, ethanol-treated N1E-115 cells show the largest up-regulation of the δ-opioid receptor, the most heterologous desensitization of adenylyl cyclase, and concentration-dependent decreases in $G\alpha_s$ and increases in $G\alpha_i$. Further analysis of these related neuronal cell lines may help to identify the molecular elements that endow some, but not all, neuronal cells with the capacity to adapt to ethanol.
Key words. Endogenous opioid systems; ethanol; Gα-proteins; receptor signalling; up-regulation; μ-opioid receptor; δ-opioid receptor.

Alcohol and endogenous opioid systems

There is considerable evidence that ethanol interacts with endogenous opioid systems to produce some of its central nervous system (CNS) effects[4, 5, 81]. ICI 174864, a selective δ-opioid receptor antagonist, can block ethanol-induced hypothermia and sedation when microinjected into discrete brain regions[81]. Moreover, the opiate antagonist naloxone can attenuate the ethanol withdrawal syndrome when given during and after the administration of ethanol[3, 4]. Heritable differences in susceptibility to alcoholism may also be related to an ethanol-opioid interaction. In inbred strains of mice, ethanol consumption correlates inversely with brain levels of [Met]enkephalin[5].

Ethanol could modulate the activity of endogenous opioid systems through effects on the synthesis, processing, or release of opioid peptides, or on opioid receptor signalling (fig. 1). Effects of ethanol on the biosynthesis and regulation of opioid peptides are described elsewhere in this volume[18]. Here, I will review the effects of ethanol on opioid receptor signalling in brain and in neuronal cells.

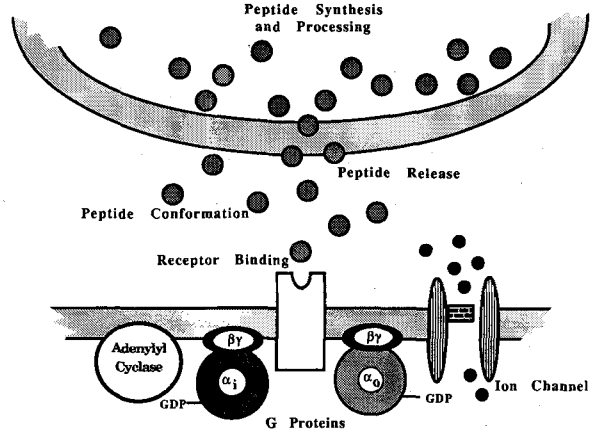

Figure 1. Possible sites of action of ethanol on endogenous opioid systems. Ethanol could alter the synthesis, release, conformation, or receptor binding of opioid peptides, or the events that follow receptor activation. Opioid receptors couple with G proteins to regulate adenylyl cyclase or ion channels and ethanol could disrupt opioid signalling at any of these loci.

Opioid receptor signalling

Multiple opioid receptors

Pharmacological, anatomical, and physiological studies suggest that opioids interact with at least four different receptor subtypes, designated μ, δ, κ, and σ[49, 52, 58, 68, 69]. Morphine and dihydromorphine (DHM) preferentially recognize the μ-opioid receptor; binding to this receptor is inhibited by nanomolar concentrations of the opiate antagonist naloxone[49, 56]. The synthetic opioid peptide *H*-Tyr-D-Ala(Me)Phe-NH-CH_2-OH (DAGO) shows far greater μ receptor selectivity than morphine, whereas [D-Pen^2,D-Pen^5]enkephalin has excellent δ receptor selectivity[68]. The peptidase-resistant derivative of [Leu^5]enkephalin, [D-Ala^2,D-Leu^5]enkephalin (DADLE), is frequently used as a δ-selective ligand but has only 2–10-fold greater affinity for δ sites than μ sites[58]. Naloxone binds δ sites 20-fold less potently than μ sites[49, 58]. The compound U50,488 binds κ sites more than 1000-fold more potently than μ or δ sites[58]. The existence of a σ-opioid receptor was first suggested by the pharmacology of *N*-allylnormetazocine[52]. This σ receptor may mediate the psychotomimetic effects of benzomorphan opiates, but is often considered apart from the μ-, δ- and κ-opioid receptors because of its unique pharmacological profile and anatomical distribution[69].

The three major opioid receptor subtypes differentially recognize the opioid peptides encoded by the preproenkephalin, proopiomelanocortin (POMC), and prodynorphin genes[58, 68]. Preproenkephalin encodes the pentapeptides [Met^5]enkephalin and [Leu^5]enkephalin, which bind δ-opioid receptors with 10–25-fold greater affinity than μ-opioid receptors. β-endorphin is encoded by POMC and recognizes μ and δ sites with equally high affinity. Dynorphin, a product of the preprodynorphin gene, binds selectively to the κ-opioid receptor subtype[58, 68].

Subtype-selective opioid ligands are endowed with unique pharmacological properties because of the differential regional localization of opioid receptor subtypes in the central and peripheral nervous systems and the preferential interaction of opioid receptor subtypes with specific effector systems[13, 27, 58, 68]. The co-existence of multiple opioid receptor subtypes within many brain regions, their occasional co-expression within the same neurons[13, 15, 80], and their interaction with multiple effector systems greatly complicates the study of how ethanol modifies opioid signalling. This task has been simplified somewhat by the identification of tissues that express only a single receptor subtype (such as the δ receptor in neuroblastoma cell lines[20] and the development of ligands with high selectivity for each receptor subtype, as described above.

Physiological effects of opioids

Many opioid receptors are located presynaptically and their activation decreases the release of various neurotransmitters and neuromodulators including acetylcholine, dopamine, norepinephrine, substance P, somatostatin, vasopressin and oxytocin[1, 13, 51]. Several physiological actions of opioids may account for their modulation of transmitter release[68]. Opioids increase an inwardly rectifying potassium conductance, decrease voltage-dependent calcium conductance, hyperpolarize neuronal membranes and decrease the spontaneous firing rate of neuronal cells[57, 68]. These actions have been observed in both central and peripheral nervous tissues, but vary among different brain regions and different neuronal cell types. Certain ionic conductances may be regulated by individual opioid receptor subtypes; for example, κ agonists inhibit voltage-dependent calcium conductance whereas both δ and μ agonists activate an inwardly rectifying potassium conductance[13, 57]. A single receptor subtype may also interact with more than one effector system; for example, in the neuroblastoma × glioma hybrid cell line NG108-15, which expresses only the δ receptor, opioids inhibit adenylyl cyclase and voltage-dependent calcium conductance[26, 27].

