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Ethanol Increases δ-Opioid Receptor Gene Expression in Neuronal Cell Lines

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SUMMARY

Long-term treatment with ethanol increases δ-opioid receptor (DOR) expression in the NG108–15 neuroblastoma × glioma hybrid cell line. To determine the underlying mechanism, we studied the effects of ethanol on [^{3}H]diprenorphine binding to intact cells and DOR gene expression in four related clonal neural cell lines. Incubation with 200 mM ethanol for 48 hr increased [^{3}H]diprenorphine binding by 1.4- (N18TG2), 1.8- (NG108–15), 1.9- (N4TG1), and 3.0-fold (N1E-115). Treatment with 25, 50, or 100 mM ethanol for 1 week caused a dose-dependent increase in receptor expression. Receptor up-regulation was associated with an increase in the potency of etorphine for inhibiting prostaglandin E_1-stimulated cAMP accumulation. Constitutive DOR expression differed more than 3-fold among the different cell lines and correlated positively with basal cAMP levels. Long-term ethanol treatment increased basal cAMP levels in three of the four cell lines, but did not induce cellular differentiation. Northern blot analysis demonstrated an identical pattern of multiple transcripts in the four cell lines. Ethanol increased the abundance of DOR mRNA by approximately 3-fold in N18TG2 cells and by approximately 5-fold in the remaining cell lines. These findings indicate that clinically relevant concentrations of ethanol regulate DOR expression by increasing the abundance of DOR mRNA. The disparity between the increase in gene expression and ligand binding suggests that ethanol may also modify mRNA translation or receptor processing.

Ethanol produces intoxication by interacting selectively with a limited number of neuronal signaling molecules (1). Tolerance and physical dependence develop during prolonged and heavy drinking, rendering neuronal function normal in the presence of ethanol and abnormal in its absence (2). Behavioral and biochemical studies indicate that changes in opioid neurotransmission contribute to ethanol intoxication and the adaptative neuronal responses that it provokes (3–7). Ethanol appears to modulate opioid neurotransmission by changing the processing, release, and receptor binding of opioid peptides (8–10).

Endogenous opioid peptides bind differentially to at least three major receptor subtypes, designated μ, δ, and κ (11). Acute ethanol exposure selectively inhibits opioid binding to the DOR by promoting ligand dissociation (8, 12). In contrast, long-term ethanol exposure up-regulates the DOR nearly 2-fold in NG108–15 cells (8, 9) and in mouse brain (13). Activation of the DOR in NG108–15 cells inhibits adenylyl cyclase activity through coupling to G_{i2} (14), and DOR up-regulation renders ethanol-treated NG108–15 cells three times more sensitive than untreated cells to opioid inhibition of adenosine-stimulated cAMP accumulation (9). Receptor up-regulation begins 12 to 18 hr after ethanol exposure and reaches a plateau after 48 hr. Cycloheximide prevents the induction of the DOR by ethanol, indicating that this process is dependent on protein synthesis and possibly on changes in gene expression. Ethanol has been shown recently to regulate the expression of several neuronal genes, including the heat shock cognate Hsc70 (15), tyrosine hydroxylase (16), and the α_2-adrenergic receptor (17), but the absence of cDNA probes for the DOR has hampered the study of ethanol-induced regulation of the DOR.

The NG108–15 cell line, a hybrid of the N18TG2 mouse neuroblastoma and the C6BU rat glioma cell lines, selectively expresses the DOR subtype(18, 19). Levels of DOR expression are much greater in NG108–15 cells than in N18TG2 cells and are negligible in C6BU cells, implying that a genetic contribution from the C6BU cells induces opioid receptor expression in

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ABBREVIATIONS: DOR, δ-opioid receptor; DPN, diprenorphine; HAT, 0.1 mM hypoxanthine, 1 μM aminopterin, and 12 μM thymidine; PGE_1, prostaglandin E_1; K_d, equilibrium dissociation constant; B_{max}, maximal binding capacity; IC_{50}, concentration that inhibits 50%; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

N18TG2 cells (18, 20). The related neuroblastoma cell lines N4TG1 and N1E-115 selectively express the DOR at much lower levels than in NG108–15 cells (19). Although all of these neuroblastoma cell lines are derived from a common C1300 neuroblastoma ancestor (19), they exhibit distinct patterns of adaptation to ethanol (21). Interestingly, ethanol induces heterologous desensitization of adenylyl cyclase and decreases $G\alpha_s$ levels in NG108–15 cells, but not in N18TG2 cells (21), indicating that a genetic contribution from the C6 parental cell line also confers increased sensitivity to ethanol on the hybrid.

The DOR gene was cloned recently from NG108–15 cells (22, 23). A single-copy DOR gene is derived from the mouse neuroblastoma N18TG2 parental cell line (23) and encodes a protein with seven transmembrane domains characteristic of the superfamily of G protein-linked receptors (22). Hybridization of cDNA probes for the coding and 3′-non-coding regions of the DOR gene to RNA extracted from NG108–15 cells and various brain regions reveals a common pattern of multiple transcripts (22). In this paper, we have used a DOR cDNA probe to investigate the mechanism by which ethanol regulates DOR expression in four neuronal cell lines. We show that ethanol differentially increases DOR expression in the NG108–15, N18TG2, N4TG1, and N1E-115 cell lines, thereby increasing the potency of etorphine for inhibiting PGE_1-stimulated cAMP accumulation. In each cell line, the ethanol-induced increase in DOR expression is associated with a much greater increase in the abundance of the two largest DOR transcripts.

Experimental Procedures

Cell Culture. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEH-21; Gibco BRL, Grand Island, New York) supplemented with 10% fetal bovine serum and 2 mM glutamine. The medium was supplemented further as follows: NG108–15 with HAT; N18TG2 with 100 μM thioguanine; N4TG1 with 50 U/ml penicillin and 50 μg/ml streptomycin. In some experiments (DOR gene expression), NG108–15 cells were cultured in defined medium, as described (9). Cultures were maintained at 37° in a humidified atmosphere of 10% CO_2 and 90% air. Media were replaced daily beginning 3 days after a weekly subculture. Evaporation of ethanol was prevented by tightly capping cell flasks or covering multiwell trays with Parafilm. Parallel cultures of control cells were treated in the same manner as ethanol-treated cells with respect to media changes and capping of flasks.

