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Ethanol Inhibits Muscarinic Receptor-Induced Axonal Growth in Rat Hippocampal Neurons

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Abstract

Background—*In utero* alcohol exposure can lead to fetal alcohol spectrum disorders characterized by cognitive and behavioral deficits. *In vivo* and *in vitro* studies have shown that ethanol alters neuronal development. One mechanism through which ethanol has been shown to exert its effects is the perturbation of activated signaling cascades. The cholinergic agonist carbachol has been shown to induce axonal outgrowth through intracellular calcium mobilization, PKC activation, and ERK1/2 phosphorylation. This study investigated the effect of ethanol on the differentiation of rat hippocampal pyramidal neurons induced by carbachol as a possible mechanism involved in the developmental neurotoxicity of ethanol.

Methods—Prenatal rat hippocampal pyramidal neurons were treated with ethanol (50–75 mM) in the presence or absence of carbachol for 24 h. Neurite outgrowth was assessed spectrophotometrically; axonal length was measured in neurons fixed and immunolabeled with the neuron-specific βIII tubulin antibody; cytotoxicity was analyzed using the MTT assay. The effect of ethanol on carbachol-stimulated intracellular calcium mobilization was assessed utilizing the fluorescent calcium probe, Fluo-3AM. The PepTag® Assay for Non-Radioactive Detection of Protein Kinase C from Promega was used to measure PKC activity, and ERK1/2 activation was determined by densitometric analysis of Western blots probed for phospo-ERK1/2.

Results—Ethanol treatment (50–75 mM) caused an inhibition of carbachol-induced axonal growth, without affecting neuronal viability. Neuron treatment for 15 min with ethanol did not inhibit the carbachol-stimulated rise in intracellular calcium, while inhibiting PKC activity at the highest tested concentration and ERK1/2 phosphorylation at both the concentrations used in this study. On the other hand, neuron treatment for 24 h with ethanol significantly inhibited carbachol-induced increase in intracellular calcium.

Conclusions—Ethanol inhibited carbachol-induced neurite outgrowth by inhibiting PKC and ERK1/2 activation. These effects may be, in part, responsible for some of the cognitive deficits associated with *in utero* alcohol exposure.

Keywords

hippocampus; pyramidal cells; muscarinic receptors; neurite outgrowth; ethanol

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Introduction

Maternal alcohol consumption during pregnancy can produce a variety of central nervous system abnormalities resulting in a broad spectrum of cognitive and behavioral impairments that constitute the most severe and long-lasting effects observed in fetal alcohol spectrum disorder (FASD) (Bertrand et al., 2005; Jones and Smith, 1975; Wattendorf and Muenke, 2005). Prenatal alcohol exposure affects several brain regions including the hippocampus, the cerebellum and the neocortex (Berman and Hannigan, 2000; Fabregues et al., 1985; Hammer and Scheibel, 1981; Smith et al., 1986). The hippocampus is the portion of the brain necessary for the formation, storage, and processing of memories, and animal models of FAS provide *in vivo* evidence that prenatal alcohol exposure may cause abnormal hippocampal architecture, including decreased numbers of pyramidal neurons, abnormal axon projections and dendritic arbors, and aberrant hippocampal electrophysiology (Berman and Hannigan, 2000).

Several *in vivo* studies have investigated the effect of *in utero* alcohol exposure on neuronal development. Prenatal ethanol exposure has an inhibitory effect on dendritic arbor size in the hippocampus, neocortex, and cerebellum of rodents (Davies and Smith, 1981; Hammer and Scheibel, 1981; Smith and Davies, 1990; Smith et al., 1986), and causes a decrease in dendrite number and branching in guinea-pig cortical layer V pyramidal neurons (Fabregues et al., 1985), chick spinal cord serotonergic neurons (Mendelson and Driskill, 1996), and rat dopaminergic neurons of the substantia nigra (Shetty et al., 1993). *In vivo* prenatal ethanol exposure has been shown to reduce the size of the hippocampal commissure (Livy and Elberger, 2001), but to increase the number of neurons projecting from rat somatosensory cortex to the spinal cord (Miller, 1987), to increase the total axoplasmic volume in layer V of the somatosensory cortex (al-Rabiai and Miller, 1989), and to increase axonal growth in the rat pyramidal tract (Miller and al-Rabiai, 1994). Additionally, hypertrophic axonal projections have been observed in axons extending from the granule cells of the dentate gyrus to apical dendrites of hippocampal pyramidal neurons (West et al., 1981). The different effects exerted by ethanol *in vivo* may reflect species or strain differences in sensitivity to ethanol or differences in the dose, timing, or route of ethanol administration (Lindsley, 2006).

In vitro studies further support the hypothesis that prenatal ethanol exposure may interfere with the development of neuronal processes. Indeed, ethanol has been shown to increase neurite outgrowth in cerebellar neurons (Zou et al., 1993), and in PC12 cells (Messing et al., 1991a; Roivainen et al., 1993). Only very high concentrations of ethanol inhibit neurite extension and viability of primary culture hippocampal neurons (Heaton et al., 1994). On the other hand, ethanol has been shown to inhibit neurite outgrowth in cerebellar granule neurons (Liesi, 1997), and LA-N-5 human neuroblastoma cells (Saunders et al., 1995), and to decrease neurite outgrowth and dendritic branching in primary cultures of fetal cortical neurons grown in close proximity of glial cells monolayer (Bingham et al., 2004). Ethanol has also been reported to increase the number of minor processes and the number of cells with more than one axon, and to accelerate the development of hippocampal pyramidal neurons in the early phase of development (first 24 h in culture), while inhibiting the development of dendrites and synapses of later stages of development (Clamp and Lindsley, 1998; Yanni and Lindsley, 2000). Furthermore, ethanol strongly inhibits neurite outgrowth mediated by the cell adhesion molecule L1CAM in cerebellar granule cells (Bearer et al., 1999), but not in cortical neurons (Hoffman et al., 2008).

Recently, it has also been reported that ethanol inhibits neurite outgrowth mediated by astrocytes; indeed, neuritogenesis is inhibited in cortical neurons grown in the presence of astrocytes prepared from rats prenatally exposed to ethanol in comparison to neurons incubated with astrocytes from unexposed animals (Pascual and Guerri, 2007). Ethanol also inhibits

axonal growth of cerebellar neurons induced by the active fragment of the astrocyte-released activity-dependent neuroprotective protein (Chen and Charness, 2008). In addition, we have recently reported that the stimulation of muscarinic receptors in astrocytes induces neurotogenesis in hippocampal neurons (Guizzetti et al., 2008) and that this effect is inhibited by ethanol (Guizzetti et al., manuscript in preparation).

Taken together, these studies suggest that ethanol alters neuronal morphogenesis both *in vivo* and *in vitro* with effects that range from inhibition to overstimulation of neuronal differentiation. The variety of effects exerted by ethanol depends on the brain region from which the investigated neurons derive, the developmental stage of neurons, and the presence or absence of glial cells.

In several instances, the effect of ethanol on neuronal development has been ascribed to an interference exerted by ethanol on specific signal transduction pathways. Indeed, it was recently reported that alcohol inhibition of L1CAM-induced neurite outgrowth was due to inhibition of the activation of extracellular signal-regulated kinase (ERK) 1/2 (Tang et al., 2006). Alcohol-induced inhibition of axonal growth stimulated by the active fragment of the activity-dependent neuroprotective protein is due to the inhibition of the tyrosine phosphorylation of Fyn kinase and Crk-associated substrate (Chen and Charness, 2008). Furthermore, inhibition by ethanol of retinoic acid-induced differentiation of human SH-SY5Y neuroblastoma cells is due to inhibition of protein kinase C (PKC) and ERK1/2 signaling (Hellmann et al., 2008).

