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ETHANOL INDUCES APOPTOTIC DEATH OF DEVELOPING β-ENDORPHIN NEURONS VIA SUPPRESSION OF CYCLIC ADENOSINE MONOPHOSPHATE PRODUCTION AND ACTIVATION OF TRANSFORMING GROWTH FACTOR-β1-LINKED APOPTOTIC SIGNALING

Cui Ping Chen, Peter Kuhn, Kirti Chaturvedi, Nadka Boyadjieva and Dipak K. Sarkar

Endocrine Program and Department of Animal Sciences,

Rutgers, the State University of New Jersey, New Brunswick, NJ 08901

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Running Title: cAMP-repressed TGF-β1 signaling mediates ethanol's toxicity

Corresponding author: Dr. Dipak K. Sarkar, Endocrinology Program and Department of Animal Sciences, 84 Lipman Drive, New Brunswick, NJ 08901, Tel 732-932-1529, Fax 732-932-4134, Email: sarkar@AESOP.RUTGERS.EDU

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ABSTRACT

The mechanism by which ethanol induces β-endorphin (β-EP) neurons death during the developmental period was determined using fetal rat hypothalamic cells in primary cultures. The addition of ethanol to hypothalamic cell cultures stimulated apoptotic cell death of β-EP neurons by increasing caspase-3 activity. Ethanol lowered the levels of adenylyl cyclase 7 (AC7) mRNA, AC8 mRNA and/or cAMP in hypothalamic cells, whereas a cAMP analog blocked ethanol's apoptotic action on β-EP neurons. The AC inhibitor dideoxyadenosine (DDA) increased cell apoptosis and reduced the number of β-EP neurons, and potentiated ethanol's apoptotic action on these neurons. β-EP neurons in hypothalamic cultures showed immunoreactivity to transforming growth factor-beta 1 (TGF-β1) protein. Ethanol and DDA increased TGF-β1 production and/or release from hypothalamic cells. A cAMP analog blocked ethanol's activation of TGF-β1 in these cells. TGF-β1 increased apoptosis of β-EP neurons, but it did not potentiate ethanol's or DDA's actions on these neurons. TGF-β1 neutralizing antibody blocked ethanol's apoptotic action on β-EP neurons. Determination of TGF-β1-controlled cell apoptosis regulatory genes levels in hypothalamic cell cultures and in isolated β-EP neurons indicated that ethanol, TGF-β1 and DDA similarly alter the expression of these genes in these cells. These data suggest that ethanol increases β-EP neuronal death during the developmental period by cellular mechanisms involving, at least partly, the suppression of cAMP production and activation of TGF-β1-linked apoptotic signaling.

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Embryonic exposure to ethanol reduces the number of neurons in various parts of the central nervous system (CNS) including the hypothalamus (De et al., 1994; Goodlett and Horn, 2001). Ethanol reduces this cell number by suppressing the proliferation of neuronal precursors and/or enhancing cell death. The cellular mechanisms by which ethanol induces neuronal death are unclear. One possibility is that ethanol alters neurotrophic factor availability and its receptor function, leading to impaired cell survival and increased cell death. Recent studies have shown that cAMP analogs act as trophic signals for β-EP neurons in the hypothalamus (Yang et al., 1993). Interestingly, chronic ethanol treatment causes plasticity of the cAMP–adenylyl cyclase (AC) system in various neurons (Gordon and Diamond, 1993; Boyadjieva et al., 1997). However, cAMP also promotes apoptosis in cortical neurons (Takadera et al., 2002) and non-neuronal cells (Hur et al., 2003). Whether ethanol causes apoptosis of β-EP and other cell populations in the hypothalamus by reducing the activity of the cAMP–AC system is not known. Also not apparent is how cAMP reduction induces apoptotic death of neurons.

One possibility is that reduced cellular levels of cAMP activate transforming growth factor-beta 1 (TGF-β1) to induce neuronal apoptosis, since cAMP reduces TGF-β1 gene transcription in pituitary cells (Pastorcic and Sarkar, 1997) and inhibits TGF-β1-induced Smad3/4-dependent transcription in keratinocytes (Schiller et al., 2003). Also, TGF-β1 induces apoptosis of cerebellar granule neurons (De Luca et al., 1996) and the developing chick retina (Schuster et al., 2002). However, TGF-β1 can also be neuroprotective in hippocampal and cortical neurons (Henrich-Noack et al., 1996; Scorziello et al., 1997). Furthermore, TGF-β1 knockout mice showed increased neuronal cell death and microgliosis in mouse brain (Brionne et al., 2003). Hence, TGF-β1's mediatory role in ethanol's apoptotic action on hypothalamic neurons needed to be demonstrated.

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Three different isoforms of TGF-βs, TGF-β1–3, have been described in mammalian cells and have been shown to have similar biological activities in many cells. In the developing nervous system, TGF-βs are identified in many populations of postmitotic, differentiating neurons (Krieglstein et al., 2000). TGF-β1 is secreted in an inactive, latent form and is activated by acidification, alkalization, proteases or heat (Roberts and Sporn, 1990). TGF-β receptors are present in the hypothalamus and on β-EP cells (Bouret et al., 2001). TGF-β1-induced apoptosis in non-neuronal cells is associated with increased mitochondrial apoptotic proteins bcl-xs, bak, and bax, and decreased mitochondrial anti-apoptotic proteins bcl-xL and bcl-2. It is believed that increased activity of pro-apoptotic peptides causes cytochrome C release to activate caspases and to cause cell death (Nass et al., 1996; Francis et al., 2000; Lee et al., 2002). It is not known whether TGF-β1-induced signaling is required for apoptosis of hypothalamic cells, particularly β-EP neurons.

In this study, we demonstrated that ethanol decreased cellular levels of cAMP and TGF-β1-regulated apoptotic signaling to induce death of developing hypothalamic cells in cultures. Furthermore, we identified that β-EP neurons are one of the hypothalamic cell types that were a target of ethanol.

Materials and Methods

Animal use. Pregnant Sprague-Dawley female rats were obtained from Simonsen Laboratories (Gilroy, CA) and were used as the source of fetal rat brains for hypothalamic cell cultures. Animal surgery and care were performed in accordance with institutional guidelines and complied with the National Institutes of Health policy. The animal protocol (#99-005) was

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approved by the Rutgers Animal Care and Facilities Committee.