The precise molecular mechanisms by which opioids produce their physiological effects are not fully understood. The opioid receptors belong to a family of receptors that interact with GTP-binding proteins (G proteins) to regulate second messenger systems or ion channels[68]. So far, all members of this family have been shown to be homologous proteins that form 7 transmembrane loops and are encoded by intronless genes. By analogy with other G protein-coupled receptors, individual opioid receptor subtypes may prove to be the products of different genes encoding proteins with different profiles for ligand recognition and effector interaction. For example, the genes for five human muscarinic cholinergic receptor subtypes encode homologous but distinct proteins, that differentially recognize muscarinic ligands and selectively activate phosphoinositide hydrolysis or inhibit adenylyl cyclase[59].

Interaction of opioid receptors with G proteins

G proteins appear to mediate the effects of many neurotransmitters, neuromodulators, and growth factors through their interactions with adenylyl cyclase, phospholipases A and C, and ion channels[21]. All G proteins are heterotrimeric membrane-associated proteins comprising α, β and γ subunits. The α subunits of different G proteins contain homologous domains for GTP binding and hydrolysis, and variable domains for receptor recognition and effector interactions. The $\beta\gamma$ subunits show greater sequence homology and appear to interact with multiple α-subunits. Receptor activation promotes the exchange of GTP for GDP within the α subunit, leading to subunit dissociation (fig. 2). Free $\alpha \cdot$ GTP and perhaps $\beta\gamma$ then interact with specific effectors to regulate second messenger systems and ion channels[21]. Although the genes for numerous α-subunits have been cloned, only a few of the encoded proteins have been assigned specific

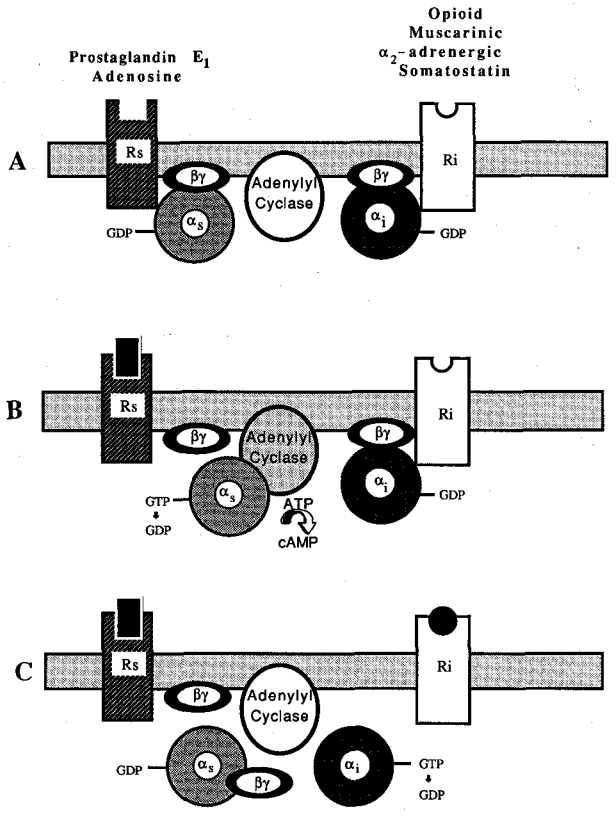

Figure 2. Coordinate regulation of adenylyl cyclase by stimulatory and inhibitory hormones. *A* Unliganded receptors and inactive G protein heterotrimers in the GDP-bound state; *B* Activation of stimulatory receptors causes exchange of GTP for GDP on $G\alpha_s$, dissociation of α_s from $\beta\gamma$, and activation of adenylyl cyclase by $\alpha_s \cdot$ GTP. *C* Subsequent activation of inhibitory receptors (e.g. δ-opioid) causes exchange of GTP for GDP on $G\alpha_i$, dissociation of α_i from $\beta\gamma$, and inactivation of $\alpha_s \cdot$ GTP by the $\beta\gamma$ subunits released from G_i.

functions. These include stimulation of adenylyl cyclase by G_s, inhibition of adenylyl cyclase by G_i, inhibition of voltage-dependent calcium conductance by G_o, and activation of potassium conductance by G_k[21,27,82].

The best characterized biochemical effect of opioids is the inhibition of adenylyl cyclase mediated by G_i (fig. 2). Adenylyl cyclase activity is coordinately regulated by stimulatory and inhibitory receptors which act through G_s and G_i, respectively. Activation of stimulatory receptors promotes the subunit dissociation of G_s, allowing free $\alpha_s \cdot$ GTP to activate adenylyl cyclase[62]. Activation of inhibitory receptors, such as the δ receptor, promotes the subunit dissociation of G_i, releasing $\beta\gamma$ subunits to complex and inactivate free $G\alpha_s$[21,43].

Opioid peptide may interact with at least two additional G proteins. In pertussis toxin-treated NG108-15 cells, in which the δ-opioid receptor is uncoupled from $G\alpha_i$ and $G\alpha_o$, opioid inhibition of voltage-dependent calcium conductance is reconstituted more potently by purified $G\alpha_o$ than $G\alpha_i$[27]. Purified μ receptors also interact with purified G_i and G_o in phospholipid vesicles[76]. Activation of potassium conductance in locus coeruleus neurons and the submucous plexus by μ and δ agonists, respectively, is dependent on GTP[57], and although a specific G protein has not yet been implicated, similar regulation of potassium channels by muscarinic receptors appears to be mediated by a G protein designated G_k[82].

Thus, opioid signal transduction requires at least three membrane components – a family of opioid receptor subtypes, a family of G proteins, and at least three effector systems: adenylyl cyclase, the voltage-dependent calcium channel, and an inwardly rectifying potassium channel. Investigations into the effects of ethanol on opioid signalling have thus far dealt largely with the first step in these signalling pathways; the binding of opioid ligands to their receptors.

