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Ethanol Withdrawal Seizures Produce Increased c-*fos* mRNA in Mouse Brain

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SUMMARY

mRNA levels for the protooncogene c-*fos*, measured by Northern blot analysis, were greatly increased in brains of mice undergoing ethanol withdrawal seizures. This increase was transient (levels were increased at the time of the seizure and returned to normal by 24 hr or less after seizure) and was larger in hippocampus (40-fold) than in cerebral cortex (10-fold) or in cerebellum (6-fold). In mice that were fed ethanol chronically and withdrawn but that did not undergo overt withdrawal seizures, c-*fos* mRNA levels were not significantly increased. The findings with ethanol withdrawal seizures are similar in many respects to results of earlier studies with chemically induced seizures or kindling, which had led to the suggestion that c-*fos* expression may play a role in neuronal adaptation. The development of ethanol withdrawal seizures has been likened to kindling, and there is evidence indicating that ethanol withdrawal symptoms become more severe after repeated episodes of withdrawal. The present data support the hypothesis that this phenomenon may involve ethanol withdrawal seizure-induced increases in c-*fos* expression in various brain areas.

The protooncogene c-*fos* is the normal cellular homologue of the oncogene found in murine osteosarcoma viruses (1–3). The c-*fos* gene encodes a phosphoprotein that, in combination with the product of another protooncogene, c-*jun*, can bind to DNA (4) and may thus influence the transcription of other gene products. In many cell types, c-*fos* expression is rapidly stimulated by growth factors and other mitogens, suggesting that the protooncogene is involved in cell growth (5–7). In neural cells, c-*fos* expression is increased by depolarization and application of neurotransmitters (8, 9), and these observations have led to the hypothesis that the c-*fos* gene product may be involved in the changes that underlie long term adaptive responses in the adult central nervous system. (8, 9).

It has been reported by several investigators that chemical- and lesion-induced seizure activity is associated with increases in c-*fos* mRNA and c-*fos* protein in hippocampus and other limbic areas, as well as in certain cerebral cortical areas (10–14). Furthermore, electrical stimulation that led to an increase in sensitivity of the brain to future seizure-inducing stimuli (i.e., kindling) produced a large increase in c-*fos* protein immunoreactivity in granule cells of the dentate gyrus and in other limbic areas (15). It was suggested in the latter study that the increased c-*fos* expression, which was transient, might serve to couple the electrical stimulus to the long term changes in the central nervous system that result in kindling.

One of the severe symptoms of ethanol withdrawal in animals and humans is the appearance of withdrawal seizures (16, 17). There is some evidence that ethanol withdrawal symptoms become more severe with repeated episodes of ethanol ingestion and withdrawal (18–20), and it has been proposed that, in this sense, generation of ethanol withdrawal seizures may be similar to a kindling process (21). In the present study, we examined c-*fos* mRNA levels in brains of mice undergoing ethanol withdrawal and withdrawal seizures.

Materials and Methods

Adult male C57BL/6NCR mice (20–25 g) were obtained from the National Cancer Institute (Frederick, MD) and were housed four per cage under controlled environmental and lighting (12-hr light/dark cycle) conditions for at least 1 week, with food and water available *ad libitum*, before being used in experiments. For chronic ethanol treatment, mice were individually housed and fed a liquid diet for 7 days (16). Experimental animals received liquid diet composed of chocolate-flavored Slender (Carnation Corporation, Los Angeles, CA), vitamin supplement (3 g/liter diet; ICN Inc., Cleveland, OH), and 7% (v/v) ethanol. Control animals were pair-fed a diet in which sucrose (108.3 g/liter equicalorically replaced the ethanol (16). The ethanol-fed mice drank 11.9 ± 1.5 g (n = 124; range, 7.5–18.7 g) of diet per day, which is similar to earlier published data (15, 21). With the consumption of this quantity of ethanol-containing diet, the mice were reported to maintain blood ethanol concentrations of 100–300 mg/100 ml (16). At the end of the 7-day ethanol ingestion period, all animals were given the control liquid diet (withdrawal). Withdrawal signs (16) were monitored hourly for the first 3 hr after withdrawal and every 30 min thereafter, up to 12 hr after withdrawal, or until the time of sacrifice

ABBREVIATIONS: SDS, sodium dodecyl sulfate; MOPS, 3-[*N*-morpholino]propanesulfonic acid; SSC, saline sodium citrate; NMDA, *N*-methyl-D-aspartate.