Primary cultures of hypothalamic cells and treatments. Primary cultures of fetal hypothalamic cells were prepared from the mediobasal part of the hypothalamus (containing neuroendocrine neurons including β-EP, dopamine, thyrotropin-releasing hormone and growth hormone releasing hormone, as well as containing glial cells; Brown, 1998). Briefly, pregnant rats of the Sprague-Dawley strain (Simonsen Laboratories, Gilroy, CA) at 18–20 days of gestation were sacrificed, and the fetuses were removed by aseptic surgical procedure. Brains from the fetuses were immediately removed; hypothalami were separated and placed in ice-cold Hanks' balanced salt solution (HBSS) containing antibiotic solution (100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B), 0.1% bovine serum albumin (BSA) and 200 µM ascorbic acid (all from Sigma, St. Louis, MO). The block of hypothalamic tissue consisted of the mediobasal portion of the hypothalamus and extended about 1 mm rostral to the optic chiasma and just caudal to the mammillary bodies, laterally to the hypothalamic salci, and dorsally to ~2 mm deep. The hypothalamic cells were washed and then incubated at 37^{o}C for 5 min using the same medium. Following dispersion, the cells were plated at a density of 3.0 x 10^{6} cells per 25-mm^{2} flask and at a density of 1.0 x 10^{6} cells per well in a 24-well plate. Both the flask and plate were coated with polyornithine at a concentration of 100 µg/ml, and then incubated for 3 hr. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) at 37^{o}C and 7.5% CO_2 in a humidified water-jacketed incubator for 2 d. After 2 d of plating, the medium was changed at one-day intervals with serum-free, chemically-defined medium (consisting of 30 nM selenium, 20 nM progesterone, 1 µM iron-free human transferrin, 100 µM putrescine and 5 µg/ml insulin). On the third day the medium was removed, and the cultures were treated with vehicle or various doses of ethanol, TGF-β1,

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dideoxyadenosine (DDA), dibutyryl cAMP (dbcAMP) or a caspase 3 blocker Ac-ASP-Glu-Val-Asp-CHO (Ac-DEVD-CHO), staurosporin (STS) for 1, 2 or 4 d. All of the culture reagents were purchased from Sigma (St. Louis, MO) with the exception of FCS, TGF-β1 and Ac-DEVD-CHO which were purchased from Hyclone Laboratories (Logan, UT), R&D Systems (Minneapolis, MN), and Calbiochem (San Diego, CA) respectively.

Immunocytochemistry. To identify apoptotic β-EP neurons, hypothalamic cell cultures were double-stained with terminal deoxynucleotideyl transferase-mediated ddUTP-biotin labeling (TUNEL; Apoptosis kit, Roche Diagnostic Corporation, Indianapolis, IN) and immunoreactive β-EP using an ABC kit (Vector Laboratories Inc., Burlingame, CA) as described by us previously (De et al., 1994). The antibody for β-EP was Y-10 (a gift from Dr. S.S.C. Yen, University of California at San Diego) and used at a dilution of 1:1000. The immunoreactivities of Y-10 have been well-characterized and found to be specific for β-EP. Previously we have shown that pre-incubation of the antiserum with an excess (100 μg/ml) of β-EP antigens eliminated immunoreactive staining in hypothalamic cultures (De et al., 1994). Routine counts of cells exhibiting β-EP immunoreactivities or combined TUNEL and β-EP-like immunoreactivities were completed by two independent investigators. Approximately 200–500 total cells in each culture were counted, and the percentages of β-EP or TUNEL and β-EP-positive cells in each culture were determined. Co-localization of TGF-β1 in β-EP staining was carried out using a double-label method as described by us previously (Burns and Sarkar, 1993) using β-EP antibody Y-10, TGF-β1 goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1 μg/ml), biotinylated ABC reagents (Vector Laboratories), 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (NBT/BCIP) as a coloring reagent for β-EP staining, and diaminobenzedine (DAB) as a coloring agent for TGF-β1 staining. Pre-incubation of the antisera

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with an excess (100 µg/ml) of TGF-β1 antigens reduced immunoreactive staining.

Proteins and apoptotic enzymes assays. Hypothalamic cell extracts were used to measure levels of nucleosome, caspase-3, and cAMP using a Nucleosome ELISA kit (Oncogene Research Products, Boston, MA), a Caspase-3 ELISA kit (Calbiochem) and a cAMP kit (Amersham; Piscataway, NJ) following instructions from the manufacturers. The supernatant samples of hypothalamic cell cultures treated with vehicle, ethanol or DDA were used to assess TGF-β1 release. The media samples were acidified using 4 mM HCl to activate a latent form of TGF-β prior to measuring the peptide. The TGF-β1 levels were determined by a Quantikine ELISA kit (R&D Systems) according to the kit's instructions. Protein contents of the cell extracts were determined using Bio-Rad DC Protein Assay Reagents (Bio-Rad Laboratories, Hercules, CA). Protein values were used to calculate the amount of cellular cAMP as pmol/µg protein and caspase-3 activity as pmol/min/mg protein.

Laser Capture Microdissection (LCM) of β-EP neurons. A rapid immunohistochemical staining protocol for β-EP was developed to prevent the significant degradation of RNA due to prolonged incubation in aqueous media during the standard staining process. Briefly, hypothalamic cell cultures maintained on glass slides were fixed with 4% paraformaldehyde for 5 min. The endogenous peroxidase activity in these cells was inhibited by incubating with 1% H_2O_2 in methanol for 10 min. In the presence of an RNase inhibitor (1 unit/µl), the culture slides were incubated with 10% blocking serum in 0.1 M phosphate buffer saline (PBS) for 15 min, primary antibody (1:400 anti-β-EP; Y-10) for 90 min followed by secondary antibody (1:200; biotinylated anti-rabbit) for 30 min. The slides were then incubated with ABC solution (Vector Laboratories) made in 0.1 M PBS for 30 min and stained using DAB. After β-EP immunoreactivity was developed, the slides were completely dehydrated by incubating in graded

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ethanol solutions (75%, 30 sec; 95%, 30 sec; 100% twice, 30 sec) and xylene (twice, 1 min). From each culture slide, approximately 1000 individual positive cells were captured using the PixCell LCM system (Arcturus, Mountain View, CA). Laser spot size was set to 7.5 μm. The power amplitude and pulse duration of the PixCell laser were adjusted for each slide (65–75 mW, 750–850 ms). The thermoplastic film-coated caps containing the captured cells were incubated in proteinase K (2 mg/ml) solution made in lysis buffer (20 mmol/L Tris-HCl pH 8.0, 20 mmol/L EDTA, 2% sodium dodecyl sulfate) and examined under the microscope to ensure complete cell lysis. Total RNA from the LCM-captured cells was extracted using a Micro RNA isolation Kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. The genomic DNA from the RNA solution was removed by DNase I treatment, and the remaining RNA was amplified using a single round of linear amplification technique using the standard protocol with the RiboAmp RNA Amplification Kit (Arcturus). To assess the quality of RNA, culture slides from which the β-EP cells had been captured were scraped and extracted for RNA. Obtained RNA was run on 1.2% agarose gel, which displayed 18S and 28S bands with slight smear, indicating the prominent RNA remained intact (data was not shown). An additional sample with 2000 randomly captured cells, isolated using a procedure identical to that of the tested samples, was used to assess the quality of amplified antisense RNA (aRNA). After amplification, the aRNA from this sample was run on 1.2% agarose gel. The bulk of aRNA ranged from around 200 to 1700 bases, which was within the expected base length for amplified aRNA with the RiboAmp Kit (Arcturus). The mRNA levels of ACs, TGF-β1, and apoptosis regulatory genes in enriched β-EP neurons were measured using quantitative real-time RT-PCR. Four independent samples were used for each group.