We have recently reported that the cholinergic agonist carbachol induces axonal growth in prenatal rat hippocampal pyramidal neurons through the stimulation of M1 muscarinic receptors that lead to an increase in intracellular calcium and activation of PKC and ERK1/2 (VanDeMark et al., 2009). In previous studies we have found that ethanol inhibits muscarinic receptor signaling both in vitro and in vivo and that this effect is associated with microencephaly and inhibition of astrocytes proliferation (Balduini and Costa, 1989; Balduini et al., 1994; Costa and Guizzetti, 1999; Guizzetti and Costa, 2000; Guizzetti and Costa, 2002; Guizzetti et al., 2003). In this study, we investigated the hypothesis that ethanol may inhibit the neuritogenic effect of carbachol by inhibiting the signaling pathway activated by cholinergic stimulation in hippocampal neurons.

Materials and Methods

Materials

Time-pregnant Sprague-Dawley rats were purchased from Charles River (Wilmington, MA). Neurobasal-A medium, Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and trypsin were from Invitrogen (Carlsbad, CA). Cell culture inserts and nylon mesh filters were from BD Falcon™ (Franklin Lakes, NJ); glass coverslips were from Fisher Scientific (Federal Way, WA), and plastic coverslips from Nunc™ (Rochester, NY). The antibodies against tubulin, βIII isoform, microtubule-associated protein 2 (MAP2), and Tau1 were purchased from Chemicon® (Temecula, CA). The Alexa Fluor® 488 and 555 secondary antibodies, Hoechst 33342, and Fluo-3AM were purchased from Molecular Probes™ Invitrogen (Carlsbad, CA). The antibodies against phospho-ERK1/2 and ERK1/2 were purchased from Cell Signaling (Danvers, MA). The PepTag® Assay for Non-Radioactive Detection of Protein Kinase C was from Promega (Madison, WI). Ethanol determination kits were purchased from r-Biopharm (Marshall, MI). Carbachol and all other reagents were purchased from Sigma Chemical Company (St. Louis, MO)

Culture of hippocampal neurons

Primary cultures of hippocampal neurons were prepared from 21 day-old rat fetuses as previously described (Brewer et al., 1993; Guizzetti et al., 2008; VanDeMark et al., 2009), with minor modifications. Briefly, a pregnant dam was euthanized with carbon dioxide, and the uterine horns were removed by using a protocol approved by the University of Washington Institutional Animal Care and Use Committee. Fetuses were removed and sacrificed by decapitation. The hippocampi were removed from the cerebral hemispheres, stripped of meninges, placed in Hank's balanced salt solution, cut into small pieces, and treated with papain (2 mg/ml HBSS) in the presence of DNase (40 μg/ml) and $MgCl_2$ (5 mM) for 30 min at 37°C. The tissue was spun down, and resuspended in Neurobasal-A complete media (Neurobasal-A medium supplemented with 10% FBS, 30 mM glucose, 3 mM GlutaMAX, 1% gentamicin, and 0.5% fungizone) and DNase (40 μg/ml). Tissue was further dissociated by repeated passages through a Pasteur pipette, and cells were filtered through a nylon mesh of 40 μm pore size. Cells were then spun down and resuspended in Neurobasal-A complete media. For the neurite extension assays, cells were seeded on cell culture porous inserts at the density 2×10^5 per insert. For morphometric analysis, cells were seeded on round glass coverslips placed in 24 well plates at 1×10^4 cells per coverslip. Inserts and coverslips were coated overnight with 100 μg/ml poly-D-lysine at 37°C. Neurons were allowed to attach in Neurobasal-A complete media for at least 30 min, after which they were switched to astrocyte-conditioned medium (ACM) containing the various treatments for 24 hours. No differences in cell survival were noticed between neurons maintained for 24 hours in ACM compared to cells maintained in Neurobasal-A medium.

For Western blot analysis and PKC activity, cells were plated in 35 mm dishes (2×10^6 cells/dish for Western blot experiments and 5×10^6 cells/dish for PKC activity assays). Cells were allowed to attach overnight in Neurobasal-A complete and then switched to ACM containing the different treatments.

Preparation of Astrocyte-Conditioned Medium

Primary astrocyte cultures were prepared from cerebral cortex of 21-day-old rat fetuses as previously described (Guizzetti et al., 1996; 2008), and maintained for 10–14 days in DMEM/10% FBS. Cells were passed in 100 mm dishes and cultured for 4 days in DMEM/10%FBS. Two days before astrocyte-conditioned medium was needed, astrocytes were switched to DMEM supplemented with 0.1% bovine serum albumin (BSA). After 48 hours, the medium was collected and spun for 10 min at 200 g to remove any debris and floating cells. This medium was utilized for treatment of neurons.

Carbachol and Ethanol Treatments

The general experimental design adopted in this study is the following: hippocampal neurons were incubated in ACM alone (control) or in ACM containing 100 μM carbachol without or with 50 or 75 mM ethanol, or ethanol alone (50 or 75 mM). In neuritogenesis and cytotoxicity experiments, incubations with carbachol and/or ethanol were carried out for 24 h (unless otherwise indicated). The release in intracellular Ca^{++}, an event occurring rapidly after muscarinic receptor stimulation, was measured every 10 sec during the first 200 sec after carbachol addition; PKC activation was measured 15 min after carbachol treatments; ERK1/2 activation was measured after 30 min treatments with carbachol. Ethanol was added to the treatment medium; each plate was treated with only one concentration of ethanol, as ethanol redistributes in all the wells within the same plate uniformly over the course of the incubation. To minimize ethanol evaporation from 24-well plates, water containing ethanol at the same concentration present in the treatments was added in the interwell spaces and to all the wells that did not have cells, and Parafilm was wrapped around the two long sides of the plates closing part of the space between the lid and the dish without totally stopping the air exchange. In order

to reduce ethanol evaporation from dishes during short term exposure to ethanol (less than 1 hour), Parafilm was wrapped around two third of the perimeter of the Petri dishes, closing part of the space between the lid and the dish without totally stopping the air exchange. We found that in these conditions ethanol loss does not exceed 15%, cells do not suffer from lack of oxygen, and no pH changes occur. In our hands, this method of ethanol exposure is comparable to the use of sealed chambers (also in use in our laboratory) for treatments up to 24 h (Guizzetti et al., 2007; Guizzetti and Costa, 1996; Guizzetti and Costa, 2002). Ethanol concentrations at the beginning and the end of the incubation were determined in some experiments using a commercially available kit from r-Biopharm (Marshall, MI).

Neurite Extension Assay

Neurite extension was assessed spectrophotometrically following a previously described method (Guizzetti et al., 2008; Smit et al., 2003; VanDeMark et al., 2009). Briefly, hippocampal neurons were plated on inserts with a 3 μm pore membrane at their base coated with poly-D-lysine; the inserts were then placed into 24-well plates. During the 24 h incubation with carbachol and/or ethanol, neurons extended their neurites through the microporous membrane to the lower chamber. At the end of the incubation, membrane inserts were then removed, neurons were fixed in methanol and neurites were stained with a cresyl violet solution (0.09%) (Jin et al., 2006). Cresyl violet is a synthetic dye that is widely used to stain neuronal tissue. It binds to the acidic components of the neuronal cytoplasm, such as RNA-rich ribosomes, and to the nuclei and nucleoli. Cell bodies are removed from the top of the membrane using a cotton swab and the dye is extracted from the neurites in the underside of the porous inserts and quantified at 562 nm on a spectrophotometer using a SPECTRAmax® PLUS microplate spectrophotometer. Because cell bodies are separated from neurites by the porous membrane, they are easily removed from the top of the membrane while leaving undisturbed the neurites on the lower side of the membrane. By the time cell bodies are removed, cells are not anymore in medium and are fixed in methanol. Because of all these reasons, it is very unlikely that the dye from the cell body will “leak” into the neurites. With this method, neurite outgrowth is not measured in individual cells, but in the whole well and therefore the absorbance value represents an average of all the cells plated..