Measurements of δ-opioid receptors. [^{3}H]DPN (9–25 Ci/mmol, Amersham, Arlington Heights, IL) binding to confluent intact cells was measured as described previously (9) with minor modifications. Cells were detached by gentle shaking after incubation for 20 min at 37° in 137 mM NaCl, 5 mM KC1, 0.17 mM $NaHPO_4$, 0.22 mM KH_2PO_4, 6 mM glucose, 59 mM sucrose, and 5 mM HEPES, pH 7.6 (buffer A) and pelleted by centrifugation at 150 × *g* for 5 min. Cells were then washed twice by resuspension and centrifugation in 50 ml of buffer A, and resuspended at a final concentration of 3 to 10 × 10^5 cells/ml in buffer B (110 mM NaCl, 5 mM KC1, 1 mM MgCl, 1.8 mM $CaCl_2$, 25 mM glucose, 55 mM sucrose, and 10 mM HEPES, pH 7.4), or in buffer C (Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 25 mM HEPES, pH 7.4). Buffer C was supplemented with HAT for NG108–15 cells. Binding did not differ when measured in the two buffers. For each experimental condition, specific binding at a saturating concentration of [^{3}H]DPN (5–7 nM) was determined in triplicate samples from two separate flasks by incubating suspended cells for 15 min at 37° in the absence (total binding) or presence (nonspecific binding) of 10 μM naloxone. Measured in this way, changes in specific binding (total binding minus nonspecific) reflect changes in receptor number. In some experiments binding of 0.2 to 8 nM [^{3}H]DPN was measured to determine the B_{max} and K_d. Bound and free ligand were separated by rapid filtration under reduced pressure over Whatman GF/B glass fiber filters using a Brandel cell harvester. Filters were washed 3 times with 5 ml of ice-cold phosphate-buffered saline, and bound radioactivity was determined by liquid scintillation counting. Saturation binding data for [^{3}H]DPN, analyzed by the computer program LIGAND, best fit a one-site ligand-receptor interaction. Therefore, in most experiments, B_{max} and K_d were calculated by linear regression analysis according to the method of Scatchard (9). [^{3}H]DPN binding to whole cells was saturable and linear with respect to cell number and protein concentration within the range used in these experiments. Protein content of a trichloroacetic acid cell precipitate was determined by the method of Lowry *et al.* (24) using bovine serum albumin as a standard. In NG108–15 and N1E-115 cells, curves for etorphine inhibition of 2 nM [^{3}H]DPN binding were best fit by a two-site interaction. However, estimates of the high affinity K_d were associated with high coefficients of covariance. For N18TG2 and N4TG1 cells, the data were best fit by a one-site model. To permit comparisons among the cell lines, data in all cell lines were analyzed according to a one-site model.

cAMP measurements. Cells attached to 4.5-cm^2 multiwell trays were preincubated for 30 min at 37° in buffer C containing 100 μM 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone, a phosphodiesterase inhibitor (buffer D). cAMP synthesis was stimulated by incubating cells for 15 min at 37° in 0.5 ml of buffer D containing 10 μM PGE_1. Basal cAMP was determined during a similar incubation in the absence of PGE_1. Opioid inhibition of PGE_1-stimulated cAMP accumulation was determined by co-incubating cells with PGE_1 and various concentrations of etorphine, a stable and potent DOR agonist (25). cAMP was measured by radioimmunoassay (9). The IC_{50} for etorphine inhibition of cAMP accumulation was calculated by log-logit analysis.

Northern blot analysis. Total cellular RNA was isolated from NG108–15 cells by the RNAzol B method (Biotecx Lab., Inc., Houston, TX) (26). RNA was dissolved in diethylpyrocarbonate-treated water, its concentration was measured by absorbance at 260 nm, and it was stored at −70° until further use. RNA (10–20 μg) was denatured in 6.5% formaldehyde, 50% formamide, 20 mM MOPS, pH 7.0 and fractionated by electrophoresis on 1.2% agarose gels containing 0.36 M formaldehyde, 20 mM MOPS, pH 7.0. Gels were stained for 15 min with 33 μg/ml acridine orange in phosphate-buffered saline, pH 6.5, and photographed to verify the integrity and amount of RNA. Gels were then destained and incubated for 20 min in 50 mM NaOH to facilitate the transfer of high molecular weight transcripts. The RNA was transferred to nylon membranes (Biorad Zeta-probe GT) by capillary blotting using a BIOS blotting apparatus (New Haven, CT) and crosslinked by baking for 1 hr at 80°. Examination of acridine orange-stained gels after blotting revealed complete transfer of RNA. Membranes were prehybridized for 2 hr at 42° with 50% formamide, 20 μg/ml salmon sperm DNA, 2.5× Denhardt's solution (1× Denhardt's = 50 mg/ml Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 0.5% SDS, and 5× SSPE (1× SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA) and then hybridized overnight at 42° with 1 to 5 × 10^6cpm/ml of the ^{32}P-labeled cDNA probe DKI4, which includes the entire coding region of the DOR gene cloned from NG108–15 cells. Probes were radiolabeled by the random primer method (27) and purified on Select D G50 spin columns (5′ → 3′ Inc; Boulder, CO). After hybridization, the filters were washed successively at 65° in 2× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 1× SSC and 0.5× SSC, each containing 0.2% SDS. Filters were then exposed to radiographic film (Kodak X-OMAT AR) with two intensifying screens for 1 to 7 days at −70°. Autoradiograms were analyzed by computerized scanning densitometry.

Materials. Cell lines were generously provided as follows: N4TG1 from Dr. Kwen Jen Chang (Burroughs Welcome, Research Triangle, NC), N18TG2 and NG108–15 from Dr. Ping Yee Law (University of Minnesota, Minneapolis, MN), and N1E-115 cells from Dr. Elliott Richelson (Mayo Clinic, Jacksonville, FL).

Statistical methods. Dose-response curves were compared using

analysis of variance and differences between means were evaluated with the Sheffé F-test using the program Statview II. The significance of correlations was tested with Pearson's product-moment correlation coefficient. Statistical significance was defined by $p < 0.05$.

Results

Effect of ethanol on [^{3}H]DPN binding in four cell lines. Equilibrium saturation binding isotherms for [^{3}H]DPN were performed using whole cells suspended in a physiologic buffer. Because these cells differed in size, binding was expressed in units of fmol/mg protein to facilitate comparisons. [^{3}H]DPN B_{max} in NG108–15 cells (9) was approximately three times that in the other three cell lines. Receptor affinity was similar in the four cell lines, consistent with their expression of a single opioid receptor subtype (19). Treatment of each cell line with 200 mM ethanol for 48 hr differentially increased [^{3}H]DPN B_{max} without changing binding affinity (Fig. 1 and Table 1). The magnitude of receptor up-regulation did not correlate with constitutive levels of receptor expression. For example, ethanol increased DOR expression by 3-fold in N1E-115 cells and by 1.9-fold in N4TG1 cells ($F = 8.25$, $p < 0.01$), yet constitutive receptor expression was similar in the two cell lines (Table 1). Conversely, although constitutive receptor expression was more than 3-fold higher in NG108–15 cells than in N4TG1 cells, ethanol caused a similar increase (considered as a ratio to control values) in DOR expression in these two cell lines. Ethanol-induced up-regulation of the DOR was greater in NG108–15 cells than in N18TG2 cells ($F = 6.15$, $p = 0.038$), in keeping with previous findings that the hybrid cell line is more sensitive to ethanol than the neuroblastoma parent (21).