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Real-time reverse transcription (RT)-PCR. Expression levels of various apoptotic and anti-apoptotic genes in cultured cells were measured by quantitative real-time RT-PCR (TaqMan assay) using an ABI PRISM 7700 Sequence Detector (PerkinElmer Applied Biosystems, Foster City, CA). This assay is based on the 5' nuclease activity of Taq DNA polymerase for fragmentation of a dual-labeled fluorogenic hybridization probe. Total RNA was isolated from hypothalamic cultures treated with vehicle, ethanol, TGF-β1, or DDA using the RNeasy Mini Kit (Qiagen, Valencia, CA) and following the manufacturer's instructions. Total RNA (1 μg) was subjected to first-strand cDNA synthesis using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). cDNA was subjected to real-time RT-PCR using either the fluorogenic 5' nuclease assay (all the genes except AC7) or SYBR Green I double-stranded DNA binding dye chemistry (AC7), which were both provided by Applied Biosystems Sequence Detection Systems. Gene-specific primers and fluorescent-labeled probes were designed using the application-based primer design software, Primer Express, version 1.5, (PerkinElmer Applied Biosystems) and were based on published GenBank sequences. These primers and probes are gene-specific as confirmed by the BLAST search and are listed in Table 1. We could not reliably detect the level of p27/kip mRNA in the cell extracts, therefore this gene was not used in this study. Amplification was performed for one cycle of a sequential incubation at 50°C for 2 min and 95°C for 10 min, and subsequent 40 cycles of a consecutive incubation at 95°C for 15 sec and 60°C for 1 min, except for AC7 which was detected by running 40 cycles at 95°C for 15 sec and 52°C for 1 min, followed by 72°C for 1 min. The PCR products were run on a 1.5% agarose gel to verify the appropriate size of the amplicons. Relative quantity of mRNA was calculated by relating the PCR threshold cycle obtained from the tested samples to relative standard curves generated from a serial dilution of cDNA prepared from the total RNA. The mRNA level in each

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sample was normalized with the level of GAPDH mRNA which was measured by a control reagent (PerkinElmer Applied Biosystems). Data shown in the tables are mean ± SEM percentage of control values. Six to eight independent samples were used for each group.

Statistical analysis. The data shown in the figures and text are mean ± SEM. Data comparisons between two groups were made using *t*-tests, while comparisons among multiple groups were made using one-way ANOVA. Post-hoc tests involved the Student-Newmann-Keuls test. A value of $p < 0.05$ was considered significant.

Results

Using fetal hypothalamic cells in primary cultures and DNA fragmentation assay, we have previously shown that long-term exposure to ethanol induces apoptotic death of hypothalamic cells during the developmental period (De et al., 1994). In this study, using cells from the mediobasal hypothalamus of fetal rats, we also found that exposure to ethanol doses of 50–150 mM for 2 d or 4 d dose-dependently increased the amount of DNA damage in the cells as determined by the nucleosome activity (a marker for DNA damage in the cells; Allen et al., 1997; Fig. 1A). Studies were conducted to determine whether ethanol-induced cell apoptosis in hypothalamic cultures involved activation of caspase-3. This enzyme is known to increase endonuclease activity to cause cell apoptosis (Cohen, 1997). As shown in Fig. 1B, ethanol dose-dependently increased the activity of caspase-3 in hypothalamic cells after 2 and 4 d of treatment (Fig. 1B). Previously we have shown that chronic treatment with 200 mM ethanol increases the number of apoptotic β-EP neurons (as determined by Nissl and TUNEL stains) in developing hypothalamic cell cultures (De et al., 1994). In this study, we show that treatment with a

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moderate dose of ethanol (100 mM) for 2 d or 4 d reduced the number of β-EP-immunoreactive neurons in the hypothalamic cell cultures (Fig. 1C-E). We also counted the number of β-EP-immunoreactive cells stained with TUNEL (which identifies apoptotic cells; Allen et al., 1997) following 100 mM ethanol for different time periods. As shown in Figure 1F,G, many TUNEL-positive cells in cultures were β-EP-positive (green color appeared yellow when co-localized in red-colored β-EP cells). Furthermore, the number of TUNEL-positive-β-EP-immunoreactive cells was low in control cultures and in ethanol-treated cultures after 1 d of treatment but increased significantly after 2 d and 4 d of ethanol treatment as compared to controls (Fig. 1H). These results suggest that ethanol induces apoptotic death of hypothalamic cells, many of which are β-EP neurons.

Additional experiments using the potent caspase inhibitor Ac-DEVD-CHO revealed that this blocker reduced ethanol's effect on hypothalamic cell caspase-3 activity 2d after treatment (pmol/min/mg; n = 5-6; control, 25 ± 2; 150 mM ethanol, 125 ± 6; ethanol + 1.0 μM Ac-DEVD-CHO, 90 ± 5; ethanol + 10.0 μM Ac-DEVD-CHO, 38 ± 2; ethanol + 100.0 μM Ac-DEVD-CHO, 5 ± 1; $p < 0.01$, ethanol vs. the rest of the groups), indicating that the blocker is effective in reducing the function of the enzyme in these cells. The blocker also reduced ethanol-induced nucleosome activity at 2 d in hypothalamic cells (unit/ml; n = 6; control, 0.4 ± 0.2; 150 mM ethanol, 3.3 ± 0.4; ethanol + 1.0 μM Ac-DEVD-CHO, 2.8 ± 0.3; ethanol + 10.0 μM Ac-DEVD-CHO, 0.8 ± 0.3; ethanol + 100.0 μM Ac-DEVD-CHO, 0.6 ± 0.1; $p < 0.001$, ethanol vs. the rest of the groups, except ethanol + 1.0 μM Ac-DEVD-CHO). Furthermore, the caspase blocker reduced the ethanol inhibitory action on the number of β-EP neurons (Fig. 1I) and on ethanol's stimulatory action on the number of TUNEL-positive β-EP neurons (Fig. 1J). These data suggest that ethanol induces death of β-EP neurons via a caspase-3-dependent mechanism.

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We tested the hypothesis that ethanol reduces cAMP activity to cause apoptosis in β-EP neurons, by determining the effect of ethanol and the AC inhibitor DDA (Shoshani et al., 1996) on intracellular levels of cAMP, mRNA levels of AC6, 7 and 8, β-EP cell numbers, and TUNEL-positive β-EP neurons in hypothalamic cell cultures. We have chosen these isoforms of AC, since our preliminary study identified AC6, 7 and 8 as the major isoforms of AC in the hypothalamic cells that are ethanol sensitive (Yoshimura and Tabakoff, 199; Chandler et al., 2004; Mass et al., 2005). We have also determined the effect of the cAMP analog dbcAMP on ethanol-induced alterations in the number of β-EP neurons and TUNEL-positive β-EP neurons in hypothalamic cell cultures. Measurement of intracellular levels of cAMP revealed that the dose (100 mM) of ethanol that induced apoptosis of β-EP neurons significantly reduced cellular levels of cAMP in hypothalamic cell cultures after 2 or 4 d of treatment (Fig. 2A). Like ethanol, DDA also reduced the cellular levels of cAMP in these cell cultures. Both ethanol and DDA reduced the mRNA levels of AC6 after 4 d of treatment, but after 2 or 4 d of treatment they reduced adenylyl cyclases 7 and 8 in hypothalamic cells (Fig. 2B-D).