Immunocytochemistry

In order to measure cell morphology, neurons were plated on coverslips placed in 24-well tissue culture plates. After treatment, the cells were fixed in 4% paraformaldehyde, then permeablized in 0.1% Triton X-100 for 10 minutes at room temperature, and blocked in 3% BSA for 30 minutes. The coverslips were then incubated for at least 18 hours with the neuron-specific mouse anti-tubulin, beta III isoform monoclonal antibody (Chemicon®, Temecula, CA). After primary antibody incubations, coverslips were incubated for 1 hour with Alexa Fluor® 488. Nuclei were then stained with 5 μg/ml Hoescht for 5 minutes and coverslips were mounted onto glass slides with Vectashield® mounting gel (Vector Laboratories, Inc, Burlingame, CA), covered with cover glass (Corning, Acton, MA), and sealed with nail polish.

Morphometric Analysis

The slides were viewed with a fluorescence microscope (Nikon™, Melville, NY) and pictures were obtained using a SPOT-RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). The images were analyzed with the MetaMorph 6.1 software (Molecular Devices, Sunnyvale, CA). Hippocampal neuron analysis was limited to cells that were identifiable as pyramidal, and were not in contact with any other cells. The criteria used for distinguishing pyramidal neurons from non-pyramidal cells and for determining the developmental stage of the pyramidal cell were based on nomenclature described previously (Dotti et al., 1988), used by others (Clamp and Lindsley, 1998), and by us (VanDeMark et al., 2009). Briefly,

hippocampal pyramidal neurons were those with 3 or more extensions after 24 hours in culture. Further characterization into stage 3 pyramidal neurons required a cell body diameter of 10–15 μm, 2–5 undifferentiated neurites, and a single axon with length ≥ 40 μm. In addition, the extensions had to be visible for unambiguous measurement for the duration of the analysis, and neurites that overlapped with other cells were excluded (Lindsley et al., 2003).

MTT assay

Cell viability was measured using Thiazolyl Blue Tetrazolium Bromide (MTT). At the end of ethanol treatments, hippocampal neurons plated in 24-well plates (2×10^5 cells/well) were washed and incubated with 250 μg/ml MTT in PBS for 1 hour at 37°C followed by a 5 min-wash in DMSO to solubilize the MTT-formazan. The absorbance of the MTT-fomazan complex was measured by a spectrophotometer set to detect 562 nm wavelengths.

Intracellular Calcium Mobilization

Changes in the levels of intracellular Ca^{++} were measured as previously described (Giordano et al., 2007; VanDeMark et al., 2009). Briefly, neurons were plated in 35-mm glass bottom dishes and were loaded with the intracellular Ca^{++}-sensitive fluorescent dye Fluo-3/AM (3 μM) for 30 min. Fluo-3 is a fluorescein-based calcium indicator that exhibits large intensity increases in fluorescence upon Ca^{++} binding. In this study the cell-permeable acetoxymethyl (AM) ester form of Fluo-3 was utilized. Once Fluo-3/AM enters the cell, the ester bond is cleaved by endogenous esterases in the cytosol and the dye is trapped within the cytosol as it is no longer cell permeable. At the end of the incubation with Fluo-3/AM, cells were washed and incubated for an additional 30 min in a Fluo-3/AM–free Locke's buffer to remove extracellular traces of the dye and to complete intracellular de-esterification. The cells were then placed on the stage of a fluorescence microscope and the dye was excited with a continuous fluorescence light source (excitation wavelength: 488nm); images of the emitted fluorescence at wavelength 520 nm were captured at 10-s intervals by a charge-coupled device camera (Princeton Instruments, Trenton NJ) using MetaMorph software (Molecular Devices, Sunnyvale, CA). 50 cells in each treatment group were analyzed. Fluorescence measurements were normalized as ΔF/F (Manning and Sontheimer, 1999). Cells were classified as responders or non-responders depending on the size of the carbachol-induced rise in intracellular calcium. Only cells responding with a 10% or greater rise in intracellular calcium were analyzed for their average peak response. Data are expressed as the mean of three separate experiments.

PKC Activity

PKC activity was determined using a non-radioactive method as previously described (VanDeMark et al., 2009). Briefly, after treatment, neurons were lysed in a modified PKC extraction buffer (25 mM Tris, 0.05% Triton X-100, 10 mM β-mercaptoethanol, protease and phosphatase inhibitors). After protein extraction and quantification, equal amounts of protein were used in each PKC reaction following the PepTag® Assay for Non-Radioactive Detection of Protein Kinase C protocol (Promega, Madison, WI). Samples were then incubated with a positively-charged, fluorescent, PKC-specific peptide for 30 min and separated on agarose gels. The phosphorylated, negatively-charged peptide was separated from the non-phosphorylated, positively-charged peptide and visualized under UV light. Resulting bands were quantified by densitometry and normalized to controls.

Protein Isolation and Western Blot

Neurons were plated and treated as described for PKC activity assays. After treatment, cells were lysed with cell lysis buffer in the presence of protease and phosphatase inhibitors, and the protein content was quantified. Equal amounts of proteins were loaded in 10% Bi-Tris gels and separated by electrophoresis. Proteins were then transferred to PVDF membranes.

Membranes were blocked in 5% milk and immunolabeled with an anti- phospho-ERK1/2 antibody, followed by a HRP-conjugated secondary antibody. Membranes were stripped and reprobed with a β-actin antibody to control for loading and an ERK1/2 antibody to control for total ERK1/2 levels Resulting bands were quantified by densitometry and normalized to controls.

Statistical Analysis

Each experiment was carried out at least three times. In the morphometric analyses at least 60 cells per treatment were measured ($n \geq 60$). All statistical tests were carried out using KaleidaGraph 4.0. Unless otherwise stated, for comparing multiple treatments to controls, one–way ANOVA followed by Dunnett's test was used to determine significant differences. Values are expressed as mean ± S.E.M.

Results

In order to determine the effect of ethanol on carbachol-induced neurite outgrowth, primary hippocampal neurons plated in transwell cell culture inserts were treated in ACM for 24 hours with or without carbachol (100 μM) in the presence of ethanol (50–75 mM) and neurite outgrowth was assessed using a spectrophotometric method (see Materials and Methods). Carbachol alone increased neurite outgrowth by 2.5-fold, and this effect was significantly inhibited by both ethanol concentrations (Fig. 1A), whereas ethanol alone did not affect neurite length (Fig. 1B). The effect of ethanol was not due to increased cytotoxicity, measured by the MTT assay; indeed, neurons incubated for 24 h in the presence of 75 mM ethanol exhibited 94 % ± 8 of the incorporation of MTT-formazan observed in control cells (n=3; p=0.542).