Acute effects of ethanol on opioid signalling in brain

Acute effects of ethanol on opioid receptor binding

Hiller and co-workers were the first to demonstrate that ethanol can selectively inhibit opioid receptor binding[28,29]. Brief exposure to ethanol reversibly decreased the binding to rat brain membranes of the δ-preferring peptide [^{3}H]DADLE, but did not inhibit the binding of μ-, κ-, or σ-selective ligands. Ethanol appeared to be acting on the δ receptor rather than on the ligands. There was less inhibition of [^{3}H]DADLE binding in brain preparations enriched in μ receptors, and more inhibition of the binding of [^{3}H]naltrexone, a μ-selective ligand, in a neuroblastoma cell line that expresses only the δ-opioid receptor. Hence, for ligands that recognize more than one opioid receptor subtype, the magnitude of inhibition by ethanol was a property of the predominant receptor subtype expressed by a tissue, rather than the ligand used for binding. A series of alcohols inhibited [^{3}H]DADLE binding in proportion to their chain length and lipid partition coefficients, giving rise to the hypothesis that δ receptors are more significantly affected than μ receptors by membrane lipid perturbations. In these experiments, n-butanol decreased the affinity but not the maximal binding capacity (B_{max}) of [^{3}H]DADLE, apparently by increasing the rate of ligand dissociation[29]. Inhibition of [^{3}H]DADLE binding by n-butanol was potentiated by sodium and by increases in temperature from 24 °C to 37 °C.

In general, the concentrations of ethanol required to inhibit δ receptor binding are higher than those attained during drinking. The concentration of ethanol that produces half-maximal inhibition (IC_{50}) is commonly around 200–400 mM[8,28,39], and levels achieved in animals by in vivo administration of ethanol (25–100 mM) cause, at most, slight inhibition in vitro[17,35,37,38,50,60]. Concentrations of ethanol much higher than those that inhibit δ receptor binding also decrease the affinity of certain μ receptor ligands[17,28,39,46,72]. By contrast, low concentrations of ethanol (25–100 mM) can increase μ receptor binding in a variety of tissues[17,28,30,46,72] by increasing maximal binding capacity[17,46].

Not surprisingly, the acute effects of ethanol on opioid receptor binding are dependent on experimental conditions, ligand, tissue, and species. The failure to observe any effect of ethanol may result from the use of high ligand concentrations, the coexistence in a tissue of receptor subtypes affected oppositely by ethanol, or the use of brain regions that are relatively insensitive to ethanol.

Effects of ethanol on opioid ligands

High concentrations of ethanol can induce conformational changes in opioid peptides, as determined by Fourier-transform infrared spectroscopy (FT-IR)[2] or nuclear magnetic resonance (NMR) spectroscopy[63, 64]. This has led to speculation that ethanol may inhibit opioid receptor binding by disrupting both the ligand and the receptor[2]. Tyr-D-Ser-Gly-Phe-Leu-Thr-OH (DSTLE) and DADLE each bind the δ receptor with high affinity. Ethanol (514 mM) induced a larger shift in the FT-IR spectrum and inhibited more potently the binding to rat brain membranes of DADLE compared to DSTLE. Similarly, the binding and FT-IR spectrum of naltrexone, a structurally-rigid μ-selective antagonist, were affected by ethanol less than those of [Met]enkephalinamide, a more conformationally-labile μ receptor agonist[2, 64].

To what extent inhibition of binding results from conformational changes in opioid ligands remains to be determined. The ethanol dose-response curves for spectral shift and binding inhibition may differ significantly; for example, binding of [^{3}H]DSTLE was inhibited by concentrations of ethanol that did not alter ligand conformation[2]. Moreover, ethanol significantly inhibited naltrexone binding in tissues enriched in δ receptors (N4TG1 cells) but not μ receptors (toad brain)[28]. In any case, concentrations of ethanol that are attained during drinking have not yet been shown to induce conformational changes in opioid peptides; hence, the clinical significance of this phenomenon is uncertain.

Acute effects of ethanol on opioid inhibition of brain adenylyl cyclase

Even at concentrations that inhibit δ receptor binding, ethanol does not acutely decrease the inhibition of adenylyl cyclase mediated by opioid receptor activation or direct activation of G_i. Short-term treatment with 300 mM ethanol did not shift the dose-response curves for inhibition of rat striatal adenylyl cyclase activity by morphine, DADLE, or acetylcholine[61]. Hoffman and Tabakoff[32] also reported that 50 mM or 250 mM ethanol did not change the inhibition of mouse striatal adenylyl cyclase by morphine or DADLE. Ethanol likewise did not affect the inhibition of adenylyl cyclase mediated by the direct activation of G_i by non-hydrolyzable GTP analogs[6].

These observations have several possible explanations. First, ethanol may inhibit opioid binding to a population of δ receptors that does not couple with adenylyl cyclase[61]. Second, neurons may express greater numbers of opioid receptors than are required for maximal opioid inhibition of adenylyl cyclase. Third, the effect of ethanol on opioid inhibition of adenylyl cyclase activity might be lost in broken cell preparations. To examine the latter possibility, we compared the acute effects of ethanol on δ-opioid receptor binding and opioid inhibition of cAMP accumulation using intact NG108-15 cells. Ethanol inhibited [3H]DADLE binding with an IC_{50} of approximately 200 mM (unpublished observations). However, ethanol did not inhibit maximal DADLE inhibition of cAMP accumulation at concentrations up to 600 mM, nor did ethanol decrease the potency of DADLE (unpublished observations). For the moment, this finding remains enigmatic. It will be interesting to learn whether ethanol-induced decreases in opioid receptor affinity alter receptor regulation of inwardly rectifying potassium channels or voltage-dependent calcium channels.

Chronic effects of ethanol on brain opioid signalling

The effects of chronic ethanol administration on brain opioid receptor binding have depended on experimental conditions, species, brain region, and route of administration. These studies are complicated further by the coexistence in tissue preparations of multiple opioid receptor subtypes, the use of ligands (such as [^{3}H]DADLE) that recognize more than one receptor subtype, and the application of linear regression analysis to non-linear binding curves.