The adenylate cyclase inhibitor DDA increased TUNEL-positive β-EP neurons (Fig. 2E) and reduced the number of β-EP neurons (% of total cell; n = 3-4; 0-dose, 9.8 ± 0.9; 10 μM dose, 5.4 ± 0.5; 100 μM dose, 2.7 ± 0.3; $p < 0.01$, 0-dose vs. 10 μM dose; 10 μM dose vs. 100 μM dose) 2d after treatment in hypothalamic cultures. DDA at low dose (10 μm), but not at high dose (100 μM), potentiated ethanol action on TUNEL-positive β-EP neurons (Fig. 2F) and the number of β-EP neurons (% of total cell; n = 3-4; 100 mM ethanol, 5.2 ± 0.3; 100 mM ethanol + 10 μM DDA, 2.9 ± 0.4; 100 mM ethanol + 100 μM DDA, 1.6 ± 0.4; $p < 0.01$, ethanol vs. ethanol + 10 μM DDA; ethanol vs. ethanol + 100 μM DDA). These data suggest the possibility that reduced cellular levels of cAMP following DDA or ethanol leads to increased death of β-EP neurons.

The cAMP analog dbcAMP, when simultaneously treated with ethanol, decreased ethanol's ability to increase the number of TUNEL-positive β-EP neurons (Fig. 2G) and to reduce the number of β-EP neurons (Fig. 2H). dbcAMP has also been shown to reduce ethanol-induced apoptosis of β-EP neurons (De et al., 1994). These results suggest that ethanol reduces intracellular levels of cAMP to induce apoptosis of β-EP neurons.

The mediatory role of TGF-β1 in ethanol's and DDA's apoptotic actions on hypothalamic cells and β-EP neurons was studied by determining the TGF-β1 immunoreactivity in hypothalamic cells and β-EP neurons; by measuring changes in the levels of TGF-β1 mRNA and the release of the TGF-β1 peptide following treatments with ethanol and DDA in hypothalamic cells; by studying the effects of dbcAMP on ethanol-induced TGF-β1 release; by evaluating the effect of TGF-β1 with or without ethanol/DDA on β-EP neurons' apoptosis; and by determining the effect of a TGF-β1 neutralizing antibody on ethanol- and STS-induced apoptosis of hypothalamic cells and β-EP neurons. In fetal hypothalamic cultures, many cells showed TGF-β1 immunoreactivity, some of which were co-localized in β-EP immunoreactive cells and some other uncharacterized cells (Fig. 3A,B). Measurement of TGF-β1 mRNA levels in cell extracts and TGF-β1 protein levels in media samples from these cultures revealed that hypothalamic cells produced and secreted TGF-β1 (Fig. 3C-D). Both mRNA expression and the TGF-β1 release were elevated 2 d after ethanol or DDA treatment, and the release of the peptide remained elevated 4 d after ethanol or DDA treatment. Since many β-EP cells were TGF-β1-immunoreactive, the observed increase in TGF-β1 release from hypothalamic cells following ethanol or DDA treatment may represent changes in TGF-β1 release from β-EP neurons.

The data shown in Fig. 2 indicate that ethanol reduced the hypothalamic cell content of AC-cAMP. Ethanol also increased TGF-β1 mRNA levels and release from these cells (Fig. 3C,D). Similarly, DDA, which decreased the cell contents of AC-cAMP, also increased TGF-β1 levels from these cells (Figs. 2 and 3C,D). Based on these results, we decided to determine if the ethanol-stimulated-TGF-β1 release resulted from reduction of cAMP levels. As shown in Fig. 3E, dbcAMP prevented ethanol's stimulatory action on TGF-β1 release, suggesting that ethanol may increase TGF-β1 release by repressing the inhibitory action of cAMP on TGF-β1.

Determination of β-EP neuronal viability indicated that these cells were very sensitive to TGF-β1, since treatment of TGF-β1 in a dose range of 0.05-2.0 ng/ml for 2 d concentrations-dependently increased the number of apoptotic β-EP neurons as determined by counting the number of TUNEL-positive β-EP-stained cells (Fig. 3F). This concentration-range of TGF-β1 that caused β-EP neuronal apoptosis is within the range that was released (0.3-1.0 ng/ml released during a period of 2 d) after the treatment with a neurotoxic dose of ethanol (Fig. 3D,E). When the dose-response effects of TGF-β1 on β-EP neurons were determined in the presence of high dose of ethanol (100 mM), TGF-β1 failed to maximize the ethanol's ability to induce apoptosis of β-EP neurons (Fig. 3G), supporting the view that ethanol and TGF-β1 may share common pathway to induce apoptosis of these neurons. Similarly, determination of the TGF-β1 action on β-EP neurons in the presence of DDA indicated that the peptide failed to potentiate DDA's ability to induce apoptosis of β-EP neurons (Fig. 3H), supporting the view that TGF-β1 and DDA may use common pathway to induce apoptosis of these neurons. These data suggest that TGF-β1 produced by hypothalamic cells during ethanol or DDA challenge has the ability to increase the apoptotic death of β-EP neurons.

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Further studies were conducted to determine TGF-β1's mediatory role in ethanol's apoptotic action by determining the effect of its neutralizing antibody on basal and ethanol-induced changes in nucleosome levels in hypothalamic cells, number of β-EP neurons and in TUNEL-positive β-EP neurons. In hypothalamic cell cultures, the TGF-β1 neutralizing antibody completely blocked ethanol's apoptotic action (Fig. 4A), suggesting that TGF-β1 might be important in mediating ethanol's apoptotic action on these cells. Fig. 4B and 4C show that TGF-β1 neutralizing antibody blocked ethanol-stimulated loss of β-EP neurons and the number of TUNEL-positive β-EP neurons. TGF-β1 neutralizing antibody alone did not affect the number of β-EP neurons or the TUNEL-positive β-EP neurons. TGF-β1 neutralizing antibody also failed to alter staurosporine-induced increase in nucleosome activity in hypothalamic cells (Fig. 4D), suggesting that the antibody has a specific action on blocking ethanol-induced apoptotic death of β-EP neurons. Together, these data suggest that the increased TGF-β1 release caused by ethanol may be important in the mediation of ethanol's apoptotic action on β-EP neurons.