As the described method used for the determination of neurite outgrowth does not provide information on neuronal morphology, we also confirmed these results using a morphometric approach. The axonal length in neurons incubated for 24 h in the presence of carbachol and/or ethanol (50 and 75 mM) and then fixed and immunolabeled with a β-tubulin antibody was measured using the MetaMorph software. We limited this analysis to axonal length because we have previously shown that carbachol increases the length of axons without affecting the length of minor neurites (VanDeMark et al., 2009). We confirmed that carbachol induced a significant increase in the length of axons when compared to control cells, and found that this effect was inhibited by ethanol in a concentration-dependent manner (Fig. 2A; solid bars). We also calculated the percentage of neurons whose axons were longer than 150 μm as an additional index of carbachol-induced hippocampal neuron differentiation, and found that carbachol increased by more than two fold the number of cells with longer axons when compared to control cells (first 2 bars in Fig. 2B), thus confirming our previous results (VanDeMark et al., 2009); this effect of carbachol was also inhibited by ethanol (Fig. 2B; solid bars). Ethanol alone had no significant effects on either axon length or the percentage of cells with axons longer than 150 μm (Figs. 2A, B; striped bars). Representative fields from untreated (C), carbachol-treated (D) and carbachol-treated in the presence of 75 mM ethanol (E) neurons are shown.

In our in vitro system, neurons continue to develop over several days. To test whether the effect of ethanol was related to the developmental stage of neurons or whether its effect was exerted on muscarinic signaling, we investigated the effect of ethanol on unstimulated neurons cultured for 48 h in the presence or absence of ethanol. The length of the axons in neurons incubated for 48 h in astrocyte-conditioned medium was similar to the length of the axons of neurons incubated for 24 h in the presence of carbachol (Fig. 2F). Ethanol, while inhibiting axonal elongation stimulated by carbachol (Fig. 2F and 2 A), did not affect the maturation of neurons occurring after 48 h in the absence of stimuli (Fig. 2F). These results suggest that the effect of ethanol on carbachol-induced axonal growth is likely due to the inhibition of signaling pathways activated by carbachol leading to neuronal maturation.

We have previously shown that in pyramidal hippocampal neurons the mobilization of Ca^{++} from intracellular stores, the activation of PKC, and the phosphorylation of ERK1/2 are involved in the neuritogenic effect of carbachol (VanDeMark et al., 2009).

To measure calcium mobilization, neurons were pre-incubated with the intracellular calcium indicator Fluo3-AM. Images of the cells were captured every 10 sec by an inverted fluorescent microscope attached to a digital camera. Ethanol (50 and 75 mM) was added to the cultures 15 min before beginning recording; carbachol (100 μM) was added to the cultures after 100 sec of recording and image capture continued for an additional 200 sec; the fluorescence intensity of the Ca^{++}-bound dye Fluo3 was quantified in at least 50 cells/experiment. Figure 3A shows the average normalized fluorescence intensity before and after carbachol treatment. Seventy percent of the cells (+/− 8.84) (Fig. 3B) responded to carbachol with a rapid increase in intracellular Ca^{++} that returned to control levels within 200 sec. Ethanol did not affect the percentage of responding neurons, nor the average peak response after 15 min pre-incubation at either of the tested concentrations (Fig. 3B, C), suggesting that ethanol does not directly interfere with carbachol-stimulated intracellular calcium mobilization. On the other hand, pre-incubation with 75 mM ethanol for 24 hr significantly inhibited the percentage of neurons responding to carbachol and the average peak response (Fig. 7D, E), similarly to what has been reported in astrocyte purified cultures and in astrocyte/neuron mixed cultures (Catlin et al., 2000; Kovacs et al., 1995).

We also tested the effect of ethanol on PKC activation, as we have previously shown that it is involved in carbachol-induced axonal growth (VanDeMark et al., 2009). To measure PKC activation, a non-radioactive kit that quantifies the ability of PKC to phosphorylate a fluorescent substrate was used. Pre-incubation of neurons with 75 mM ethanol for 15 min inhibited PKC activation induced by 100 μM carbachol, while 50 mM ethanol slightly reduced PKC activation in comparison to carbachol alone, but not in a statistically significant manner (Fig. 4, solid bars). Ethanol (50 and 75 mM) alone, in the absence of carbachol, caused an increase in PKC activity in comparison to control cells (Fig. 4, striped bars).

Finally, we previously identified ERK1/2 activation as the key event in carbachol-induced axon growth in hippocampal neurons (VanDeMark et al., 2009). We thus investigated the effect of ethanol on carbachol-stimulated ERK1/2 phosphorylation in hippocampal pyramidal neurons by Western blot, using ERK1/2 phospho-specific antibodies. Densitometric analysis revealed that carbachol (100 μM) caused a 2-fold increase in the levels of phosphorylated (active) ERK1/2 in hippocampal neurons after 30 min incubation. Ethanol inhibited carbachol-induced phosphorylation of ERK1/2 at both tested concentrations (Fig. 5A, solid bars), but did not have any effect on ERK1/2 activity in the absence of carbachol stimulation (Fig. 5A, striped bars). Figure 5B shows a representative immunoblot of the effect of ethanol on carbachol-stimulated ERK1/2 phosphorylation.

Discussion

While it is generally accepted that alcohol exposure affects neuronal development both *in vivo* and *in vitro*, a review of the literature reveals that contrasting effects of ethanol have been reported, with studies indicating that ethanol increases the elongation of neurites and studies showing an inhibitory effect of alcohol. Increases in axonal growth in the rat pyramidal tract (Miller and al-Rabiai, 1994) and in the corpus callosum of nonhuman primates (Miller et al., 1999) have been observed, along with increases in the density of callosal neurons (Miller, 1997). In contrast, other *in vivo* animal models have shown decreased callosal size with prenatal ethanol exposure (Moreland et al., 2002; Zimmerberg and Mickus, 1990), similarly to what has been observed clinically in FAS children. Indeed, magnetic resonance images and autopsies of children with known prenatal alcohol exposure have shown significantly smaller callosal

areas, especially of the posterior region, and agenesis of the corpus callosum (Coulter et al., 1993; Pratt and Doshi, 1984; Riley et al., 1995; Sowell et al., 2001; Swayze et al., 1997).

In addition, prenatal alcohol exposure has been shown to cause optic nerve hypoplasia in children (Stromland, 1987). This effect has been reproduced in animal models in which alcohol exposure during myelination reduces the number of myelinated fibers (Phillips, 1989). *In utero* ethanol exposure leads to a loss of myelinated axons in the optic nerve of mice (Parson and Sojitra, 1995), and to smaller optic nerves, degenerating and atrophic optic axons, reduced axonal densities, and decreased numbers of small axonal-diameter myelinated fibers in ethanol-exposed rats (Pinazo-Duran et al., 1997; Sawada et al., 2002). The results of these studies suggest that axonal growth is affected by ethanol exposure in both human FAS cases and *in vivo* models of FAS.

The effect of ethanol on neurite outgrowth has been investigated in several *in vitro* systems, that also reported inhibitory and stimulatory effects (Bingham et al., 2004; Liesi, 1997; Messing et al., 1991a; Roivainen et al., 1993; Saunders et al., 1995; Zou et al., 1993). It is likely that the contrasting results reported in different studies reflect the heterogeneity of brain regions, as suggested by the observation that ethanol inhibits neurite outgrowth stimulated by L1CAM in cerebellar granule neurons but not in cortical neurons (Bearer et al., 1999; Hoffman et al., 2008). Furthermore, the developing brain undergoes continuous and rapid changes; therefore, the timing of ethanol exposure plays a pivotal role in the type of response ethanol may trigger. For instance, some of the inhibitory effects of ethanol on neuronal development have been shown to be mediated by astrocytes (Chen and Charness, 2008; Guizzetti et al., manuscript in preparation; Pascual and Guerri, 2007), which, however, proliferate and increase in number relatively late in development (during the third trimester of gestation in human and postnatally in rats); therefore, the effects of earlier ethanol exposure are unlikely to be mediated by astrocytes. Finally, ethanol has been shown to activate signaling molecules involved in neuronal development, such as PKC and ERK1/2 (Chaturvedi and Sarkar, 2005; Messing et al., 1991b), but to inhibit the activation of these enzymes mediated by membrane receptors, such is the case of L1CAM and Brain-Derived Neurotrophic Factor (Hellmann et al., 2008; Tang et al., 2006).