Pfeiffer and colleagues[60] force-fed ethanol to rats for 3 weeks and then measured the binding to striatal membranes of [^{3}H]DHM or [^{3}H]DADLE. Chronic ethanol treatment decreased the equilibrium dissociation constant (K_d) of the high-affinity [^{3}H]DADLE site by about one third without affecting the maximal binding capacity (B_{max}). By contrast, chronic ethanol administration did not alter either the B_{max} or K_d for [^{3}H]DHM.

Gianoulakis[17] fed ethanol to rats for 2 or 3 weeks and measured the binding to whole brain membranes of [^{3}H]DHM, [^{3}H]DADLE, and [^{3}H]naloxone. These ligands also recognize several classes of binding sites and only high-affinity binding was analyzed. Chronic ethanol treatment increased the B_{max} of each ligand by approximately 10–20%. Ethanol treatment also increased slightly the affinity of [^{3}H]DADLE and decreased slightly the affinity of [^{3}H]DHM, consistent with previous findings[60].

Hynes and colleagues[35] used non-linear least squares analysis[56] to approach the problem of ligand interactions with multiple opioid binding sites. [^{3}H]DADLE recognized a high- and low-affinity binding site in whole brain homogenates from control CF-1 mice. Brain homogenates from mice maintained for 5 days on a liquid diet supplemented with ethanol showed only a single intermediate-affinity [^{3}H]DADLE binding site with a B_{max} 82% higher than the sum of the two binding sites

observed in control mice. There were no associated changes in the binding of [^{3}H]naloxone.

Khatami and colleagues[39] employed combinations of selective ligands to study the differential effects of ethanol on μ, κ, and δ receptors in membranes from the frontal cortex of C57BL mice. [^{3}H]DAGO, a highly selective μ-opioid ligand, identified a single population of μ receptors. In the presence of excess unlabeled DAGO, [^{3}H]DADLE recognized a single class of δ receptors. Specific κ-opioid receptor binding was determined by binding [^{3}H]bremazocine in the presence of excess unlabeled DAGO and DADLE, to block μ and δ receptors, respectively. μ and δ receptor binding were equally inhibited by acute exposure to high concentrations of ethanol, whereas κ receptor binding was unaffected. Low concentrations of ethanol did not acutely potentiate μ receptor binding, in contrast to earlier findings in mouse striatum[72]. The failure to show selective inhibition by ethanol of δ compared to μ receptor binding may relate to differences in receptor structure, distribution, or lipid milieu between frontal cortex and other brain regions.

The C57BL mice were then made dependent on ethanol by administering a liquid diet containing ethanol for 7 days[39]. The potency of ethanol in inhibiting μ and δ receptor binding was not diminished 24 h after ethanol withdrawal; however, there was a selective decrease in μ receptor density and no change in the affinity of any of the opioid receptor subtypes. The lack of change in δ receptors may reflect differences among brain regions in the capacity to adapt to ethanol; alternatively, δ receptor density may have returned to normal in the 24 h following ethanol withdrawal[8].

There is little known about the effects of ethanol-induced changes in brain opioid receptor binding on receptor-effector function. Hoffman and Tabakoff[32] showed that there was no difference in morphine or DADLE inhibition of striatal adenylyl cyclase activity between control and ethanol-treated mice; however, these measurements were made 24 h after ethanol withdrawal, at which time there was also no change in δ receptor binding[39]. The same workers observed a decrease in high-affinity [^{3}H]DHM binding, a loss of inhibition of [^{3}H]DHM binding by sodium, and a rightward shift in the dose-response curve for morphine stimulation of DOPA and DOPAC accumulation in the striata of ethanol-withdrawn mice[33, 73].

Despite differing greatly in methodology, these studies allow certain conclusions. Long-term administration of ethanol to animals increases the affinity or density of δ receptor binding sites in whole brain and in certain brain regions. Chronic treatment of animals with ethanol more variably reduces μ receptor affinity or density, depending on experimental conditions and the brain region examined. The consequences of these receptor changes on various opioid receptor-effector interactions remain to be investigated, and a role for such changes in the development of tolerance to and physical dependence on ethanol is still conjectural.

A cellular model system to study the short- and long-term effects of ethanol on opioid receptor signalling

The experiments described thus far emphasize the complexities inherent in using brain homogenates or membranes to study the effects of ethanol on opioid receptor signalling. Large biochemical effects of ethanol in small, but important, brain regions may be masked by the insensitivity to ethanol of adjacent neurons. Multiple opioid receptor subtypes are present even in discrete brain regions. Neuronal elements must be studied together with contaminating glial and endothelial elements. Normal opioid signalling is likely to be deranged by neuronal disruption and fractionation.

The use of cultured neuronal cell lines overcomes many of these difficulties[8, 24, 65, 70, 71]. Large quantities of homogeneous intact cells may be studied under precisely controlled conditions. This makes it possible to investigate certain aspects of receptor regulation, such as receptor-mediated endocytosis or phosphorylation of receptors by cytoplasmic kinases[67], that can only be studied in intact cells. Moreover, many cell lines express only the δ receptor subtype[20], eliminating the possibility of competing effects of ethanol on δ and μ receptors.

One potential disadvantage in the use of cultured neuronal cells is the possibility that cellular transformation has altered the biochemical substrate on which ethanol acts. Some reassurance in this regard is provided by the similarities between cultured neuronal cells and brain in the pharmacology, biochemistry, physiology, and response to ethanol of the δ receptor[26, 44, 45, 68]. Another problem is the fact that the behavioral concomitants of molecular events cannot be defined. This can be overcome, in part, by identifying molecular events induced by ethanol whose time course and direction of change mirror the clinical events of ethanol intoxication, tolerance, and physical dependence.

Ethanol intoxication, tolerance and physical dependence in humans

Ethanol produces dose-dependent depression of central nervous system (CNS) function[7]. Incoordination can be measured at blood ethanol concentrations of 7 mM[22]. A blood ethanol concentration of 22 mM (100 mg/dl), which legally defines intoxication in many states, causes incoordination, ataxia, and sedation. In non-alcoholic individuals, blood ethanol concentrations in excess of 100 mM (461 mg/dl) are frequently lethal, with death resulting from inhibition of medullary control of respiration.