Effect of low concentrations of ethanol on DOR regulation. In most of our experiments we have exposed cells to 200 mM ethanol for 48 hr to produce large effects over a short period of time. However, this concentration of ethanol is achieved only rarely in humans (2). To determine the effects of ethanol concentrations measured more commonly after heavy drinking, we incubated cells for one week with 25, 50, or 100 mM ethanol. In these experiments, binding was measured using a single saturating concentration of [^{3}H]DPN. Ethanol caused a dose-dependent increase in DOR binding ($F = 8.31$; $p = 0.0006$) that differed significantly among the four cell lines ($F = 6.64$, $p = 0.0006$) (Fig. 2). The dose-response curve for N18TG2 cells was relatively flat, whereas the curve for N1E-115 cells was steep and those for NG108–15 and N4TG1 cells were intermediate. These data indicate that clinically relevant concentrations of ethanol differentially up-regulate the DOR in neuronal cells. The magnitude of this effect varies as a function of the dose and duration of exposure to ethanol.

Effect of ethanol on receptor-effector coupling. In NG108–15 cells, ethanol-induced up-regulation of the DOR was associated with an increase in the potency and maximal effect of etorphine in inhibiting phenylisopropyladenosine-stimulated cAMP accumulation (9). We asked next whether ethanol-induced up-regulation of the DOR would modify receptor-effector coupling in the other cell lines. We determined the K_d and IC_{50} values, respectively for etorphine binding and inhibition of PGE_1-stimulated cAMP accumulation using intact cells maintained at 37° in a physiologic buffer. Table 2 shows that in control cells the ratio of the K_d to IC_{50} (coupling efficiency) for etorphine varied among the four cell lines. In N1E-115 cells, the coupling efficiency was close to 1, indicating a 1:1 relationship between receptor occupancy and biologic response. The corresponding ratios in NG108–15, N4TG1, and N18TG2 cells were greater than 1, indicating that in these cell lines, the maximal response to etorphine occurred after only fractional receptor occupancy.

Curves for etorphine inhibition of [^{3}H]DPN binding in control and ethanol-treated cells were superimposable (not shown).

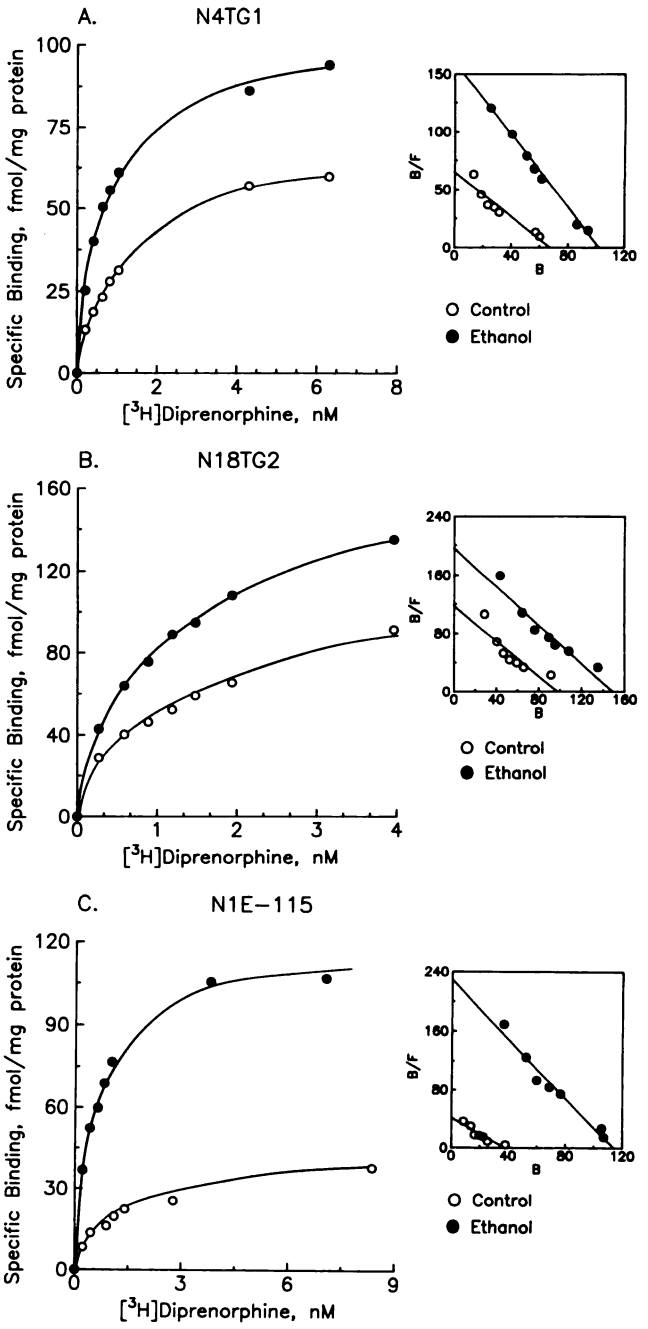

Fig. 1. Effect of ethanol on [^{3}H]DPN saturation binding isotherms. A) N4TG1, B) N18TG2, and C) N1E-115 cells were cultured for 48 hr in the absence (O) and presence (●) of 200 mM ethanol, washed, and suspended in physiologic buffer (buffer B, see Methods). Intact cells were incubated for 15 min at 37° with 0.2 to 8 nM [^{3}H]DPN in the absence and presence of 10 μM naloxone. Each figure depicts specific binding as a function of free ligand concentration and a Scatchard transformation of the data for a representative experiment. Data points are the means of triplicate determinations. Mean values for B_{max} and K_d appear in Table 1.