To determine whether ethanol-induced apoptosis follows pathways similar to those of TGF-β1-induced cellular apoptosis, we ran tests comparing mRNA expression of cell apoptosis regulatory genes in β-EP cells from the hypothalamic cultures treated with ethanol and TGF-β1. Because the hypothalamic cells were mixed cells, it was difficult to ascertain whether the changes in the cell signaling genes in the hypothalamic cells reflected those of β-EP cells following ethanol, TGF-β1, and DDA treatment. Hence, we employed an LCM approach to isolate β-EP cells from the hypothalamic cultures after ethanol, TGF-β1, and DDA treatments. Assuming that ethanol exposure induced apoptosis by reducing the levels of cAMP and increasing TGF-β1 levels, we compared ethanol's, TGF-β1's and DDA's actions on mRNA

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expression of cell apoptosis regulatory genes in β-EP cells in cultures. We chose a 2-d treatment period since the number of apoptotic cells was highest than any other days studied. Furthermore, preliminary study indicated similar gene expression profiles after 2 and 4 d of ethanol treatment. Figure 5A-C shows the capture of individual β-EP positive cells from hypothalamic cell cultures in thermoplastic film-coated caps using the PixCell LCM system. The quality of RNA obtained from these captured cells was tested and found to be prominent and intact (data not shown). The RNA of the captured β-EP neurons was used for measurements of genes that responded in hypothalamic cells to the treatment of ethanol, TGF-β1, and DDA using real-time RT-PCR. The mRNA levels of several key apoptotic regulatory genes in these captured cells collected from the ethanol-, TGF-β1- and DDA-treated cultures are shown in Fig. 5D–L. As expected, treatment with ethanol, TGF-β1, and DDA caused a significant decrease in adenylyl cyclase 7 and 8 mRNA levels but an increase in TGF-β1 mRNA levels in β-EP neurons (Fig. 5D–F). Ethanol, TGF-β1, and DDA treatments also decreased mRNA levels of the anti-apoptotic gene bcl-2 in these neurons (Fig. 5G). TGF-β1 also significantly inhibited the anti-apoptotic gene bcl-xL in these neurons (Fig. 5H). The β-EP neurons exposed to ethanol or DDA showed lower levels of bcl-xL mRNA than those exposed to control treatment, but the differences were not significant. Ethanol, TGF-β1, and DDA treatments increased mRNA levels of apoptotic genes bcl-xs, bax and bak in β-EP neurons (Fig. 5I–K). As expected, ethanol, TGF-β1, and DDA treatments all increased caspase-3 mRNA levels. These data suggest that cell apoptosis regulatory genes in β-EP neurons respond similarly to ethanol, DDA, and TGF-β1 treatments.

Discussion

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The data presented here provide evidence that ethanol treatment enhances apoptosis of fetal hypothalamic cells possibly by increasing caspase-3 activity. We identified that the β-EP neuron is one of the hypothalamic cells that undergoes apoptosis following ethanol treatment. We showed that ethanol suppresses cellular levels of AC-cAMP and causes TGF-β1 release, and that a TGF-β1 blocking antibody and a cAMP analog inhibit ethanol-induced apoptosis of β-EP neurons. We further showed that ethanol exposure causes changes in apoptotic and survival proteins that are consistent with changes following TGF-β1 induction or cAMP reduction.

In this study, both AC7 and AC8 mRNA levels were reduced in β-EP neurons and in hypothalamic cells following ethanol treatment for 2 and 4 d. Similar treatments with ethanol reduced cAMP levels in hypothalamic cells. Ethanol has been shown to acutely increase the effect of cAMP analogs on β-EP production and release in hypothalamic cells, but it chronically (> 24 h) prevents the effects of cAMP analogs and hormones that affect various AC-bound receptors and β-EP neuronal functions (De et al., 1994;Boyadjieva et al, 1997). Hence, chronic ethanol might affect the activity of adenylate cyclases to reduce the levels of cAMP production in β-EP neurons and other hypothalamic cells. It should be noted that ethanol also alters adenosine release to affect intracellular levels of cAMP in β-EP neurons in hypothalamic cultures (Boyadjieva and Sarkar, 1999). Chronic ethanol has been shown to desensitize the adenosine-regulated cAMP production and β-EP release from hypothalamic neurons. Hence, ethanol might reduce AC-bound receptors and desensitize the adenosine-regulated cAMP production to reduce intracellular levels of cAMP. These data of the present study are consistent with the concept that one of the cellular adaptations to long-term ethanol use is the downregulation of signaling pathways driven by AC-cAMP in several brain regions (Yoshimura

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and Tabakoff, 1999; Chandler et al., 2004; Mass et al., 2005). Ethanol is known to interact directly with the adenosine system by blocking nucleoside transporters in the cell membrane. The effect of this inhibition is an increase in extracellular adenosine levels and adenosine receptor activation (Diamond and Gordon, 1997). Chronic ethanol treatment has been shown to reduce cAMP levels as a consequence of the desensitization of stimulatory G protein-coupled receptors (such as adenosine A2 receptors) seen following prolonged receptor activation (Hack and Christie, 2003). Hence, ethanol's inhibitory actions on cAMP might involve G protein-coupled adenosine receptors in β-EP neurons and other cells in the hypothalamic.

In this study, we found that ethanol and DDA reduced the level of AC-cAMP while it increased gene expression and/or release of TGF-β1 in hypothalamic cells. Furthermore, the cAMP analog prevented ethanol-induced TGF-β1 release in hypothalamic cells. We found that β-EP neurons in hypothalamic cultures express TGF-β1 mRNA and proteins, and both ethanol and DDA increase the gene levels of this peptide in these neurons. These data provide the first evidence that ethanol increases TGF-β1 production and/or release by reducing AC-cAMP levels in β-EP neurons and other cells in the hypothalamic. How cAMP represses the TGF-β1 system in hypothalamic cells is not clearly understood. Most of cAMP's gene targets identified to date contain one or more cAMP-responsive elements (CREs; Borrelli et al., 1992). We do not know whether the effects of cAMP on TGF-β1 represent CREs binding to the negative elements of TGF-β1 gene promoters. One should note that the TGF-β1 promoter contains AP-2-like sequence elements (Geiser et al., 1991) which could potentially mediate cAMP responses (Imagawa et al., 1987). Alternatively, the TGF-β1 promoter includes at least three AP-1 binding sites that appear to mediate the induction of the genes activated by phorbol esters (Kim et al., 1990). The distinction between 12-O-tetradecanoyl phorbol acetate responsive elements (TREs)

and CREs has been blurred by many instances of "cross-talk" between protein kinase A and protein kinase C pathways, and it is possible that cAMP signals affect expression of the TGF-β1 gene through AP-1 binding sites present on its promoter.

We've shown here that TGF-β1 induces apoptosis of immature β-EP neurons. The apoptotic action of TGF-β1 on these neurons was similar in magnitude to those found after ethanol and DDA. TGF-β1 was ineffective in inducing apoptosis in the presence of a maximal dose of ethanol or DDA, suggesting the possibility of utilizing a common cellular mechanism by these agents in causing apoptosis of β-EP neurons. We also showed that a TGF-β1 neutralizing antibody blocks ethanol's apoptotic action in β-EP neurons. Hence, ethanol's apoptotic action on these cells appears to be resulted from increased TGF-β1 production. The role of TGF-β1 in the regulation of apoptosis has been reported in only a few nervous system structures: cultured rat immature cerebellar neurons maintained in low potassium (De Luca et al., 1996); the chick ciliary, dorsal root, retina and spinal motor neurons (Krieglstein et al., 2000; Dunker, 2001); and now rat hypothalamic cells.