Chronic consumption of ethanol produces significant tolerance to its intoxicating effects. Urso and colleagues[75] identified a large cohort of sober alcoholics whose blood alcohol concentrations ranged from ap-

proximately 25–125 mM (120–540 mg/dl). Likewise, Lindblad and Olsson[48] measured blood alcohol levels between 115 and 170 mM in consecutive alcoholics seen in an emergency room. The highest blood alcohol level ever reported, 327 mM, was measured in an alcoholic described as 'agitated and slightly confused but alert, responsive to questioning, and oriented to person and place (though unclear as to time)'[36]. The fact that some alcoholics show few signs of intoxication at blood alcohol concentrations that would prove lethal in non-alcoholic individuals attests to the remarkable ability of the CNS to adapt to ethanol.

CNS adaptation to ethanol probably begins during the first hours of drinking. Mirsky and colleagues[53] showed that after a single bout of drinking, naive subjects become sober at blood ethanol concentrations higher than those associated with initial intoxication. Animal studies confirm that some tolerance to ethanol may occur after as little as 30 min of ethanol administration[47].

Physical dependence is a state in which ethanol is required to maintain normal CNS function[7]. Adaptations that render CNS function normal in the presence of ethanol might be expected to cause dysfunction when ethanol intake is abruptly discontinued. Thus, the hyperactive state associated with ethanol withdrawal is in many ways opposite to the sedated state associated with acute intoxication.

Adaptation of the CNS to ethanol is probably accomplished by changes at multiple CNS levels including the integrated neural systems that mediate state-dependent tolerance[79], neural pathways that utilize specific neuromodulators, such as vasopressin[31], and individual neuronal cells. Considerable evidence suggests that cellular changes may play an important role in adaptation to ethanol, either by limiting its entry into the plasma membrane[66] or by compensating for its effects within the membrane[34]. It is these cellular aspects of CNS adaptation to ethanol that can best be studied in cultured neuronal cells.

Effects of ethanol on δ-opioid receptor binding in NG108-15 cells

We chose to develop our model system using the neuroblastoma × glioma hybrid cell line NG108-15[8]. This well-characterized cell line expresses many neuronal properties including neurotransmitter synthesis, membrane excitability, and the coupling of neurotransmitter receptors to cAMP synthesis, phosphoinositide hydrolysis, and ion channels[26,27,41,46]. Of at least four opioid receptor subtypes expressed in brain, only one – the δ-opioid receptor – is expressed in NG108-15[20,44].

Acute ethanol exposure reversibly inhibited [^{3}H][D-Ala^2,Met^5]enkephalinamide (DAMEA) binding to the δ receptor of intact NG108-15 cells[8]. Relatively high concentrations of ethanol were required to inhibit binding – the IC_{50} of 173 mM is achieved only occasionally during drinking. Saturation binding isotherms indicated that ethanol acutely reduced the affinity of opioid peptides for the δ receptor (unpublished results), as demonstrated earlier for brain by Hiller and colleagues[28].

To test whether NG108-15 cells could adapt to the acute inhibitory effect of ethanol on δ receptor binding, cells were incubated for 4 days in the absence or presence of 200 mM ethanol, a concentration close to the IC_{50} for the acute effect. Long-term incubation with ethanol did not significantly change the viability, morphology, size, or protein content of NG108-15 cells[8,12]. There was likewise no change in the potency or efficacy of ethanol in inhibiting [^{3}H]DAMEA binding; however, ethanol-treated cells did show an 85% increase in the specific binding of [^{3}H]DAMEA, reflecting an increase in maximal binding capacity but no change in binding affinity[8]. The magnitude of this increase in δ receptor binding was nearly identical to that observed in whole brain homogenates from ethanol-dependent CF-1 mice[35].

We next asked whether ethanol increases δ receptor expression in NG108-15 cells over a time course that mirrors the development of tolerance and physical dependence. [^{3}H]DAMEA binding was measured in NG108-15 cells incubated with 200 mM ethanol for 0–96 h. Binding in ethanol-treated cells increased significantly after 18 h and plateaued after 48 h at a level 85% higher than control values. By contrast, treatment of NG108-15 cells with 25 mM ethanol for 2 weeks increased receptor density by 50%[8]. This relatively rapid increase in receptor expression is consistent with both animal and human studies that demonstrate the development of tolerance to ethanol within hours of its administration[47,53]. As in intact animals, adaptation in NG108-15 cells is a function of both ethanol dose and duration of exposure.

The biochemical state at the onset of withdrawal can be readily assessed in ethanol-adapted NG108-15 cells by replacing ethanol-containing medium with control medium. Following ethanol withdrawal, δ receptor expression remains heightened for 12–24 h before returning to normal levels[8]; during this withdrawal period, endogenous opioid peptides could activate an augmented population of δ receptors, perhaps leading to heightened opioid effects. The time course for restoration of normal δ receptor expression resembles that for the occurrence of withdrawal convulsions in man, which are most frequent 12–48 h after the cessation of drinking[7,78]. In this regard, it is of interest that application of opioid peptides to discrete brain regions can produce convulsions in experimental animals[25]. Conceivably, the presence of increased numbers of δ receptors in ethanol-dependent animals[17,35] plays a role in the genesis of ethanol-withdrawal convulsions.

Thus, this cellular model system has certain parallels in the clinical events of intoxication, tolerance, and withdrawal. The most important of these is the development during chronic ethanol exposure of a biochemical change – increased δ receptor expression – that could compensate for an acute effect of ethanol; decreased δ receptor

affinity. Following ethanol withdrawal, the adaptive response that restored normal physiological function could then become the basis for an abnormal physiological state. To support this hypothesis, one must show that the opioid binding sites induced by ethanol are functional receptors that could indeed alter the cell's biochemical responses.