TABLE 1

Effect of ethanol on [^{3}H]DPN binding, basal cAMP, and cell protein in neural cell lines

Cells were cultured for 48 hr in serum-containing medium in the absence (control) or presence (ethanol) of 200 mM ethanol. Binding to intact cells was determined as described (see Methods). Basal cAMP accumulation was measured as described (see Methods) in the absence and presence of 1 U/ml adenosine deaminase (ADA). All assays were performed in the absence of ethanol. The mean ratio (E/C) of binding, cAMP levels, and cell protein in ethanol treated cells compared with control cells was calculated from paired experiments and differs from the ratio of the means. The number of experiments is indicated in parentheses.

Cell line and condition	[^{3}H]DPN			Basal cAMP				Cell protein	
	B_{max}		K_d	− ADA		+ ADA			
	fmol/mg protein	E/C	nM	pmol/mg/min	E/C	pmol/mg/min	E/C	mg/10^6 cells	E/C
NG108-15[a]									
Control	204 ± 15 (4)	1.80 ± 0.12	0.60 ± 0.02 (4)	56.3 ± 5 (31)	1.93 ± 15	6.8 ± 1.0 (25)	1.35 ± 11	0.89 ± 0.04 (10)	1.08 ± 0.05
Ethanol	371 ± 31 (4)		0.60 ± 0.08 (4)	103.3 ± 8.4 (29)		7.6 ± 1.5 (19)		0.95 ± 0.05 (10)	
N18TG2									
Control	88 ± 13 (4)	1.42 ± 0.04	0.95 ± 0.07 (4)	11.0 ± 0.1 (10)	2.62 ± 0.38	1.8 ± 0.5 (16)	1.03 ± 0.15	0.49 ± 0.02 (3)	1.06 ± 0.02
Ethanol	123 ± 19 (4)		0.81 ± 0.17 (4)	27.0 ± 6.4 (10)		2.5 ± 1.2 (13)		0.53 ± 0.02 (3)	
N4TG1									
Control	63 ± 8 (3)	1.92 ± 0.19	0.71 ± 0.13 (3)	2.9 ± 0.5 (8)	3.05 ± 0.77	0.9 ± 0.2 (15)	0.99 ± 0.10	0.48 ± 0.07 (3)	1.00 ± 0.02
Ethanol	118 ± 16 (3)		0.61 ± 0.04 (3)	7.5 ± 2.2 (8)		0.8 ± 0.2 (12)		0.46 ± 0.09 (3)	
N1E-115									
Control	51 ± 7 (6)	2.96 ± 0.15	0.59 ± 0.12 (6)	5.4 ± 0.7 (14)	1.15 ± 0.17	1.4 ± 0.3 (14)	1.08 ± 0.15	0.95 ± 0.06 (5)	1.17 ± 0.11
Ethanol	149 ± 17 (6)		0.77 ± 0.14 (6)	5.5 ± 0.7 (14)		1.6 ± 0.4 (14)		1.11 ± 0.05 (5)	

[a] Values for [^{3}H]DPN binding in NG108-15 cells are from (9).

Although long-term treatment with ethanol did not change the K_d for etorphine inhibition of [^{3}H]DPN binding, it did decrease the IC_{50} for etorphine inhibition of PGE_1-stimulated cAMP accumulation (Fig. 3, Table 2). Therefore, ethanol treatment increased the coupling efficiency in each cell line. Maximal inhibition of cAMP accumulation by etorphine ranged from 40% in control N18TG2 cells to 72% in control N4TG1 cells. Ethanol treatment significantly increased the maximal effect of etorphine in NG108–15 cells but did not change that for the other cell lines. These findings indicate that ethanol-induced up-regulation of the DOR is associated in each cell line with an increase in the physiologic response to a DOR agonist.

The relationship between DOR expression and basal cAMP. Basal cAMP accumulation was measured in the presence of a phosphodiesterase inhibitor and in the absence of ethanol. Basal levels of cAMP varied among the four cell lines and were greatly reduced when cells were assayed in the presence of adenosine deaminase (Table 1). All of the cell lines except N1E-115 showed increased basal levels of cAMP after long-term ethanol exposure (Table 1). Basal cAMP accumulation did not differ significantly between control and ethanol-treated cells when assayed in the presence of adenosine deaminase, consistent with recent findings in NG108–15 cells (28). These data indicate that spontaneous release of adenosine contributes significantly to basal cAMP production in these cells and that long-term treatment with ethanol increases basal cAMP accumulation in NG108–15, N18TG2, and N4TG1 cells by changing the release or reuptake of adenosine. Levels of DOR expression correlated significantly with mean basal cAMP levels in the combined group of control and ethanol-treated cells ($r = 0.94$; $n = 8$; $p = 0.01$). This association suggests that DOR expression may be regulated in some neural cell lines by cAMP or a factor that co-varies with cAMP.

Effects of ethanol on cellular morphology. DOR expression increases when dividing NG108–15 cells are differentiated (29) or synchronized (30). Ethanol increased basal cAMP levels in three of the four cell lines, and elevated levels of cAMP can induce differentiation in neuronal cell lines. Moreover, Williams *et al.* (28) reported recently that treatment of NG108–15 cells wth 200 mM ethanol for 48 hr significantly increased cell protein, slowed cell division, and induced a differentiated phenotype. These findings raise the possibility that ethanol up-regulates the DOR by inducing cellular differentiation. To investigate the effects of ethanol on cell morphology, we cultured each cell line at low densities on poly-D-lysine-coated plastic dishes in the absence and presence of ethanol. The use of a poly-D-lysine substrate promotes neurite extension in differentiating NG108–15 cells,[1] and keeping cells at a low density facilitates morphologic observations. Chronic ethanol exposure did not induce neurite outgrowth in any of the cell lines (Fig. 4). Incubation of cells with 200 mM ethanol for 48 hr reduced cell yield by 10 to 30%, but much of this loss occurred when viable cells detached from the substrate during medium changes (data not shown). There was no significant increase in the protein content per cell after chronic ethanol exposure (Table 1), and we have shown previously that treatment of NG108–15 cells for 48 hr with 200 mM ethanol does not significantly change cell volume, as determined by particle sizing (8). The failure of ethanol to promote neurite extension or increase cellular protein under these experimental conditions makes it unlikely that ethanol up-regulates the DOR by inducing cellular differentiation.

Effect of ethanol on DOR gene expression. To determine whether ethanol regulates DOR gene expression, we hybridized a cDNA probe for the full coding region of the DOR gene to total RNA extracted from cells treated for 48 hr in the absence and presence of different concentrations of ethanol. Northern blot analysis of control cells demonstrated an identical pattern

[1] M.E. Charness, unpublished observations.