Because of the diversity of effects triggered by ethanol in developing neurons, it is important to examine the consequences of ethanol exposures under different conditions that mimic processes occurring during specific phases of brain development, in order to fully understand the relationship between *in utero* alcohol and neuronal development.

We and others have been pursuing the hypothesis that acetylcholine, through the activation of muscarinic receptors, may play an important role in brain development, and particularly in neuronal maturation (Costa, 1993; Lauder and Schambra, 1999; Mount et al., 1994; Owen and Bird, 1995). We have shown that the activation of M3 muscarinic receptors in astrocytes induces neuritogenesis in primary hippocampal neurons, through the release of neuritogenic factors (Guizzetti et al., 2008), and that the direct stimulation of M1 muscarinic receptors in hippocampal neurons increases axonal length (VanDeMark et al., 2009). In our cell culture system, hippocampal neurons maintained for 24 h in vitro have not developed synapses yet (Dotti et al., 1988), therefore, the reported effect of carbachol is extrasynaptic, and is likely localized at the level of the growing axon. The concentration of carbachol that induces neurite outgrowth (100 μM) is lower than the concentration of acetylcholine found in synaptic vesicles, which are in the millimolar range (Dunant and Israel, 2000). This would be in agreement with the hypothesis that acetylcholine released by growing axons before synaptogenesis occurs (Yao et al., 2000) may be responsible for neuritogenesis of neighboring neurons in the developing brain.

As we have previously shown that ethanol inhibits muscarinic receptor signaling (Balduini and Costa, 1989; Balduini et al., 1994; Costa and Guizzetti, 1999; Guizzetti and Costa, 2000; Guizzetti and Costa, 2002; Guizzetti et al., 2003), we hypothesized that ethanol may reduce axonal growth induced by cholinergic stimulation by interfering with muscarinic signaling. In this study we report that ethanol inhibited carbachol-stimulated axonal growth. The alcohol concentrations used in this study, 50 and 75 mM, corresponding 0.23 g/dl and 0.35 g/dl respectively, are found in the blood of individuals after moderate to high ethanol intake and are in the range of concentrations recommended for *in vitro* studies (Deitrich and Harris, 1996), underlying the relevance of this *in vitro* study to the pathological effects caused by prenatal alcohol exposure. Both ethanol concentrations inhibited carbachol-induced neurite outgrowth measured by a spectrophotometric method, while only the higher ethanol concentration significantly inhibited the effect of ethanol on axonal growth, measured by morphometric analysis. These differences may reflect a difference in sensitivity of the two methods, while supporting each other in the conclusion that ethanol inhibits neuronal differentiation stimulated by carbachol.

The inhibition of carbachol-stimulated neurite outgrowth by ethanol was not a result of cytotoxicity, in agreement with previous results (Clamp and Lindsley, 1998; Heaton et al., 1994; Saunders et al., 1997) in which hippocampal neurons survived concentrations of 2.4 g/dl, 1.0 g/dl, and 0.8 g/dl of ethanol, respectively.

Ethanol alone did not inhibit neuronal development occurring in the absence of cholinergic stimulation after 48 h *in vitro*, when neurons were more developed and similar in morphology to the neurons cultured for 24 h in the presence of carbachol, suggesting that the effect of ethanol was not dependent on the differentiation stage of neurons, but rather that it may interfere with the muscarinic M1 receptor-stimulated signaling cascade. As we have previously shown that carbachol induces increase in intracellular Ca^{++}, and PKC and ERK1/2 activation in hippocampal neurons, and that these events are important for carbachol-induced axonal growth (VanDeMark et al., 2009), we investigated the effect of ethanol on each of these signaling molecules. The increase in intracellular Ca^{++} was not affected by a 15 min incubation with ethanol, similarly to what previously reported in astrocytes and mixed cultures (Catlin et al., 2000; Kovacs et al., 1995). In contrast, ethanol pre-incubation for 24 h inhibited the percentage of cells responding to carbachol with an increase in intracellular Ca^{++}, and the amplitude of the response, also in agreement with previous findings (Catlin et al., 2000; Kovacs et al., 1995). This may suggest that the mechanisms involved in Ca^{++} release are not directly affected by ethanol, but that alcohol, instead, may alter the levels or activity of proteins involved in Ca^{++} release through changes in their gene expression or post-translational modification, as these effects are not immediate and require several hours to occur. This delayed effect of ethanol on Ca^{++} release may also contribute to the inhibition of carbachol-stimulated axonal growth by alcohol.

Both PKC and ERK1/2 activation were inhibited by ethanol, although PKC activation was inhibited only at 75 mM ethanol, while ERK1/2 activation was inhibited at both the ethanol concentrations. We have shown that PKC activation is up-stream to ERK1/2 activation in pyramidal hippocampal neurons stimulated by carbachol and that the activation of both these kinases is involved in carbachol-induced neuritogenesis (VanDeMark et al., 2009), therefore suggesting that ethanol may inhibit neurite outgrowth by inhibiting PKC or up-stream activators of PKC leading then to inhibition of ERK1/2. However, we cannot exclude that additional effects of ethanol may be involved in the inhibition by ethanol of carbachol-induced neuritogenesis in hippocampal pyramidal neurons. The fact that ERK1/2 appears to be more sensitive to ethanol inhibition than PKC may be due to a difference in the sensitivity of the methods used for measuring activation. Another possibility is that more than one mechanism may be involved in the effect of ethanol on ERK1/2, one acting on PKC or upstream to PKC,

another acting downstream. Our findings are in agreement with previous results showing that ethanol inhibits neuronal differentiation by inhibiting PKC (Hellmann et al., 2008) and ERK1/2 (Tang et al., 2006).

It should be also noted that, while ethanol inhibited the activation of PKC stimulated by carbachol, it activated PKC when present alone. However, ethanol-induced PKC activation did not lead to MAPK activation. Ethanol-induced activation of PKC has previously been reported, and it has been implicated in ethanol-induced neuritogenesis in PC12 cells (Messing et al., 1991b; Roivainen et al., 1993). Similarly, we also observed a trend toward an increase of axonal length in neurons exposed to ethanol (in the absence of carbachol), although this effect was not statistically significant in our experiments. These observations underscore the complexity of the responses triggered by ethanol in developing neurons, and the fact that these responses depend largely on the factors and stimuli to which neurons are exposed, which, in vivo, change widely during the different phases of brain development.

Our results suggest that the inhibition of neurite outgrowth induced by M1 muscarinic receptor activated signaling in hippocampal neurons may be responsible, at least in part, for the cognitive deficits associated with *in utero* alcohol exposure. In support to this hypothesis, M1 knockout mice have been found to present impairments in certain memory functions, such as spatial working memory and social discrimination, which are processes dependent on the interaction between the neocortex and the hippocampus, although no global deficits in hippocampal-dependent tasks were reported (Anagnostaras et al., 2003). M1 knockout mice also present behavioral abnormalities associated with memory dysfunction, which are also observed in FAS. It has been proposed that the lack of dramatic effects in cognitive functions observed in these mice may be due to compensating mechanisms that may intervene; if this is the case, the full effect of M1 receptor elimination on hippocampal functions could be evident only after disruption of these compensatory processes (Bymaster et al., 2003). The effect of ethanol on brain development appears to be more severe than the effect of knocking out M1 muscarinic receptors, likely because ethanol may interfere with multiple signaling pathways at the same time, thereby reducing the aforementioned compensatory processes that take place when a single receptor is deleted. Finally, it should also be pointed out that the effects of ethanol on muscarinic receptor-mediated neuritogenesis in hippocampal neurons may provide a mechanistic explanation to the observations that choline supplementation appears to ameliorate learning deficits caused by ethanol exposure during brain development (Ryan et al., 2008).