Effects of ethanol on opioid inhibition of cAMP accumulation in NG108-15 cells

Activation of the δ receptor in NG108-15 cells is coupled to inhibition of adenylyl cyclase by G_i. Maximal opioid inhibition follows activation of only a fraction of the δ receptors expressed by NG108-15 cells[16]; in this situation, increases in the population of 'spare' or 'reserve' receptors will increase the potency but not the maximal effect of receptor agonists[40].

Etorphine is a potent agonist at δ, μ and κ receptors[44, 58]. Saturating concentrations of etorphine inhibited cAMP synthesis by 73% in control NG108-15 cells with an IC_{50} of 2.7 nM (fig. 3). In ethanol-treated NG108-15 cells, the IC_{50} for etorphine was reduced to 0.77 nM. Thus, in ethanol-treated cells, an 85% increase in δ receptor expression, demonstrated in the studies described above, rendered cAMP synthesis 3.5-fold more sensitive to etorphine inhibition[10]. Unexpectedly, saturating concentrations of etorphine inhibited 89% of cAMP synthesis in ethanol-treated cells – a significant increase over control levels[10]. This increase in maximal inhibition could not have resulted from an increase in space receptors[40] and suggests that long-term treatment with ethanol also modified opioid signal transduction at a site distal to δ receptor activation.

Differential effects of ethanol on G proteins in NG108-15 cells

Ethanol treatment of NG108-15 cells could potentiate maximal opioid inhibition of cAMP accumulation by increasing $G\alpha_i$, decreasing $G\alpha_s$, or both. We measured the abundance of these G proteins in membranes prepared from control and ethanol-treated NG108-15 cells using antisera directed against synthetic peptides derived from the cloned sequences of $G\alpha_s$ and $G\alpha_i$[11, 55]. Ethanol treatment caused a 42% reduction in $G\alpha_s$ and no significant change in $G\alpha_i$[11] (fig. 4A). This decrease in $G\alpha_s$ relative to $G\alpha_i$ could have potentiated maximal opioid inhibition of cAMP accumulation by allowing a given

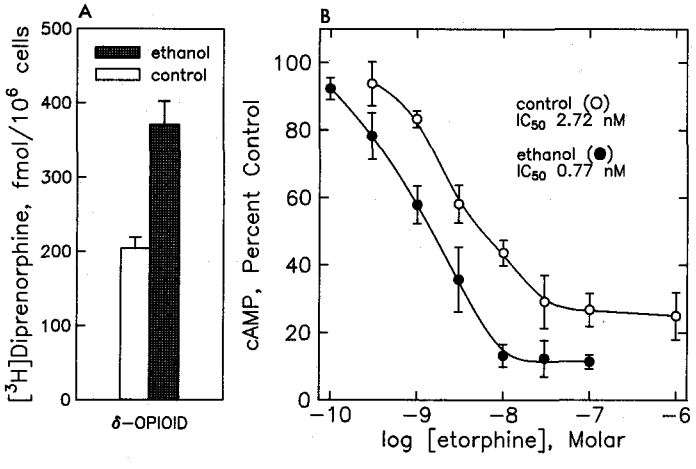

Figure 3. Effects of long-term ethanol treatment on δ-opioid receptor binding and opioid inhibition of cAMP accumulation in intact NG108-15 cells. NG108-15 cells were cultured with 200 mM ethanol for 48 h. *A* Binding of [^{3}H]diprenorphine to control and ethanol-treated cells. *B* Inhibition by etorphine of phenylisopropyladenosine-stimulated cAMP accumulation in control (○) and ethanol-treated cells (●). Adapted with permission from Charness et al.[10].

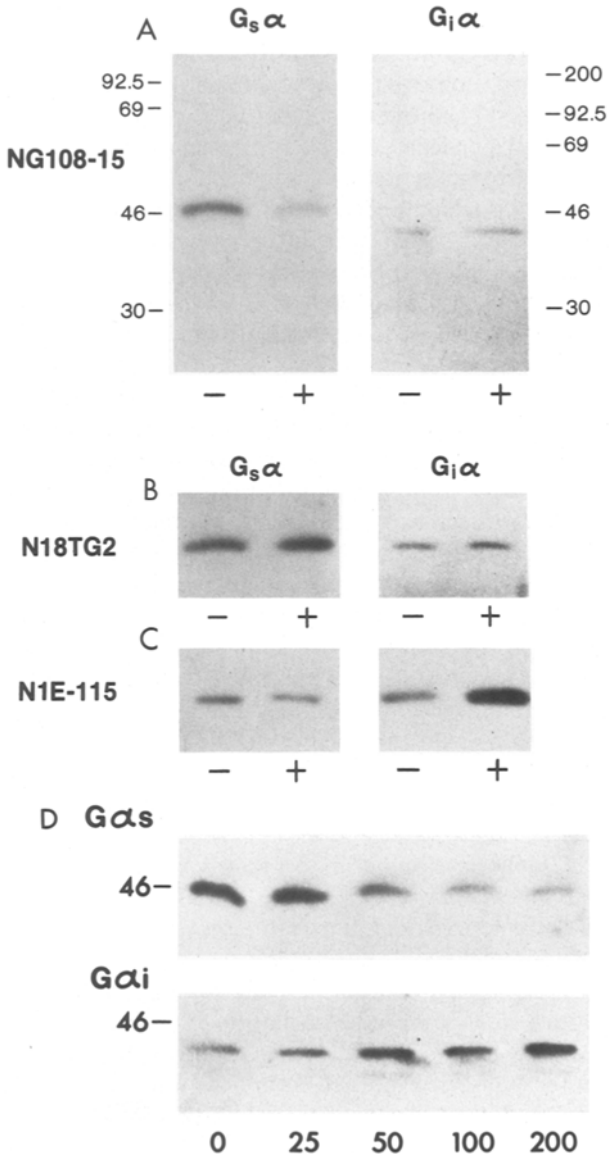

Figure 4. Effects of long-term ethanol treatment on the abundance of $G\alpha_s$ and $G\alpha_i$ in neuronal cells. Western blots of membrane preparations from cells were stained with antisera as described[11]. NG108-15 (*A*), N18TG2 (*B*), and N1E-115 (*C*) cells were treated with 0 or 200 mM ethanol for 48 h. *D* N1E-115 cells were treated with 0–200 mM ethanol for 5 days. Reproduced from Charness et al.[11] with permission.

amount of $\beta\gamma$ derived from G_i to inactivate a larger proportion of $G\alpha_s$ (see fig. 2). Also associated with this ethanol-induced decrease in $G\alpha_s$ was a reduction in the stimulation of cAMP accumulation by G_s-coupled hormones[9, 24, 54] and cholera toxin[9], a direct activator of G_s. Mochly-Rosen and colleagues have shown that ethanol treatment of NG108-15 cells causes comparable reductions in $G\alpha_s$ and its mRNA[54].