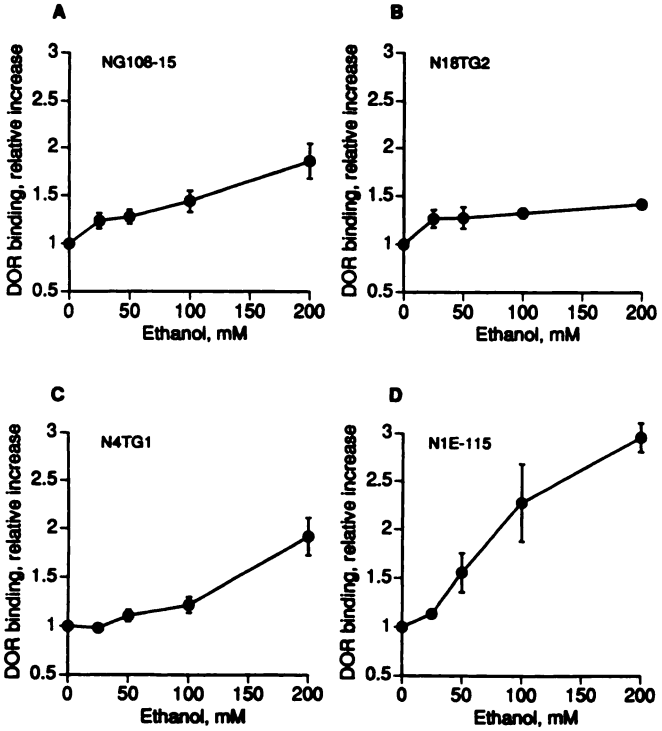

Fig. 2. Effect of low concentrations of ethanol on [^{3}H]DPN binding. Cells were cultured for 7 days with 0, 25, 50, or 100 mM ethanol, washed, and suspended in physiologic buffer (buffer B). A) Intact NG108–15, B) N18TG2, C) N4TG1, and D) N1E-115 cells were incubated for 15 min at 37° with 5 to 7 nM [^{3}H]DPN in the absence and presence of 10 μM naloxone. In each experiment, the percentage increase over control [^{3}H]DPN-specific binding was determined by comparing the mean values obtained from duplicate flasks. Each point represents the mean percentage increase determined from 4 to 9 experiments. The 200 mM points represent data from paired experiments in which specific binding of 0.2 to 8 nM [^{3}H]DPN (Table 1) was measured in cells that were exposed to 0 or 200 mM ethanol for 2 days.

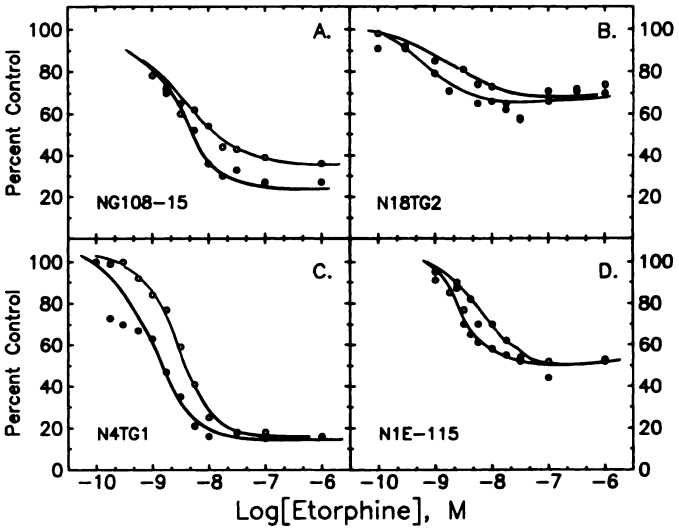

Fig. 3. Effect of long-term ethanol treatment on etorphine inhibition of PGE_1-stimulated cAMP accumulation. A) NG108–15, B) N18TG2, C) N4TG1, and D) N1E-115 cells were cultured for 48 hr in the absence (○) and presence (●) of 200 mM ethanol, washed, and assayed as described. cAMP accumulation in the absence of etorphine was normalized to 100%. Data are from representative experiments; mean values for IC_{50} and maximal inhibition appear in Table 2.

TABLE 2

Etorphine binding and inhibition of PGE_1-stimulated cAMP accumulation in control and ethanol-treated cells

Cells were cultured for 48 hr in the absence and presence of 200 mM ethanol, washed, and assayed as described in Table 1. K_d values for etorphine competition of binding were calculated by the computer program LIGAND using a one-site model and the K_d values for [^{3}H]DPN from Table 1. IC_{50} values for etorphine inhibition of PGE_1-stimulated cAMP accumulation were calculated by log-logit analysis of inhibition curves. Each value represents the mean ± SE for the number of determinations listed in parentheses. Student's *t* test comparing values for control and ethanol-treated cells.

Cell line and condition	K_d (binding)	IC_{50} (cAMP)	K_d/IC_{50}	Max. inhib.
	nM	nM		%
NG108-15				
Control	7.9 ± 0.9 (4)	4.5 ± 0.9 (7)	1.8	62.2 ± 2.0 (7)
Ethanol	8.7 ± 0.9 (4)	2.7 ± 0.6 (7)	3.2	70.3 ± 2.0 (7)[a]
N18TG2				
Control	10.4 ± 4.9 (3)	2.9 ± 0.5 (4)	3.5	40.0 ± 4.6 (7)
Ethanol	9.8 ± 1.6 (3)	0.9 ± 0.2 (4)[b]	10.8	46.5 ± 3.8 (8)
N4TG1				
Control	13.5 ± 1.2 (5)	3.0 ± 0.3 (8)	4.5	71.7 ± 3.3 (8)
Ethanol	13.9 ± 1.5 (4)	1.6 ± 0.3 (7)[b]	8.7	70.5 ± 5.0 (7)
N1E-115				
Control	5.8 ± 0.4 (6)	4.0 ± 0.5 (9)	1.5	49.0 ± 2.0 (9)
Ethanol	7.3 ± 0.7 (6)	2.0 ± 0.4 (6)[b]	3.7	43.0 ± 5.0 (8)

[a] $p < 0.05$.
[b] $p < 0.01$.

of multiple transcripts in the four cell lines (Fig. 5). The strongest signal appeared in two bands of approximately 9.1 and 7.5 kb. The abundance of DOR mRNA did not correlate with constitutive levels of [^{3}H]DPN binding. Levels of DOR mRNA were approximately equal in NG108–15, N4TG1, and N1E-115 cells, despite the fact that [^{3}H]DPN binding differed more than 3-fold among these cell lines. Conversely, levels of DOR mRNA were considerably lower in N18TG2 cells than in the other cell lines, even though [^{3}H]DPN binding was higher in N18TG2 cells than in N4TG1 and N1E-115 cells. These observations indicate that DOR expression is regulated by both transcriptional and post-transcriptional mechanisms.