The data presented here also showed activation of apoptotic molecules and inactivation of antiapoptotic molecules that are regulated by TGF-β1 in β-EP neurons after ethanol and DDA treatments. TGF-β1 has been shown to stimulate apoptosis by changing the expression of the bcl-2 class of mitochondrial proteins in non-neuronal cells (Francis et al., 2000). Of the proteins we examined, the pro-apoptotic proteins bcl-xs, bax, and bak act to stimulate apoptosis, while the anti-apoptotic peptides bcl-2 and bcl-xL inhibit apoptotic mechanisms. When the activity of the pro-apoptotic peptides predominates, cytochrome C is released to activate caspases. In our study, ethanol, TGF-β1, and DDA all reduced the mRNA levels of bcl-2 and bcl-xL while increasing the levels of bax, bak and/or bcl-xs in β-EP neurons. Furthermore, an increase in the activity of

caspase-3 in ethanol-treated cultures and caspase-3 mRNA in β-EP neurons is consistent with the amplification of apoptosis due to a loss of bcl-2 and an increase in bak. These data provide evidence that chronic ethanol exposure acting directly or via reducing cellular levels of cAMP stimulates TGF-β1 signaling that upregulates pro-apoptotic proteins but suppresses anti-apoptotic proteins to mediate ethanol's apoptotic action on β-EP neurons.

The ethanol neurotoxic action on developing hypothalamic cells may have long-term consequences, since fetal alcohol exposure is believed to cause behavioral abnormalities in alcohol-exposed offspring (Meyer and Riley, 1986). It has been shown that the neurotransmitter system that regulates the neuroendocrine response to stress is especially vulnerable to ethanol during the developmental period in rats. Behavioral and neurochemical studies indicate that defects in the ability of these rats to respond appropriately to stress appear to be due to alterations in the function of hypothalamic peptides (Weinberg et al., 1996). β-EP is one of these peptides that participates in bringing about the body's homeostasis following a stress response (Plotsky, 1986). Hence, loss of β-EP neurons can have serious consequences on the stress axis functions for the alcohol-exposed developing fetus.

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FOOTNOTES

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Reprint request to: Dr. Dipak K. Sarkar, Endocrinology Program and Department of Animal Sciences, 84 Lipman Drive, New Brunswick, NJ 08901-852, Tel 732-932-1529, Fax 732-932-4134, Email: sarkar@AESOP.RUTGERS.EDU

LEGENDS FOR FIGURES

Fig. 1. Ethanol-induced apoptosis of β-EP neurons via a caspase-3-dependent mechanism. A and B, Mean ± SEM of the nucleosome (A) and caspase-3 activity (B) in cultures that were treated with various doses of ethanol for 2 and 4 d. $^{*}P < 0.05$, compared with control. $^{**}P < 0.05$, compared with all other groups on the same day. C and D, Representative microphotographs showing β-EP immunoreactive cells in hypothalamic cultures treated 2 d with vehicle (control; C) or ethanol (100 mM; D). Arrows indicate β-EP-positive cells. "–" = 10 μm. E, Showing the change in the mean ± SEM numbers of β-EP neurons in cultures treated with ethanol (100 mM) or vehicle for 2 and 4 d. $^{*}p < 0.05$, compared with control. N = 3. F and G, Representative microphotograph showing co-localization of β-EP (red) and TUNEL (green) immunostaining in cells of hypothalamic cultures treated 2 d with vehicle (control; F) or ethanol (100 mM; G). Arrows indicate TUNEL-positive β-EP cells. "–" = 20 μm. H, Mean ± SEM of the percentage of β-EP cells that were TUNEL-positive in cultures treated with 100 mM ethanol or vehicle for various time periods. N = 3. $^{*}P < 0.05$, compared with control on the same day. J, and I The effects of various doses of caspase-3 blocker (Ac-DEVD-CHO; DEVD) on ethanol-induced alteration in the number of β-EP neurons (I) and TUNEL-positive β-EP cells (J) in hypothalamic cell cultures. $^{*}P < 0.05$, compared with control. $^{**}P < 0.05$, compared with all other doses of DEVD.

Fig. 2. Ethanol's apoptotic action is mediated by adenylyl cyclase-cAMP suppression in hypothalamic cells. A, Effects of ethanol and the AC inhibitor DDA on cellular levels of cAMP

in hypothalamic cells. Fetal hypothalamic cells were treated with ethanol (100 mM), DDA (100 μM) or vehicle (control) for 2 and 4 d. Cells were lyses and used for determination of cAMP. Data are mean ± SEM obtained from 6–8 observations. *P < 0.001, significantly different from the vehicle-treated control group on the same day. B–D, Mean ± SEM of the mRNA levels of adenylyl cyclase (AC) 6 (B), AC7 (C) and AC8 (D) in cultures treated with ethanol (100 mM), DDA (100 μM) or vehicle (control) for 2 and 4 d. *P < 0.05, compared with control. N = 6. E and F, Dose-response effects of DDA in the absence (E) or in the presence (F) of ethanol on TUNEL-positive β-EP cells. Hypothalamic cells were treated with various concentrations of DDA (10 and 100 μM) or vehicle (control) in the presence or absence of 100 mM ethanol for 2 days. Cells were processed to determine the number of TUNEL-positive β-EP cells by immunocytochemical methods. *P< 0.05, compared with control. **P < 0.01, compared with the group treated with the other dose of DDA. N = 6. G and H, Effects of dbcAMP on ethanol-induced increase in the number of TUNEL-positive β-EP cells (G) and the change in the numbers of β-EP neurons in hypothalamic cultures (H). Hypothalamic cells were treated with vehicle (control) or ethanol (100 mM) with or without dbcAMP (1.0 μM) for 2 d. Cells were used for immunocytochmical localization of the number of β-EP neurons or the number of TUNEL-positive β-EP cells. Data are mean ± SEM obtained from 4–6 observations. *P < 0.001, significantly different from the rest of the groups.

Fig. 3. Ethanol's apoptotic action is mediated by adenylyl cyclase-cAMP suppression, leading to TGF-β1 increase in β-EP cells in hypothalamic cultures. A, Representative microphotograph showing co-localization of β-EP (blue) and TGF-β1 (brown) immunostaining in hypothalamic cells in cultures. Arrows indicate TGF-β1-positive β-EP cells. B, Representative