Effects of ethanol in related clonal neuronal cell lines

The nervous system comprises hundreds of neuronal cell types, many of which exhibit intrinsically different physiological responses to ethanol[42, 74]. Moreover, there appear to be genetic factors that govern individual susceptibility to ethanol intoxication and alcoholism[14]. Hence, the ability of ethanol to disrupt transmembrane signalling and the capacity for adaptation may differ considerably among diverse neuronal cells and among individuals. We therefore chose to model the actions of ethanol in a variety of neuronal cell lines.

Figure 5 shows the genealogies of the cell lines that we studied. The mouse neuroblastoma cell lines N18TG2, N4TG1, and N1E-115 are clonal derivatives of the spontaneous mouse neuroblastoma C1300[26], all of which express moderate levels of δ receptors. The NG108-15 cell line is a hybrid of the mouse neuroblastoma N18TG2 and the rat glioma C6BU cell lines[26, 41]. Constitutive expression of the δ-opioid receptor is absent in C6BU cells, intermediate in N18TG2 and high in NG108-15[41]. Incubation of all four cell lines with 200 mM ethanol for 48 h increased the binding to intact cells of [^{3}H]diprenorphine, an opiate partial agonist[12, 44]. As was the case for NG108-15 cells, ethanol increased the maximal binding capacity, but not the affinity of the receptor ligand. The four cell lines exhibited differential sensitivity to this effect of ethanol, with N1E-115 showing the largest change (2.8-fold increase) and N18TG2 the least (1.6-fold increase). These effects of ethanol were dependent on both the dose and duration of exposure; in three of the four cell lines δ-opioid receptor expression increased significantly after incubation for one week with 25–100 mM ethanol. These experiments indicate that clinically-attainable concentrations of ethanol differentially up-regulate the δ-opioid receptor in four related clonal neuronal cell lines.

The effects of incubating three neuronal cell lines with 200 mM ethanol for 48 h on the abundance and function of signal transduction proteins

	NG108-15	N18TG2	N1E-115
↑ δ-Opioid receptor expression[10, 12]	++	+	+++
↑ Potency, opioid inhibition cAMP[10, 12]	+	+	+
↑ Maximal opioid inhibition cAMP[10, 12]	+	–	–
↓ Hormone-stimulated cAMP[9, 11]	+	–	++
↓ $G\alpha_s$[11]	++	–	+
↑ $G\alpha_i$[11]	–	–	+++

Relative changes are denoted by '+'; no effect is indicated by '–'.

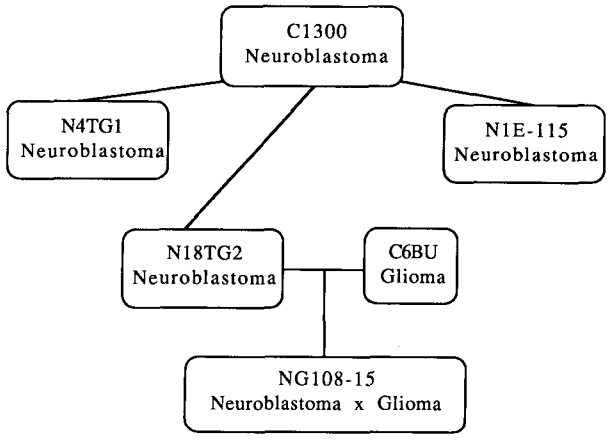

Figure 5. Lineage of neuroblastoma cell lines.

Regulation of G proteins by ethanol also differed significantly among these neuronal cell lines[11]. Ethanol-treated N18TG2 cells, which exhibited the smallest up-regulation of δ receptors[12], also showed relatively little heterologous desensitization of adenylyl cyclase[9] and no change in $G\alpha_s$ or $G\alpha_i$ (fig. 4B). By contrast, ethanol-treated N1E-115 cells, which underwent the largest up-regulation of δ receptors, also showed the greatest degree of heterologous desensitization[9]. When incubated with 25–200 mM ethanol for 5 days, N1E-115 cells showed a dose-dependent decrease in $G\alpha_s$ and increase in $G\alpha_i$ (fig. 4C, D)[11]. Thus, N18TG2 cells are less capable of adapting to ethanol than N1E-115 cells. Further analysis of these two related cell lines may help identify the molecular elements that endow some, but not all, neuronal cells with the capacity to adapt to ethanol.

We next studied the consequences of long-term ethanol treatment of N1E-115 cells on opioid inhibition of cAMP accumulation. As expected, induction of δ-opioid receptor expression increased the potency of etorphine in inhibiting cAMP accumulation[12]. However, despite causing a decrease in $G\alpha_s$ and an increase in $G\alpha_i$, long-term ethanol treatment did not increase maximal opioid inhibition in N1E-115 cells[12]. This suggests that in N1E-115 cells, the concentration of $G\alpha_i$ may not be a limiting factor in maximal opioid inhibition; alternatively, ethanol may be up-regulating a subtype of $G\alpha_i$ that does not couple with adenylyl cyclase. This enigma may be clarified by using more specific antibodies to identify selective up-regulation of the $G\alpha_i$ subtypes α_{i1}, α_{i2}, and α_{i3}[23]. It will likewise be important to learn whether up-regulation of a particular $G\alpha_i$ subtype alters other opioid actions, such as inhibition of voltage-dependent calcium conductance or activation of inwardly rectifying potassium conductance. The table summarizes the differential effects of ethanol on the abundance and function of the proteins that mediate opioid inhibition of cAMP accumulation in clonal neuronal cell lines.