Ethanol caused a large, dose-dependent increase in the abundance of the 9.1- and 7.5-kb DOR transcripts that differed significantly among the four cell lines (9.1 kb: $F = 11.54$, $p = 0.0001$; 7.5 kb: $F = 8.75$, $p = 0.0004$) (Fig. 6). The increase in the 9.1- and 7.5-kb transcripts induced by 200 mM and 100 mM ethanol was significantly less in N18TG2 cells than in the remaining cell lines (Fig. 7), consistent with the smaller effect of ethanol on DOR up-regulation in N18TG2 cells. For each cell line, ethanol caused a much larger increase in the abundance of DOR mRNA than in levels of [^{3}H]DPN binding. For example, treatment of NG108–15, N4TG1, and N1E-115 cells with 200 mM ethanol for 48 hr increased DOR mRNA by almost 5-fold, yet it increased [^{3}H]DPN binding by approximately 2-fold. Similar treatment of N18TG2 cells increased the levels of DOR mRNA and [^{3}H]DPN binding by approximately 3-fold and 1.4-fold, respectively. A similar disparity between induction of DOR mRNA and [^{3}H]DPN binding was observed after treatment with lower concentrations of ethanol. These observations suggest that the effect of increased DOR gene expression on DOR levels may be mitigated by concomitant, and perhaps compensatory, changes in mRNA translation or receptor processing. Ethanol did not consistently affect the remaining DOR transcripts, although their lower abundance made quantitative analysis difficult.

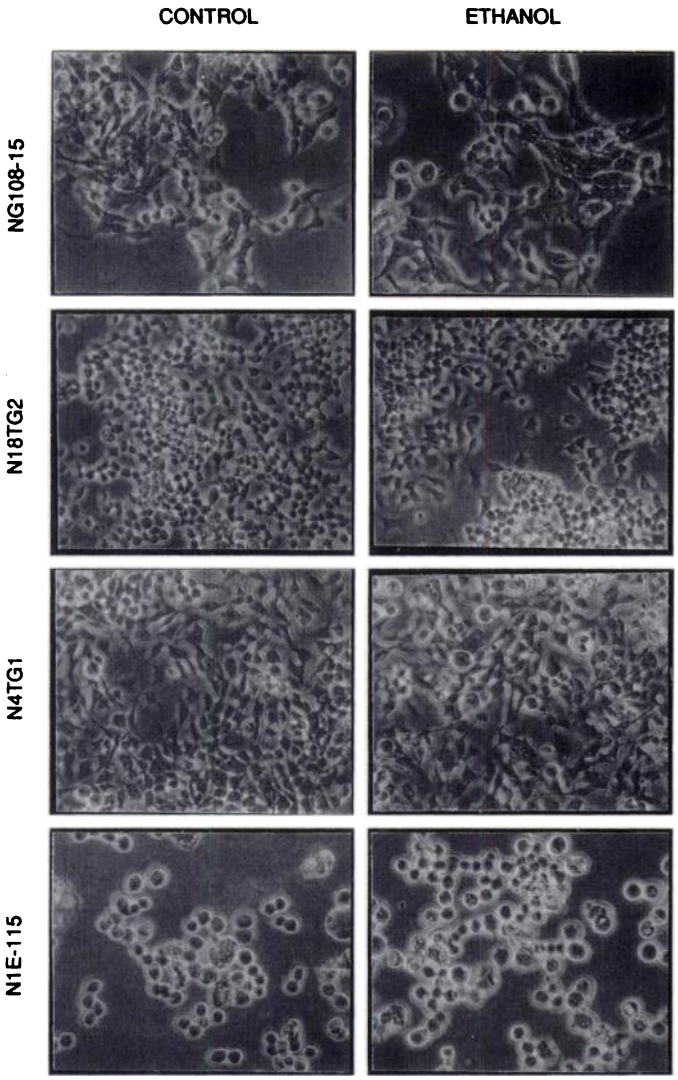

Fig. 4. Morphology of cells cultured in the absence and presence of 200 mM ethanol for 48 hr. Cells were plated at a low density on poly-D-lysine coated plastic trays and cultured for 48 hr in serum-containing medium in the absence and presence of 200 mM ethanol. Representative fields were photographed under phase contrast microscopy. Ethanol did not induce neurite formation in any of the cell lines.

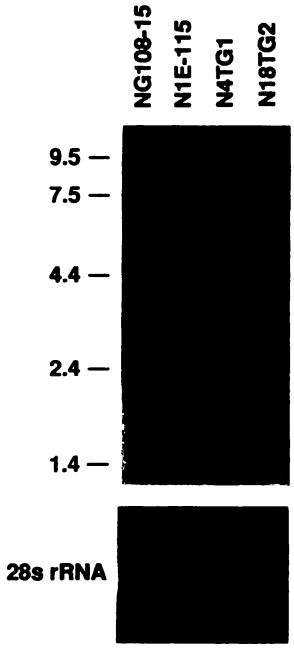

Fig. 5. Northern blot analysis of DOR gene expression in four clonal cell lines. Twenty micrograms of total RNA from the indicated cell lines were separated on 1.2% agarose gels, transferred to nylon membranes, and hybridized with the DOR cDNA probe DKI4. Equal loading of the gels was verified by staining with acridine orange, shown below the autoradiograph. The filters were exposed to Kodak X-AR film with two enhancing screens for 7 days at −70°.

Discussion

The major finding of this study is that ethanol differentially regulates DOR gene expression in neuronal cell lines. This is the first demonstration of regulation of DOR gene expression. Our findings establish that ethanol-induced up-regulation of the DOR does not depend on a genetic contribution from C6BU cells to the NG108–15 hybrid cell line. Rather, it is a general phenomenon in neuronal cell lines. Chronic ethanol administration variably increases enkephalin binding in rodent brain (13, 31–33). However, interpretation of these studies is complicated by differing experimental conditions, the co-existence in brain of multiple opioid receptor subtypes, and the use of nonselective ligands. *In situ* hybridization with specific DOR gene probes may help define the effects of ethanol on DOR expression in mammalian brain.

The effects of ethanol vary in different brain regions (34) and among different individuals (35). We have shown that responses to ethanol also vary among related clonal neural cell lines, in which molecular mechanisms of adaptation can be readily studied. Long-term ethanol treatment induces heterologous desensitization of adenylyl cyclase through regulation of different G proteins in NG108–15 and N1E-115 cells. By contrast, ethanol does not induce heterologous desensitization or regulate G proteins in N18TG2, the parental cell line of NG108–15 cells (21). Our present data indicate that N18TG2 cells are also less sensitive than several other neuronal cell lines to the induction by ethanol of DOR binding and DOR gene expression. Understanding the molecular basis for this differential induction by ethanol of cellular adaptation may provide insight into the heritable component of alcoholism (35).