(see below). Remaining mice were also examined for withdrawal signs at 24 and 48 hr after withdrawal. Two groups of ethanol-fed mice were generated in these studies. The first group consisted of animals that did not undergo spontaneous or handling-induced seizures during the first 12 hr after withdrawal. These mice were sacrificed (by decapitation) at the time of withdrawal or at 4, 8, 10, 24, or 48 hr after withdrawal. The other group of mice included those that displayed spontaneous (clonic-tonic) or handling-induced seizures. These mice were sacrificed at the time of seizure or at various times thereafter (see Fig. 2). Control mice were sacrificed at the time of withdrawal of the ethanol-fed mice.

Tissue preparation and Northern blot analysis. Mice were sacrificed by decapitation, the brains were quickly removed and the cerebral cortex, cerebellum, and hippocampus were dissected (22), frozen on dry ice, and stored at −70° until further use. Total RNA was extracted using the following modifications of a method described by Cathala *et al.* (23). Tissue from each mouse was homogenized with a Polytron (Brinkmann, Westbury, NY) for 10 sec in 700 μl of homogenizing buffer [5 M guanidine monothiocyanate containing 10 mM EDTA, 50 mM Tris-HCl, pH 7.5, and 8% (v/v) β-mercaptoethanol]. Total RNA was precipitated overnight at 4° by addition of 3.5 ml of 4 M lithium chloride and RNA was collected by centrifugation at 11,000 × *g* at 4° for 90 min. The RNA pellets were solubilized in 10 mM Tris·HCl, pH 7.5, containing 1% SDS and 1 mM EDTA. After phenol/chloroform (1:1, v/v) extraction, the total RNA was precipitated with 2.5 volumes of absolute ethanol and 0.1 volume of 4.5 M sodium acetate. The samples were stored for 14–18 hr at −20°, and the total RNA was pelleted by centrifugation at 11,000 × *g* for 30 min at 4°. The RNA pellet was dissolved in 50 μl of denaturation buffer [1× gel running buffer (20 mM MOPS, pH 7.0; 5 mM sodium acetate, pH 6.0; and 1 mM EDTA), 50% (v/v) formamide (Fluka, Ronkonkoma, NY), and 2.2 M formaldehyde] and heated to 65° for 5 min, and the RNA (10 μg/lane) was size-fractionated on a formaldehyde/agarose gel (1% agarose containing 1× gel running buffer and 2.2 M formaldehyde) at 65 V for 10–15 min and 100 V for 4–6 hr (24, 25). The gels were then stained in 1× gel running buffer containing 2 μg/ml ethidium bromide (Sigma Chemical Co., St. Louis, MO), for 15 min at room temperature, and destained overnight in 1× gel running buffer. After the gels were photographed under UV light, the RNA was electrotransferred onto a Gene Screen membrane (New England Nuclear-Dupont, Boston, MA) in 25 mM sodium phosphate buffer, pH 6.5, at 4° (15 V, overnight). Completeness of transfer was assessed by examining the gels and blots under UV light following the transfer. In some instances, negative photographs of blots were compared with those of gels taken before transfer, and densitometry revealed no substantial differences in the quantity of 28 S or 18 S RNA (i.e., transfer was complete). The blots were baked at 80° for 2 hr in a vacuum oven and were incubated for 2 hr at 42° in prehybridization buffer [50% (v/v) formamide; 2× SSC (1× SSC contains 0.15 M NaCl and 0.015 M trisodium citrate, pH 7.0); 40 mM sodium phosphate buffer, pH 6.5; 200 μg/ml tRNA (Baker's yeast) (Boehringer-Mannheim Corporation, Indianapolis, IN); 50 μg/ml herring sperm DNA (Boehringer-Mannheim); 0.8× Denhardt's solution; 10 mM EDTA; and 0.1% SDS]. Hybridization was carried out overnight at 42° in prehybridization buffer containing 10% (w/v) dextran sulfate (Sigma) and 10^6 cpm/ml ^{32}P-labeled *Sst*I/*Bam*HI restriction fragment (5.1 kilobases) of the mouse c-*fos* gene (Lofstrand Laboratories, Gaithersburg, MD). After hybridization the Northern blots were washed twice with 2× SSC for 10 min at room temperature and twice with 0.1× SSC/0.1% SDS for 30 min at 65°. The blots were then exposed (usually for 24 hr) to Kodak XAR-2 X-ray film in cassettes with intensifying screens. RNA extracted from a given brain region of control mice and mice from each of the two ethanol-fed groups was run simultaneously on gels by an investigator who was blind to the treatment. The RNA on these gels was also transferred at the same time, and the blots were hybridized simultaneously, using the same batch of probe.