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microphotograph showing decreased staining in a control section incubated with excess antigen TGF-β1 and its antiserum after staining for β-EP (blue). "–" = 20 μm. C, Showing the stimulatory effect of ethanol and DDA on TGF-β1 mRNA levels in hypothalamic cells in primary cultures. Cultures were treated with or without ethanol (100 mM) or DDA (100 μM) for 2 or 4 d; cells were extracted and used for mRNA measurements using RT-PCR. *P < 0.05, significantly different from the control group. N = 5–6 per group. D, Showing the stimulatory effect of ethanol and DDA on TGF-β1 release from fetal hypothalamic cells in primary cultures in serum-free defined medium. Cultures were treated with or without ethanol (100 mM) or DDA (100 μM) for 2 or 4 d. Media samples were obtained for a period of 24 hr prior to the end of the treatment and used in the TGF-β1 ELISA assay. *P < 0.05, significantly different from the control group. N = 5–7 per group. E, Showing the effect of dbcAMP on ethanol-induced increase in TGF-β1 release in hypothalamic cultures. Hypothalamic cells were treated with vehicle (control) or ethanol (100 mM) with or without dbcAMP (1.0 μM) for 2 d. Media samples were collected at 24-hr intervals and assayed for TGF-β1 levels. Data are mean ± SEM obtained from 4–6 observations. *P < 0.01, significantly different from the rest of the groups on the same treatment day. F, Showing the concentration-dependent effect of TGF-β1 on β-EP neuronal apoptosis as determined by calculating the percentage of the β-EP cells that were TUNEL-positive after treatment with TGF-β1 for 2 d. *P < 0.05, significantly different from the control group. **P < 0.05, significantly different from the 0.05 and 0.5 ng/ml of TGF-β□□□□□□□□□□□□□□□. n = 4 per group. G, Showing the inability of TGF-β1 to increase apoptosis of β-EP neurons in the presence of ethanol (100 mM). Hypothalamic cultures were treated with various concentrations of TGF-β1 in the presence of 100 mM ethanol for a period of 2 d. These cultures were processed for immunocytochemical localization of β-EP neurons that

are TUNEL positive. The mean ± SEM percentage of the β-EP cells that were TUNEL-positive are shown. N = 4 per group. H, Showing the inability of TGF-β1 to increase apoptosis of β-EP neurons in the presence of DDA (10 μM). Hypothalamic cultures were treated with various concentrations of TGF-β1 in the presence of 10 μM DDA for a period of 2 d. These cultures were processed for immunocytochemical localization of β-EP neurons that are TUNEL positive. The mean ± SEM percentage of the β-EP cells that were TUNEL-positive are shown. N = 4 per group.

Fig. 4. Showing the blocking effect of TGF-β1 neutralizing antibody on ethanol-induced apoptosis of β-EP neurons. Effect of TGF-β1 neutralizing antibody (anti-TGF-β1) on ethanol-induced β-EP neuronal apoptosis was determined by measuring the nucleosome levels in hypothalamic cells (A), the changes in the β-EP cell number (B) and the percentage of the β-EP cells that were TUNEL-positive (C). Cells were treated for 2 d with vehicle (control), ethanol (100 mM), TGF-β1 neutralizing antibody (2 μg/ml), or with ethanol (100 mM) and a TGF-β1 neutralizing antibody (anti-TGF-β1; 2 μg/ml). At the end of the experiment, cultures were used for the determination of nucleosome levels, β-EP neuron numbers and TUNEL-positive β-EP neurons. *P < 0.05, compared with the rest of the groups. N = 4–6 per group. D, Showing the inability of TGF-β1 neutralizing antibody to inhibit staurosporine (STS)-induced apoptosis in hypothalamic cells. Hypothalamic cells were treated with vehicle (control), anti-TGF-β1 (2 μg/ml), STS (0.1 or 0.5 μM), STS (0.1 or 0.5 μM) and anti-TGF-β1 (2 μg/ml) for 1 day. At the end of the experiment, cultures were used for the determination of nucleosome levels by ELISA. N = 4–5 per group. *P < 0.05, compared with control. **P < 0.05, compared with similar

treatment but with a low dose of STS□□□□□□□□□□□□□□□.

Fig. 5. Expression profiles of apoptotic regulatory genes following treatment with ethanol, TGF-β1 and the adenylyl cyclase antagonist DDA in LCM-captured β-EP neurons. Two-day-old hypothalamic cultures were incubated with medium containing serum supplement and 100 mM of ethanol, TGF-β1 (2 ng/ml), DDA (100 μM) or vehicle (control) for 2 d. A and B, Representative photos showing an hypothalamic culture immunostained for β-EP antigen. Photo A shows arrow points on some β-EP cells before LCM dissection in a representative culture. Photo B shows the same hypothalamic culture after microdissection. "–" = 10 μm. C, Representative photograph of the thermoplastic film-coated caps which contained the captured cells. D-L, Histograms showing changes in the levels of TGF-β1 (D), AC7 (E), AC8 (F), bcl-2 (G), bcl-xL (H), bcl-xs (I), bax (J), bak (K) and capsase-3 (L) following treatment with ethanol, TGF-β1, DDA or control. *P < 0.05, compared to the control group. N =4 per group.

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TABLES AND FIGURES

Table 1. Gene-specific Primers and Probes

mRNA species	Primer & Probe sequences	Amplicon size (bp)	Accession number
AC6	F: AGATCAAGACCATCGGTAGCACTT	150	M96160
	R: TGAAAGAGTGTTCGTTGATGTGTTT		
	P: CCTCCGGGCTAAATGCCAGCACCTAT		
AC7	F: GCTCCTACTGAAGCCCAAGTTC	256	AF184150
	R: AATCACTCCAGCAATCACAGGC		
AC8	F: ACCGGTGGCACCAAAGTCT	151	L26986
	R: TGACCCCACGGTAGCTGTATC		
	P: ACCTTGACTGTGCCCCAAGTAACTCGGA		
TGF-ß1	F: GAATACAGGGCTTTCGCTTCA	137	X52498
	R: CAGGAAGGGTCGGTTCATGT		
	P: TCAGTCCCAAACGTCGAGGTGACCTG		
Bcl-2	F: CATGTGTGTGGAGAGCGTCAA	186	U34964
	R: TTCAGAGACTGCCAGGAGAAATC		
	P: AGTACCTGAACCGGCATCTGCACACC		
Bcl-xL	F: AAGGAGATGCAGGTATTGGTGAGT	121	U34963
	R: TCCCGTAGAGATCCACAAAAGTG		
	P: TGGATGGCCACCTACCTGAATGACCA		
Bcl-xs	F: ATCAGAGCTTTGAACAGGACACTTT	127	AF279286
	R: AGAACTACACCAGCCACAGTCATG		
	P: ACAATGCAGCAGCCGAGAGCCG		
Bax	F: CTCAAGGCCCTGTGCACTAAA	171	U49729
	R: AGCCACAAAGATGGTCACTGTCT		
	P: TCCTACTTCGGGACCCCCACATGG		
Bak	F: CGACACGGAGTTCCAGAATTTAC	94	AF259504
	R: CTTAAATAGGCTGGAGGCGATCT		
	P: CGCTGGGAATGCCTACGAACTCTTCA		
Caspase-3	F: CCCGGTTACTATTCCTGGAGAA	87	U49930
	R: CTTGTGCGCGTACAGTTTCAG		
	P: TGGTTCATCCAGTCACTTTGCGCCAT		

F = forward primer; R = reverse primer; P = probe.