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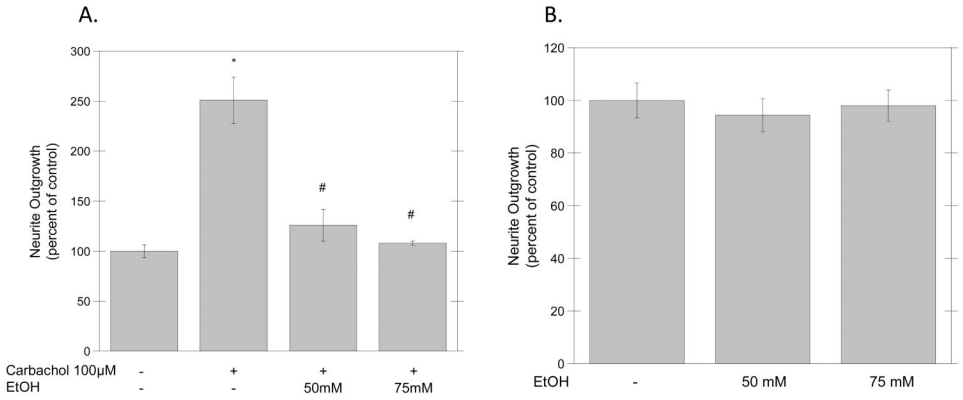
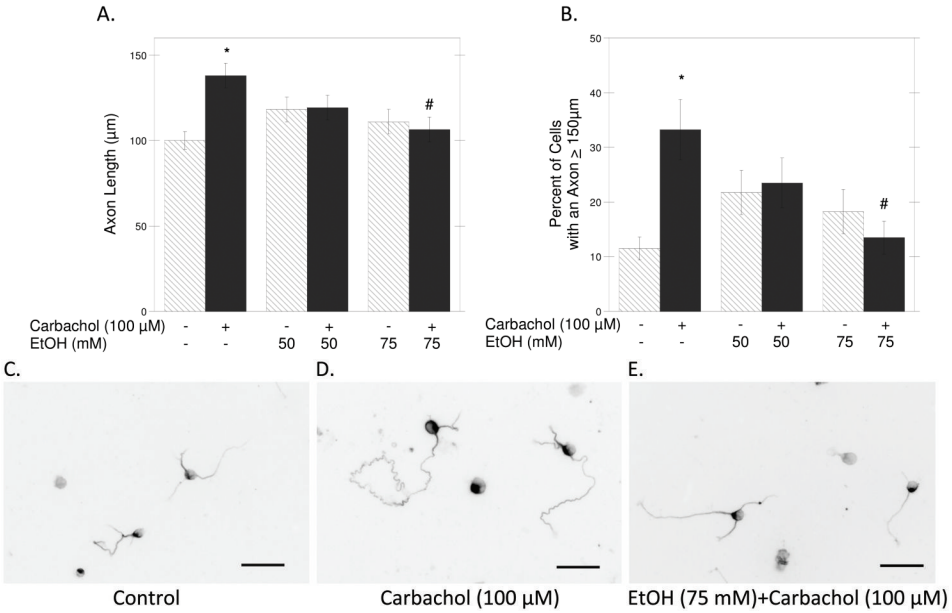


Figure 1. Effect of Ethanol on Carbachol-Stimulated Neurite Outgrowth

Hippocampal neurons plated on porous inserts were incubated for 24 hrs with (A) or without (B) carbachol (100 μM) in the presence of different concentrations of ethanol (50–75 mM), and neurite outgrowth was assessed spectrophotometrically, as described in Materials and Methods. Results represent the average of three experiments and are expressed as mean ± S.E.M. (*$p<0.05$ compared to control; #$p<0.05$, compared to carbachol alone).