After removal of the c-*fos* probe by washing the blots in a solution of 96% formamide containing 10 mM Tris, pH 7.6, and 10 mM EDTA, pH 8, some blots were hybridized with a 2.2-kilobase *Eco*RV/*Hind*III restriction fragment of c-*myc* and, following a second wash, with a 4.6-kilobase *Xho*I/*Sph*I restriction fragment of c-HA-*ras*, both labeled with ^{32}P by nick translation (Lofstrand Laboratories). In addition, blots were hybridized with an *Sca*I/*Bgl*II restriction fragment of cardiac β-actin cDNA (26) labeled with ^{32}P by nick translation, in order to quantitate messenger RNA applied in each lane. The autoradiograms were quantitated by densitometric scanning using a Gilford spectrophotometer. The integrity of RNA was assessed and the RNA was quantitated densitometrically from negative photographs of ethidium bromide-stained gels (25). The measurements of 28 S RNA and actin mRNA in several brain areas showed a significant positive correlation ($r = 0.7$, $n = 9$). Because it is possible that actin mRNA levels could be altered by chronic ethanol ingestion and/or withdrawal seizures, the quantity of mRNA was expressed on the basis of 28 S RNA levels.

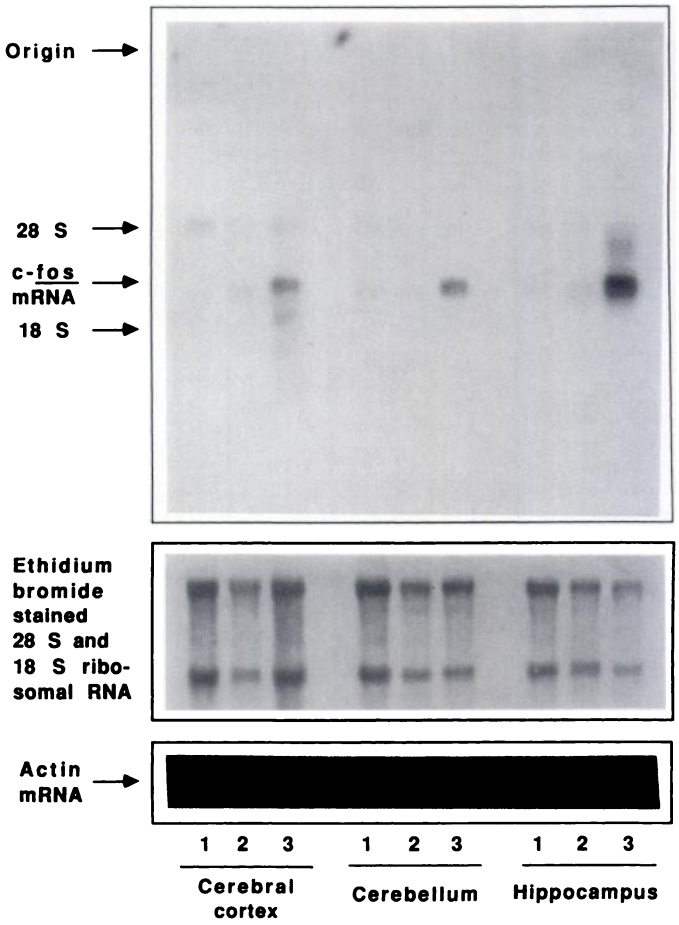

Fig. 1. Autoradiograms of Northern blots of c-*fos* mRNA in cerebral cortex, hippocampus, and cerebellum of the C57BL/6 mouse. Mice were fed ethanol in a liquid diet or control liquid diet for 7 days, as described in the text, and then all mice were given control diet (withdrawal). Control and ethanol-fed mice were sacrificed at the time of withdrawal (*lanes 1* and *2*) or the ethanol-withdrawn mice were sacrificed at the time that a withdrawal seizure occurred (*lane 3*). Brains were rapidly removed and dissected, RNA from individual brains was isolated and fractionated on agarose gels (10 μg of RNA applied/lane), and the resulting blots were probed for c-*fos* mRNA with a ^{32}P-labeled *Sst*I/*Bam*HI restriction fragment of the mouse c-*fos* gene (*upper*). After washing, the blot was rehybridized with a ^{32}P-labeled *Sca*I/*Bgl*II restriction fragment of cardiac β-actin cDNA (*lower*). *Middle*, a negative photograph (taken under UV light) of the ethidium bromide-stained gel (18 S and 28 S RNA). The blot pictured here displays mRNA from individual representative animals in a single experiment. The experiment was repeated once with similar results (see Fig. 2).