Constitutive DOR expression, receptor-effector coupling, and maximal opiate inhibition of cAMP accumulation differed among the four cell lines, yet none of these factors clearly accounted for the differential effects of ethanol on DOR expression. If there is a biologic limit to receptor expression, one might expect greater induction of the DOR in cell lines exhibiting the lowest constitutive DOR expression. However, whereas constitutive receptor expression was highest in NG108–15, intermediate in N18TG2, and lowest in N4TG1 and N1E-115 cells, ethanol-induced up-regulation of the DOR was greater in NG108–15 cells than in N18TG2 cells and equal in NG108–15 and N4TG1 cells. The magnitude of receptor induction was likewise unrelated to the efficiency of receptor-effector coupling or the maximal response to etorphine (Tables 1 and 2).

Constitutive opioid receptor expression among the four cell lines did correlate strongly with basal levels of cAMP. Moreover, long-term ethanol treatment significantly increased basal cAMP levels in all of the cell lines but N1E-115, suggesting

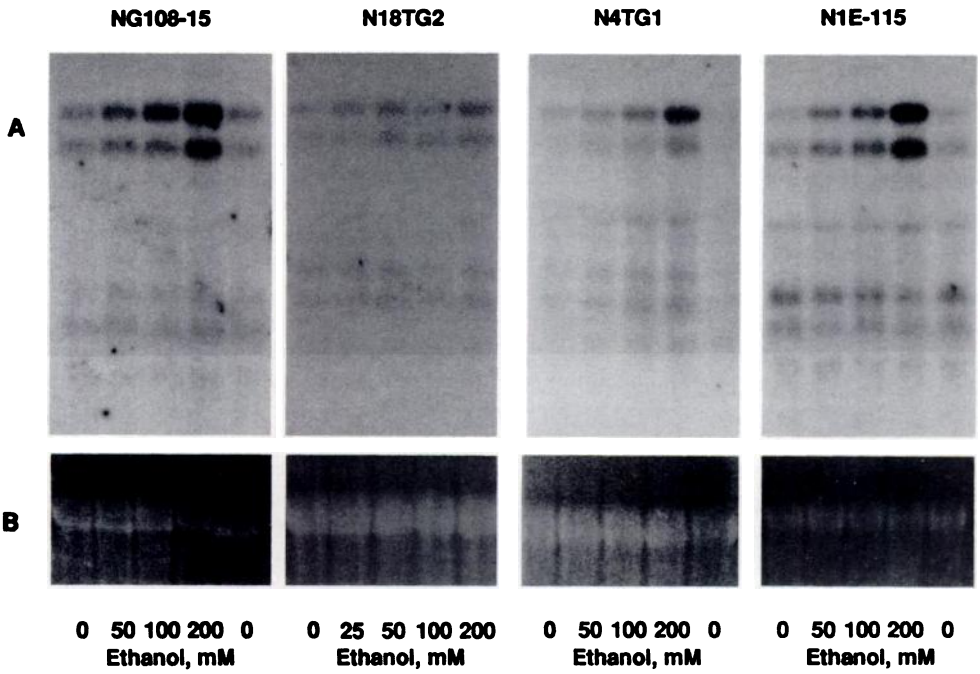

Fig. 6. Ethanol induction of DOR gene expression in 4 neuronal cell lines. Cell lines were cultured with the indicated concentrations of ethanol for 48 hr. Total RNA was purified, separated on 1.2% agarose gels, and stained with acridine orange. After destaining, the RNA was transferred to nylon membranes and hybridized with a full length cDNA probe for the DOR as described (see Methods). Complete transfer of the RNA was verified by restaining the gels after transfer. A) Representative autoradiographs from one of 3 to 4 independent experiments in each of the indicated cell lines. B) Photographs of acridine orange-stained gels from the same experiments shown above. Only the 28s rRNA band is shown.

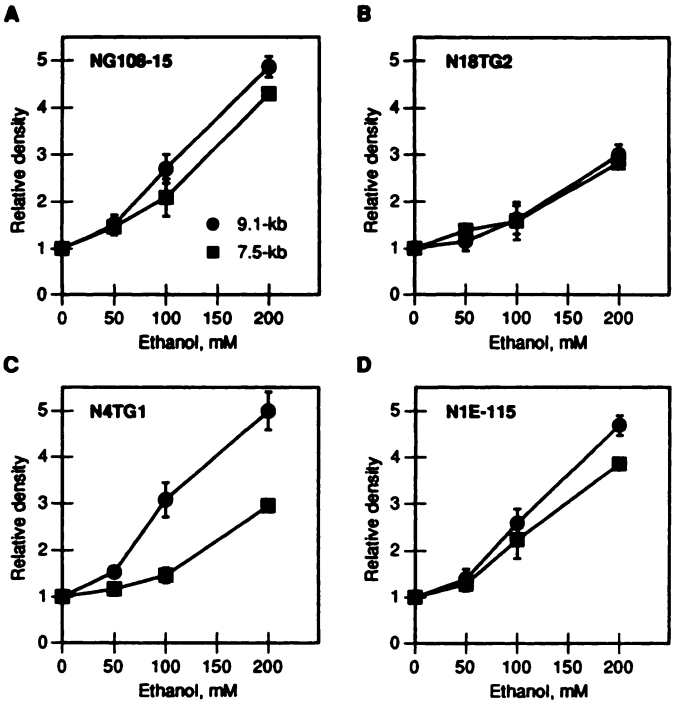

Fig. 7. Quantitative analysis of DOR gene expression. The density of the 9.1- and 7.5-kb bands was determined by computerized scanning densitometry. In each experiment, the optical density of bands from ethanol-treated cells was normalized to values obtained from control cells run on the same gel. Shown is the mean ± standard error relative density of bands from ethanol-treated cells from 3 to 5 independent experiments.

that cAMP may play a role in ethanol-induced up-regulation of the DOR in selected cell lines. Some (29), but not all (36), investigators have found that the membrane-permeable cAMP analog dibutyryl cAMP induces DOR expression and cellular differentiation in NG108–15 cells. The recent report that forskolin transiently increases and then decreases DOR expression in NG108–15 cells (37) indicates that the relationship observed in the present study between DOR expression and cAMP levels is likely to be complex. Under our experimental conditions, ethanol did not promote neurite extension or increase cell protein in any of the cell lines. Although 200 mM ethanol slightly reduced cell growth, lower concentrations of ethanol had no effect on cell growth, yet still increased DOR gene expression. These data suggest that ethanol does not regulate DOR gene expression by reducing cell proliferation or inducing cellular differentiation. However, we cannot entirely rule out the possibility that ethanol regulates DOR gene expression through toxic effects or subtle changes in the cell cycle.