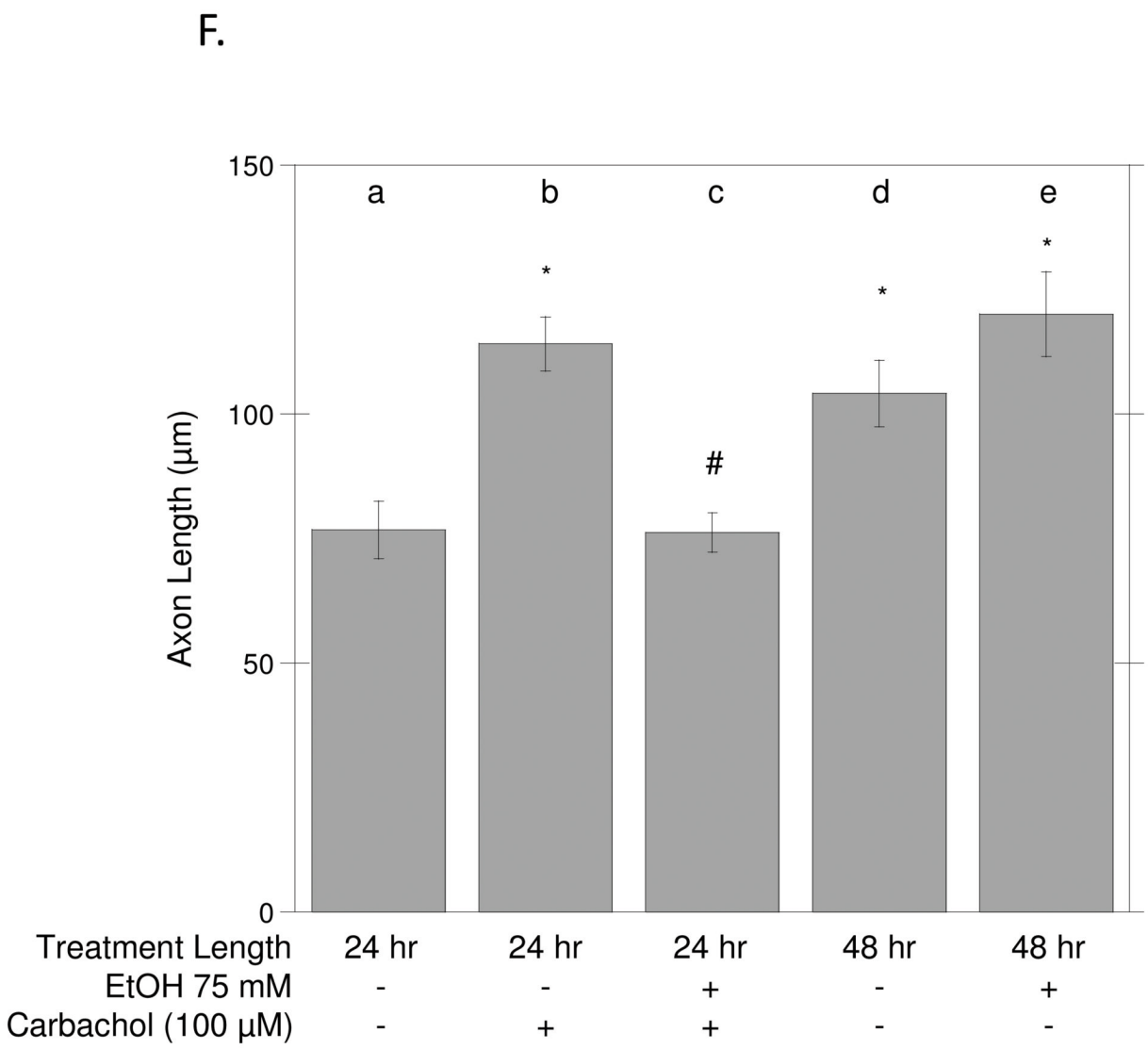
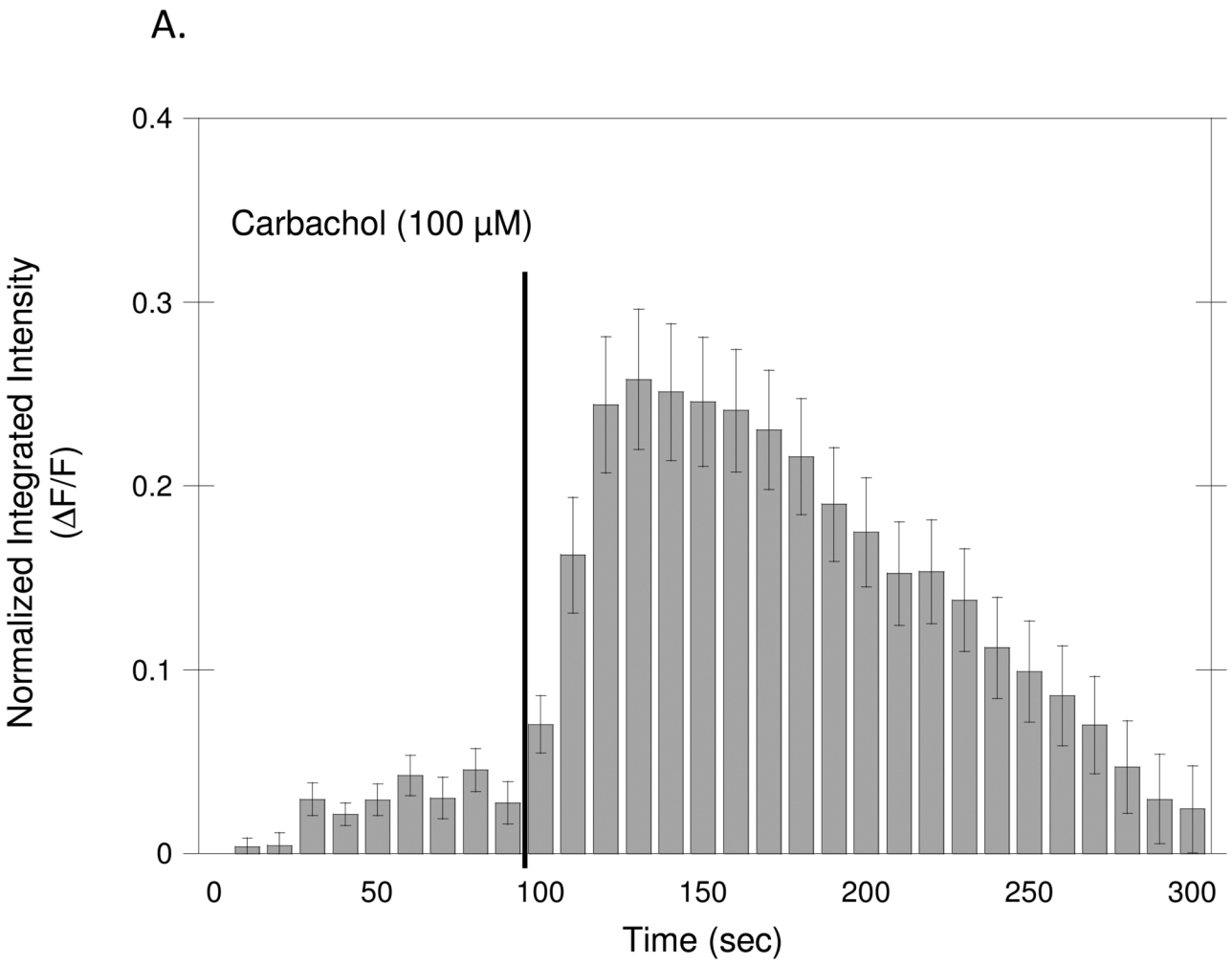


Figure 2. Effect of Ethanol on Carbachol-Stimulated Axonal Growth

(A): Hippocampal neurons were incubated for 24 h in the presence of the indicated concentrations of ethanol in the absence (striped bars) or presence (solid bars) of 100 μM carbachol. Neurons were then fixed and stained with a β-tubulin antibody followed by a fluorescent secondary, as described in Materials and Methods. Axonal length was quantified using the MetaMorph software. (B): Percent of hippocampal neurons with an axon ≥ 150 μm upon incubation with 50 or 75 mM ethanol alone (striped bars) or with 100 μM carbachol and ethanol (solid bars). Results are from 60 cells/treatment and are expressed as mean ± S.E.M. (*$p<0.05$ compared to control; #$p<0.05$, compared to carbachol alone). (C-E): Representative fields of control hippocampal neurons (C), neurons treated for 24 h with carbachol (100 μM) (D), and neurons treated for 24 h with carbachol + EtOH 75 mM (E). (Magnification 20x, scale bar = 25 μm). (F) Hippocampal neurons were incubated for 24 h in ACM (a), ACM supplemented with 100 μM carbachol (b), ACM with carbachol and 75 mM ethanol (c), or for 48 h in ACM (d) or ACM containing 75 mM ethanol (e). Neurons were then fixed and stained as described above. Results are from 60 cells/treatment, and are expressed as mean ± S.E.M. (*$p<0.05$ compared to control; #$p<0.05$, compared to carbachol alone).



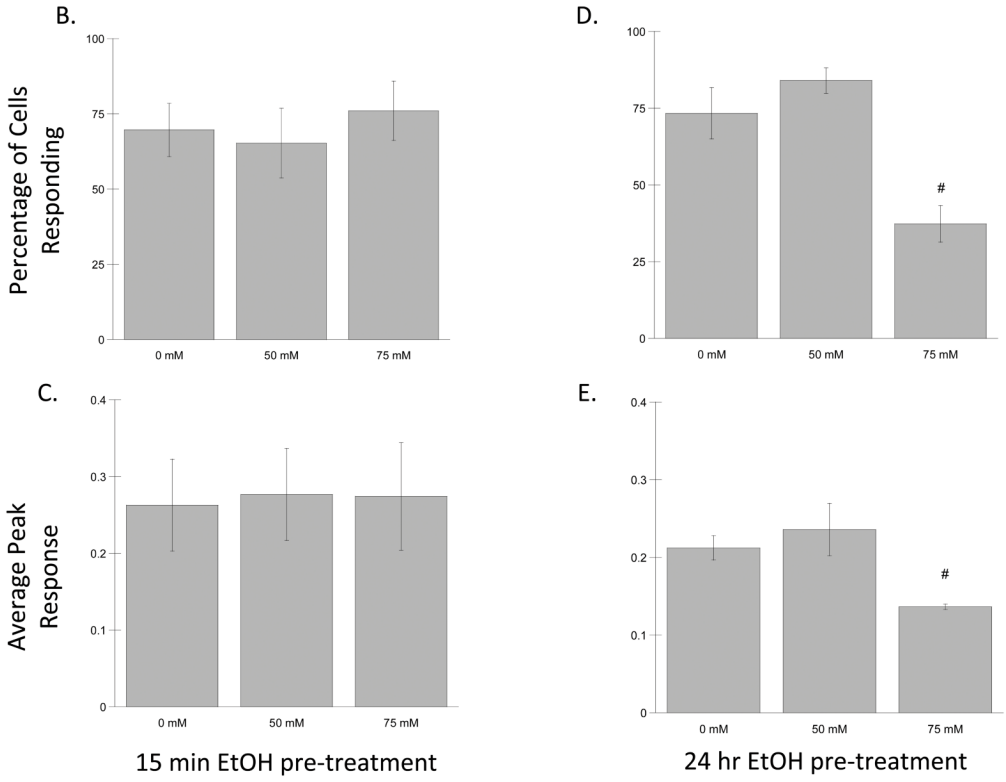


Figure 3. Effect of Ethanol on Carbachol-Stimulated Intracellular Calcium Mobilization