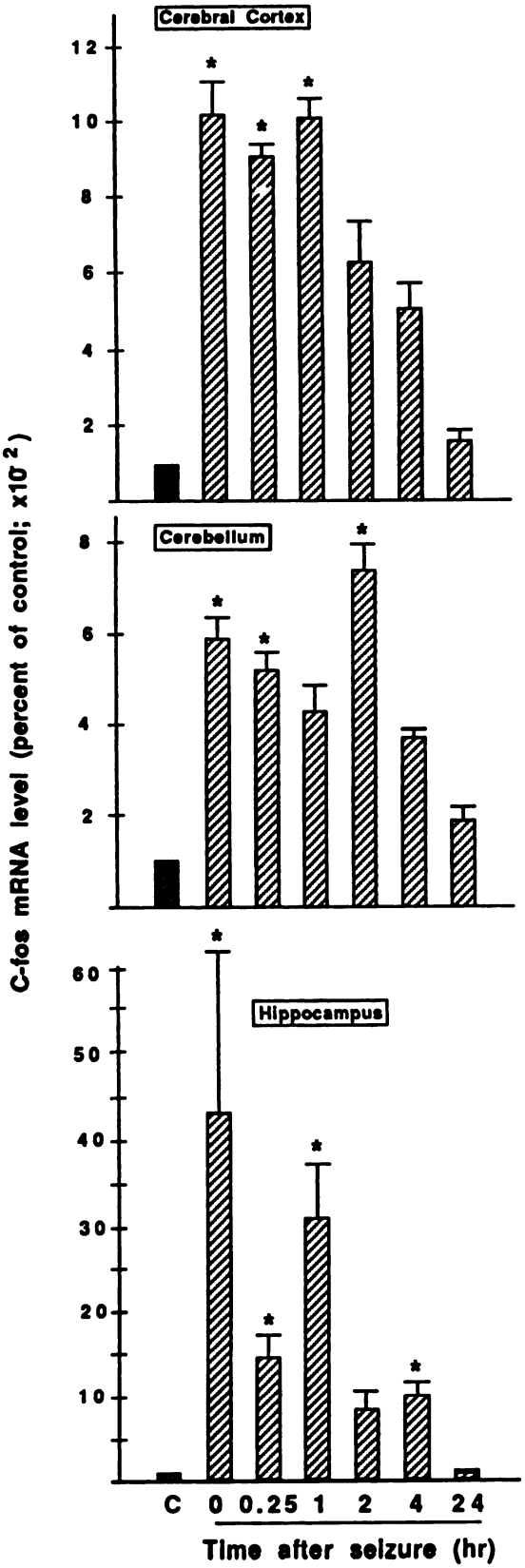

Fig. 2. Time course of changes in levels of c-*fos* mRNA in brains of ethanol-withdrawn mice that have undergone withdrawal seizure. Mice were fed ethanol chronically and withdrawn, withdrawal symptoms were monitored, and c-*fos* mRNA was quantitated by Northern blot analysis, as described in the text. The data in this figure were obtained in two independent experiments from mice that displayed spontaneous or handling-induced overt ethanol withdrawal seizures. In each experiment, RNA from at least two animals at each time point was analyzed. Values from control (C) mice ($n = 6$) were set to 100% and values for mice undergoing seizures ($n = 3$ or 4 at each time point) represent percentage of values in control mice (mean ± standard error). Note difference in scale of the *ordinate* in each *panel*. *$p < 0.05$, compared with controls (Kruskal-Wallis and Mann-Whitney *U* test).