Up-regulation of the DOR by ethanol was associated with an increase in the physiologic response to etorphine, an opiate agonist. Ethanol increased the potency of etorphine in inhibiting PGE_1-stimulated cAMP accumulation in all four cell lines and increased the maximal response to etorphine in NG108–15 cells. NG108–15 cells also express α_2-adrenergic and muscarinic acetylcholine receptors that inhibit adenylyl cyclase through coupling to pertussis toxin-sensitive G proteins (38). Long-term ethanol treatment differentially increases α_2-adrenergic (2.8-fold) and muscarinic acetylcholine receptor (1.4-fold) expression and proportionately increases the potency of α_2-adrenergic (oxymetazoline, 3.7-fold) and muscarinic acetylcholine receptor (carbachol, 1.8-fold) agonists in inhibiting cAMP accumulation (17). These observations suggest that ethanol increases agonist potency by increasing receptor expression. Maximal etorphine inhibition ranged from 40% in control N1E-115 and N18TG2 cells to 75% in N4TG1 and NG108–15 cells. The failure of DOR up-regulation to increase this maximal response in N1E-115 and N18TG2 cells suggests that the intrinsically small response to opiates in these cells is determined by events downstream from receptor activation.

Northern blot analysis of brain and neuronal cell lines reveals multiple DOR transcripts (22). Treatment of NG108–15 cells with clinically relevant concentrations of ethanol caused a large, dose-dependent increase in the abundance of the 9.1- and 7.5-kb transcripts. Because the increase in DOR expression

induced by ethanol was associated with an increase in the potency of opiates in inhibiting adenylyl cyclase, this implies that one or both of these transcripts encode proteins that recognize opioid ligands and couple to $G\alpha_{i2}$. Effects on other transcripts were not observed consistently, but their lower abundance makes it difficult to conclude that these are not also regulated by ethanol. The increase in DOR gene expression was much larger than that for DOR binding (Table 2), suggesting that ethanol may also regulate DOR processing or degradation. We previously determined the effect of ethanol on DOR degradation by treating NG108–15 cells with cycloheximide in the absence and presence of ethanol. Although ethanol did not decrease the rate of DOR degradation over the first 12 hr of cycloheximide treatment, after 18 hr, DOR binding was actually lower in ethanol-treated cells than in controls (9). It is therefore possible that NG108–15 cells compensate for ethanol-induced increases in DOR gene expression by accelerating receptor degradation.

Long-term treatment with ethanol induces or represses the expression of a large number of genes in NG108–15 cells, as determined by two-dimensional gel analysis of *in vitro* translation products (39). We found that the effects of ethanol were relatively selective among a subgroup of genes encoding proteins that regulate adenylyl cyclase activity (17). Proteins in the G_i family couple α_2-adrenergic, muscarinic acetylcholine, and DORs to inhibition of adenyly cyclase, whereas G_s mediates the stimulation of adenylyl cyclase by PGE_1 and adenosine A_2 receptors (38). Ethanol increased α_2-adrenergic (17) and DOR gene expression more than 3-fold, but had only modest effects on the m4 muscarinic acetylcholine receptor gene and no effect on the $G\alpha_s$ and $G\alpha_{i2}$ genes (17). The concurrent regulation by ethanol of the DOR and α_2-adrenergic receptor genes is of interest because these receptors are co-localized on selected neuronal populations where they mutually influence neuronal activity through convergent signaling pathways (40, 41). The need for certain neurons to integrate information from α_2-adrenergic and opioid neurotransmitter systems may have led to the evolution of common mechanisms of α_2-adrenergic and DOR regulation. This interaction may be clinically relevant, because the α_2-adrenergic receptor partial agonist clonidine can reduce the symptoms of both opiate and ethanol withdrawal (42, 43).

DORs are subject to both homologous and heterologous regulation. Exposure of NG108–15 cells to DOR agonists causes homologous down-regulation of the DOR (44, 45). Conversely, opioid antagonists induce homologous up-regulation of the DOR in neuronal cell lines, primary neuronal cultures, and intact animals (9, 46, 47). The DOR also undergoes heterologous up-regulation in response to sodium butyrate (48), ethanol, and conditioned medium from NG108–15 or NCB-20 cells (49). Homologous regulation of the DOR occurs in the presence of cycloheximide, whereas heterologous regulation by ethanol and butyrate requires new protein synthesis (9, 48).

The mechanism by which ethanol up-regulates DOR gene expression is unknown. Although neural cell lines synthesize enkephalins, it does not appear that ethanol increases receptor expression by blocking agonist-induced down-regulation of the DOR (9). Different short-chain alcohols increase DOR expression as a function of chain length and membrane lipid solubility (8), hence, it is likely that receptor up-regulation is initiated by perturbations in the hydrophobic portion of membrane proteins. Ethanol up-regulates the DOR in pertussis toxin-treated cells, indicating that the response is not triggered by changes in signaling through G_i- and G_o-coupled receptors (17). However, short-term treatment of NG108–15 cells with ethanol does increase cAMP levels by blocking the reuptake of adenosine (50), and long-term treatment also increases the activity of protein kinase C (51). Taken together, these data suggest that ethanol-induced perturbations in membrane proteins produce changes in second messengers that in turn regulate DOR gene expression.

Although the clinical presentations of ethanol and opiate intoxication are distinct, there is evidence that endogenous opioid systems may mediate some effects of ethanol. Microinjection of the selective DOR antagonist ICI 174864 into discrete brain regions blocks ethanol-induced hypothermia and sedation (7). Moreover, opiate antagonists attenuate the ethanol withdrawal syndrome when given during and after the administration of ethanol (3, 5, 52). Voluntary ethanol consumption correlates inversely with brain [Met]enkephalin levels in inbred strains of mice (4) and can be suppressed by enkephalinase inhibitors (6, 53), which prolong the actions of enkephalins. In up-regulating the DOR gene, ethanol could compensate for constitutive deficiencies of endogenous opioids that contribute to ethanol self-administration. Conceivably then, the ability of naltrexone to suppress craving for ethanol and drinking in alcoholics (54) may result in part from homologous up-regulation of the DOR.

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