Mechanisms underlying opioid receptor up-regulation by ethanol

By what mechanism does ethanol increase the expression of δ-opioid receptors in NG108-15 cells? To investigate whether receptor up-regulation was a consequence of osmotic effects of ethanol on intact cells [70], we cultured NG108-15 cells for 48 h in medium containing 25 mM ethanol, 25 mM propanol, or 25 mM butanol. These n-alkanols increased receptor density by 7, 54, and 88% respectively, demonstrating that alcohol-induced up-regulation of the δ receptor is a function of the chain length and membrane lipid partition coefficients of short-chain alcohols, rather than osmolality [8].

The δ receptor in NG108-15 cells can be down-regulated by receptor-mediated endocytosis [45]. Because NG108-15 cells synthesize and release various enkephalins [19, 26], constitutive levels of δ receptor expression may reflect tonic down-regulation of δ receptors by endogenous opioids. Ethanol-induced increases in δ receptor expression might therefore result from decreases in the release or binding of opioid peptides, or disruption of the process of receptor-mediated endocytosis.

To test whether ethanol disrupts receptor-mediated endocytosis, NG108-15 cells were incubated for 24 h with 10^{-6} M etorphine in the absence or presence of 200 mM ethanol [10]. Etorphine alone decreased δ receptor density by 60% without changing receptor affinity. Ethanol did not prevent the loss of receptors induced by etorphine; in fact, receptor down-regulation by etorphine almost completely prevented the 53% up-regulation of δ receptors induced by ethanol alone. These data suggest that ethanol does not directly disrupt those events of receptor-mediated endocytosis that follow agonist binding.

Ethanol could still interfere with receptor-mediated endocytosis by inhibiting the binding of endogeneous opioids. If so, long-term exposure to other δ receptor antagonists should also up-regulate the δ receptor. To test this hypothesis, we incubated NG108-15 cells for 24 h with 10^{-6} M naloxone in the absence or presence of 200 mM ethanol [10]. Naloxone alone increased δ receptor expression by about 20%, probably reflecting a slight inhibition of tonic down-regulation of receptors by endogenous opioids. However, naloxone up-regulated δ receptors to a lesser extent than ethanol, despite being a much better receptor antagonist than ethanol at the concentrations used. Furthermore, receptor up-regulation by naloxone and ethanol was additive. Thus, it seems likely that ethanol and naloxone up-regulate the δ receptor by different mechanisms. In the case of ethanol, up-regulation of δ receptors during chronic ethanol treatment does not seem to be causally related to the decrease in δ receptor affinity induced by acute ethanol exposure.

Ethanol might increase receptor expression by decreasing the turnover of membrane-bound receptors. This could be accomplished by either slowing the degradation or increasing the synthesis and membrane insertion of receptors. We estimated the rate of δ receptor degradation by blocking protein synthesis with cycloheximide and measuring the subsequent decline in opioid binding [10]. Cycloheximide treatment reduced opioid binding steadily over 12 h to a plateau of 42% of control levels. Ethanol did not significantly attenuate the rate or magnitude of this decline. Whereas exposure for 18 h to 200 mM ethanol increased δ receptor expression by 47% in control cells, similar ethanol exposure in cycloheximide-treated cells produced a 63% decrease in δ receptor expression. Thus, ethanol does not up-regulate the δ receptor by slowing its degradation. Moreover, normal protein synthesis appears to be a requirement for receptor up-regulation.

Ethanol might augment δ receptor expression by promoting the transcription of genes that direct receptor synthesis or processing. If so, inhibition of RNA synthesis would prevent up-regulation of the δ receptor by ethanol. NG108-15 cells treated for 24 h with actinomycin D showed a 36% reduction in δ receptor binding. Ethanol attenuated this effect, and consequently caused proportional increases in δ receptor binding in control and actinomycin D-treated cells [10]. These indirect experiments suggest that ethanol may up-regulate the δ receptor without activating the transcription of receptor genes; however, this conclusion needs to be confirmed by direct measurement of δ receptor mRNA.

Thus, ethanol increases the expression of the δ receptor by an apparently novel mechanism. Receptor up-regulation requires normal protein synthesis and does not result from ethanol's properties as a weak receptor antagonist or from an effect on receptor turnover. The increase in receptor density has important functional consequences, leading to changes in cellular levels of cAMP. The precise role these events play in physical dependence and the ethanol withdrawal syndrome remains to be determined; however, these experiments suggest that cultured neuronal cells may prove valuable in studying the molecular mechanisms of neural cellular adaptation to ethanol.

Abbreviations: B_{max}, maximal binding capacity; CNS, central nervous system; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DAGO, H-Tyr-D-Ala(Me)Phe-NH-CH_2-OH; DAMEA, [D-Ala^2,Met^5]enkephalinamide; DHM, dihydromorphine; DSTLE, Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; FT-IR, Fourier-transform infrared spectroscopy; IC_{50}, concentration of a drug producing half-maximal inhibition; K_d, equilibrium dissociation constant; NMR, nuclear magnetic resonance; POMC, proopiomelanocortin.

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The effect of ethanol on the biosynthesis and regulation of opioid peptides

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Summary. Alcoholism and alcohol abuse are serious health problems. Alcohol is known to influence the activity of a number of biological systems, for example the hormonal and neuronal systems. One of the biological systems whose activity is greatly influenced by alcohol is the endogenous opiate system. Alcohol modifies the function of both opiate receptors and opioid peptides. In fact it has been proposed that many of the effects of ethanol are mediated by its effects on the endogenous opiate system. This review will present results from various laboratories on the effects of acute and chronic ethanol treatments on various species, and on the release, biosynthesis and post-translational processing of the endorphins, enkephalins and dynorphins, the three known families of endogenous opioid peptides. Furthermore, the effect of acute and chronic ethanol consumption on the β-endorphin system in man, and the possible implications of the functional activity of the endogenous opiate system for the genetic predisposition to alcoholism will be discussed.
Key words. Acute ethanol; chronic ethanol; endorphins; enkephalin; dynorphins; release; biosynthesis.