Preliminary experiments using serial dilutions of RNA from hippocampus, cortex, and cerebellum of control mice showed that measurements of c-*fos* mRNA quantitated as described gave linear results (r = 0.96–1.0, depending on brain area) over a concentration range of 2.5 to 12.5 μg of RNA. In addition, RNA extracted from the hippocampus of ethanol-withdrawn mice at the time of seizure, when c-*fos* mRNA was greatly increased (see below), was diluted 1:10 or 1:20 before being run on gels. When the autoradiograms obtained from these gels were quantitated and the values were corrected for dilution, similar increases in c-*fos* mRNA were found for all samples (undiluted, 43-fold; 1:10 dilution, 38-fold; 1:20 dilution, 46-fold). These results indicated that the gels and autoradiograms were not overloaded or overexposed when 10 μg of RNA were used.

Statistical analysis. Data were analyzed by the Kruskal-Wallis test, and means were compared by the Mann-Whitney *U* test, using the SAS/STAT Guide for Personal Computers. Values of $p < 0.05$ were considered to be significant.

Results

Most mice fed the ethanal-containing diet for 7 days displayed withdrawal signs, which varied in severity among the animals (16), upon cessation of ethanol intake. The mice that were included in the group that did not undergo withdrawal seizures showed little or only mild withdrawal symptomatology (e.g., occasional muscle spasms) until the time of sacrifice. Mice that showed more severe withdrawal signs, which progressed to clonic-tonic seizures, were included in the group that underwent seizures. Overt withdrawal signs had dissipated in all remaining mice by 24 hr after withdrawal.

Brain levels of c-*fos* mRNA were low in control mice. However, mice that displayed withdrawal seizures had large increases in c-*fos* mRNA levels in cortex, hippocampus, and cerebellum (Figs. 1 and 2), when they were sacrificed at the time that seizures were first evidenced. There was no obvious difference in c-*fos* mRNA levels between mice that had spontaneous seizures and those that demonstrated handling-induced seizures.

Fig. 2 shows the time course of changes in c-*fos* mRNA in brains of mice that displayed overt withdrawal seizures. The majority of these seizures (approximately 70%) occurred between 5 and 8 hr after withdrawal. The mice were sacrificed at the indicated times after the seizures were observed. The greatest increase in c-*fos* mRNA levels occurred in the hippocampus, where there was an approximately 40-fold increase at the time of the withdrawal seizure. Levels of hippocampal c-*fos* mRNA were still significantly elevated at 4 hr after the seizure and had returned to baseline at 24 hr. In cerebral cortex, the change in c-*fos* mRNA was not as great as in hippocampus (i.e., approximately 10-fold) and levels of c-*fos* mRNA were no longer significantly different from those in controls by 2 hr after the seizure. The increase in cerebellar c-*fos* mRNA levels was less than that in the other brain areas, and levels had returned to control values by 4 hr after seizure.

The levels of c-*fos* mRNA were also measured in brains of ethanol-fed mice that did not display overt withdrawal seizures.

There was no significant increase in c-*fos* mRNA in any brain area studied up to 48 hr after withdrawal, although levels were slightly increased (1.5- to 2-fold) throughout withdrawal in hippocampus and cortex. In cerebellum, c-*fos* mRNA levels were increased 10- to 15-fold in two animals at 8 hr after withdrawal, whereas there was little or no increase in three other animals (data not shown).

c-*ras* mRNA was present at low levels in brains of control mice, similar to previously reported results (14) (approximately 40–70% of the level of c-*fos* mRNA, depending on brain area) but, in contrast to c-*fos* mRNA, there was no increase in c-*ras* mRNA in brains of ethanol-fed mice that had undergone withdrawal seizures. Levels of c-*myc* mRNA were even lower than those of c-*fos* and c-*ras* mRNA and could not be accurately quantitated in either brains of control mice or of mice that showed withdrawal seizures.

Discussion

The present results demonstrate that ethanol withdrawal seizures, like other types of seizure (10–14), are associated with an increase in mRNA for the protooncogene c-*fos* in brain. A substantial increase in mRNA for c-*fos* was observed in the hippocampus, cerebral cortex, and cerebellum of mice at the time that overt ethanol withdrawal seizures occurred. The greatest increase was in hippocampus and, in all brain areas, the increase in c-*fos* mRNA was transient, in that levels had returned essentially to control values by 24 hr or less after the seizure.