Hippocampal neurons plated on glass bottom dishes and incubated with the intracellular calcium indicator Fluo3-AM, as described in Materials and Methods. (A) Average normalized fluorescence intensity before and after carbachol (100 μM) treatment. Each bar represents the mean (± SEM) of 50 cells. (B–E) Effect of ethanol on carbachol-induced increases in intracellular calcium after a 15 min (B, C) or 24 h (D, E) incubation with ethanol. (B, D) Percentage of cells responding to carbachol after treatment with ethanol. (C, E) Average peak response induced by carbachol after treatment with ethanol. Results are shown as the means (± SEM) of three separate experiments. (#$p<0.05$ compared to carbachol alone).

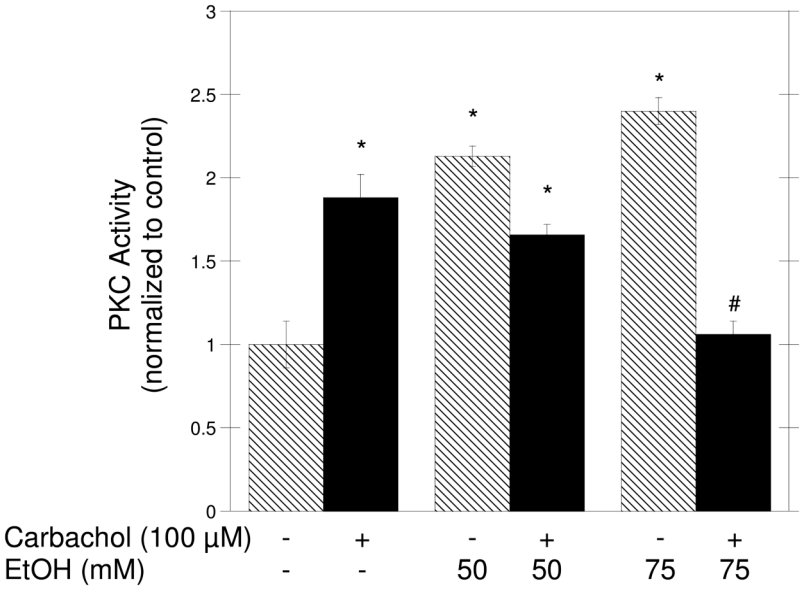


Figure 4. Effect of Ethanol on Carbachol-Stimulated PKC Activity

Hippocampal neurons were pretreated with 50 or 75 mM ethanol for 15 min; 100 µM carbachol was then added to some of the samples (solid bars), and cells were incubated for an additional 30 min. Cell lysates were collected and equal amounts of proteins were incubated with a fluorescent PKC substrate as described in Materials and Methods. At the end of the reaction the mixture was separated on an agarose gel and the bands corresponding to the phosphorylated peptide were quantified and normalized to controls. Results are the mean (± SEM) of three independent experiments. (*$p<0.05$ compared to control; #$p<0.05$, compared to carbachol alone).

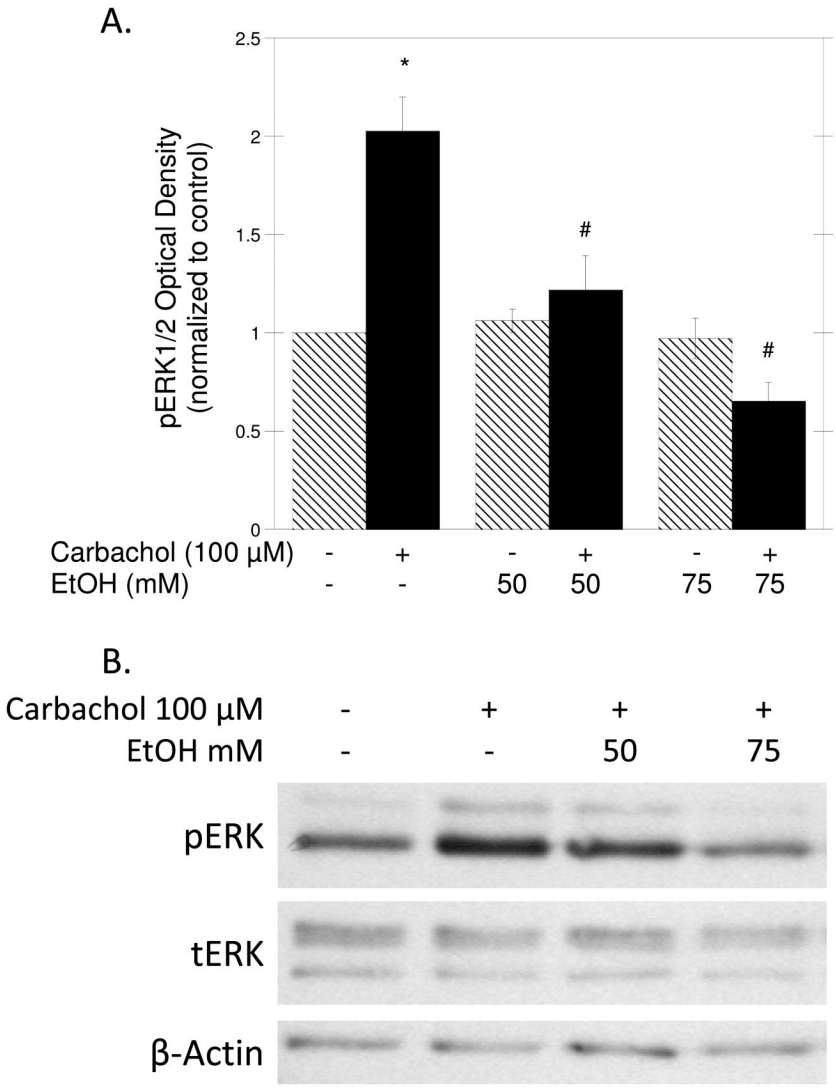


Figure 5. Effect of Ethanol on Carbachol-Induced ERK1/2 Phosphorylation

Hippocampal neurons were pretreated with 50 or 75 mM ethanol for 15 min; 100 μM carbachol was then added to some of the samples (solid bars) and cells were incubated for an additional 30 min. Cell lysates were collected, proteins were quantified, separated, transferred to PVDF membranes and incubated with phospho-ERK1/2, ERK1/2, or β-actin antibodies as described in Materials and Methods. (A) Average optical density of phosphorylated ERK1/2 normalized to controls (mean ± SEM; n=3; *$p<0.05$ compared to control; #$p<0.05$, compared to carbachol alone). (B) Representative immunoblot derived from a membrane labeled with phosphoERK1/2 (pERK), total ERK1/2 (tERK), and β-actin (loading control) antibodies.