In some ways, the changes in c-*fos* mRNA levels after ethanol withdrawal seizures are similar to changes in c-*fos* mRNA and c-*fos* protein immunoreactivity in brain after other types of seizures. For example, whether seizures were caused by pentylenetetrazole, kainic acid, or kindling stimuli, the increase in expression of c-*fos* was transient, and levels had usually returned to normal by 24 hr after treatment (10–15). Furthermore, following pentylenetetrazole-induced seizures (10) and during kainic acid-induced status epilepticus (13), as well as after ethanol withdrawal seizures, the magnitude of changes in c-*fos* expression varied among brain areas. To some extent, the differential pattern of expression of c-*fos* in various brain areas may reflect the importance of these areas in a particular type of seizure activity. Thus, c-*fos* expression was increased only in certain hippocampal and limbic areas during kainic acid-induced status epilepticus, consistent with the important role of the hippocampal formation in the response to kainate (27), whereas generalized seizures evoked by pentylenetetrazole and, in the current work, by ethanol withdrawal were associated with increased c-*fos* expression in a larger number of brain areas.

One difference between the increase in c-*fos* mRNA associated with ethanol withdrawal seizures, compared with other types of seizure, is that we observed essentially maximum increases in c-*fos* mRNA levels in all brain areas at the time of the overt withdrawal seizure. In contrast, in studies of chemically induced seizures, brain c-*fos* mRNA peaked at about 1 hr after seizure (10) and sequential increases in c-*fos* protein were observed in several brain areas (10, 11, 13), leading to the suggestion (10, 13) that, after seizure, there is a "successive recruitment" of cells expressing c-*fos* in different brain areas. A possible explanation for the difference between the response to ethanol withdrawal seizures and other seizures is the fact that, during ethanol withdrawal in mice, there was a progressive increase in electroencephalograph abnormalities (electroencephalograph slowing and decreased amplitude, single spike events, epileptic seizure discharge) in cortex, thalamus, hippocampus, and septal area (cerebellum was not examined) over a period of several hours (18). These abnormalities occurred before the appearance of overt clonic-tonic seizures (18). Because electrical stimuli can increase c-*fos* expression, as occurs during kindling (15), it is likely that during ethanol withdrawal increases in c-*fos* mRNA occurred because of abnormal electrophysiological events, even before the time of the overt seizure.

Possible mechanisms that have been proposed to explain seizure-induced increases in c-*fos* expression include membrane depolarization and enhanced firing rates of neurons or enhanced activity of neurotransmitters or neuromodulators (10–12). Because induction of c-*fos* in PC12 cells was found to be calcium dependent (8) and calcium accumulates in neurons during seizures (28), the primary event in seizure-induced increases in c-*fos* expression could be the influx of calcium. Recently, ethanol has been found to be a very potent inhibitor of responses mediated by the NMDA receptor (29, 30), and this receptor was reported to be up-regulated in hippocampus of mice that ingested ethanol chronically (31). Biochemical (32) and electrophysiological (33) studies have indicated that activation of NMDA receptors can increase levels of c-*fos* mRNA. In our study, the pattern of increases of c-*fos* mRNA in brains of mice undergoing ethanol withdrawal seizures appeared to parallel the density of NMDA receptor-gated channels in brain (34). Thus, it is possible that the increase in c-*fos* mRNA associated with ethanol withdrawal seizures may, in part, be mediated by NMDA receptor-gated calcium flux.

In addition to increases in neuronal calcium uptake, changes in intracellular calcium from other sources may also contribute to the increase in c-*fos* mRNA observed after ethanol withdrawal seizures. We have previously noted an increase in muscarinic cholinergic receptor sites in hippocampus and cortex of ethanol-fed mice at the time of withdrawal and at 8 hr after withdrawal (35). In addition, in cortex of the ethanol-fed mice, there was an increase in carbachol-stimulated polyphosphoinositide breakdown (36), which would presumably lead to a rise in intracellular calcium accumulation. Because cholinergic agonists can stimulate c-*fos* expression (see Ref. 9), these changes might also contribute to the induction of c-*fos* in certain brain areas of mice undergoing ethanol withdrawal seizures.

As mentioned in the introduction, ethanol withdrawal has been compared to kindling (21), and there is evidence that ethanol withdrawal seizures become more severe with repeated episodes of chronic ethanol ingestion and withdrawal (18–20). The increases in c-*fos* mRNA that are produced by ethanol withdrawal seizures may contribute to the changes in neuronal function that are involved in these phenomena.

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