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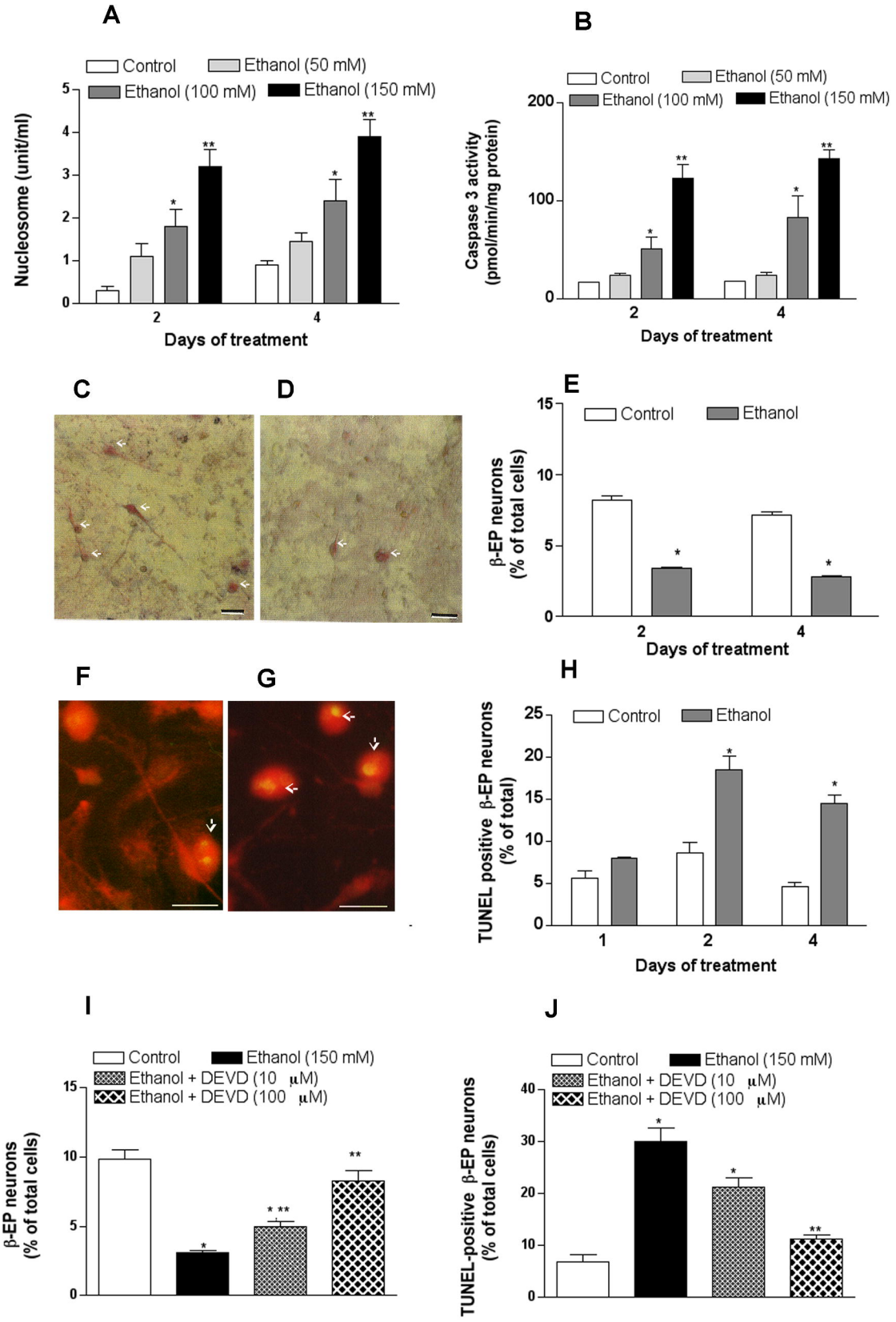

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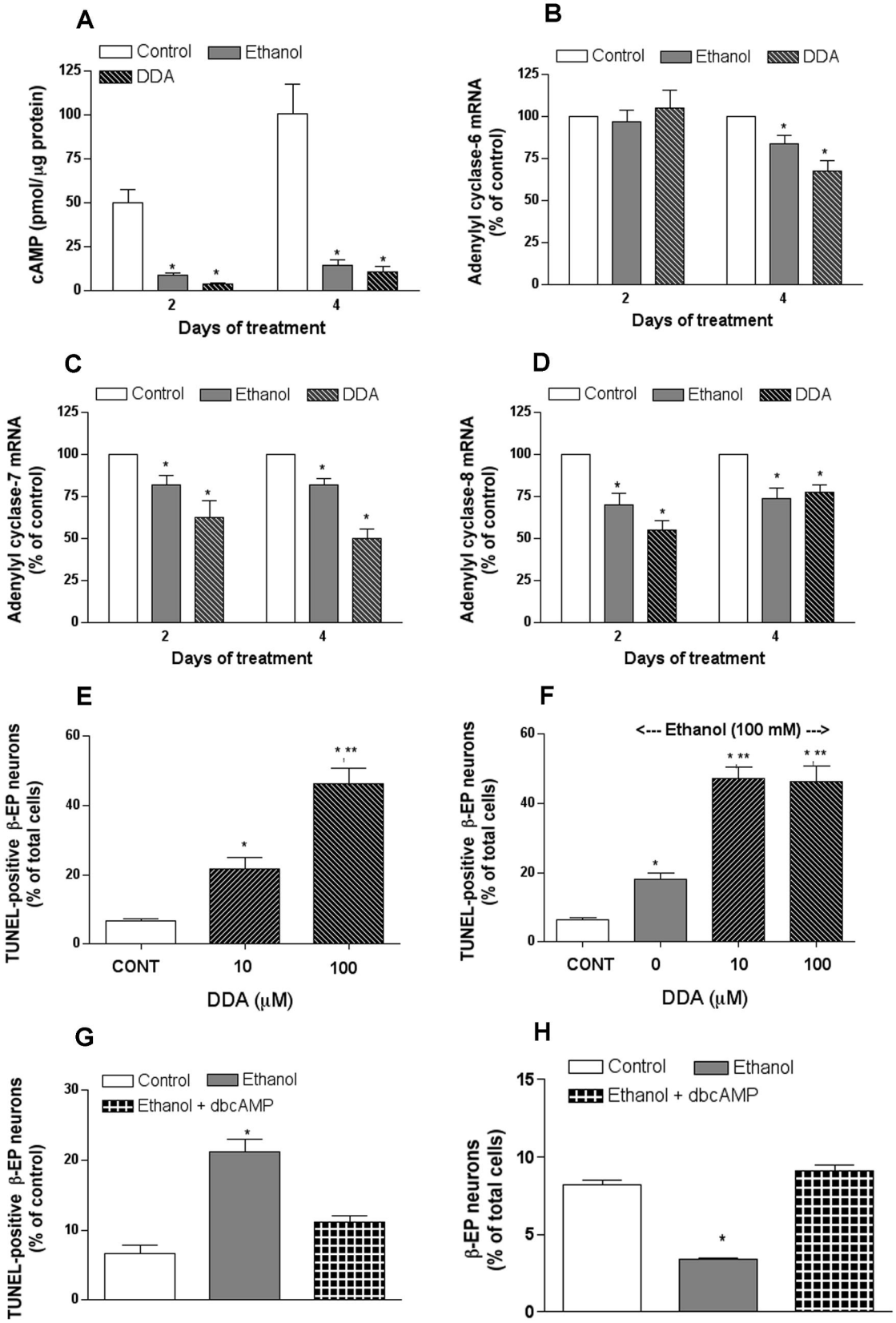

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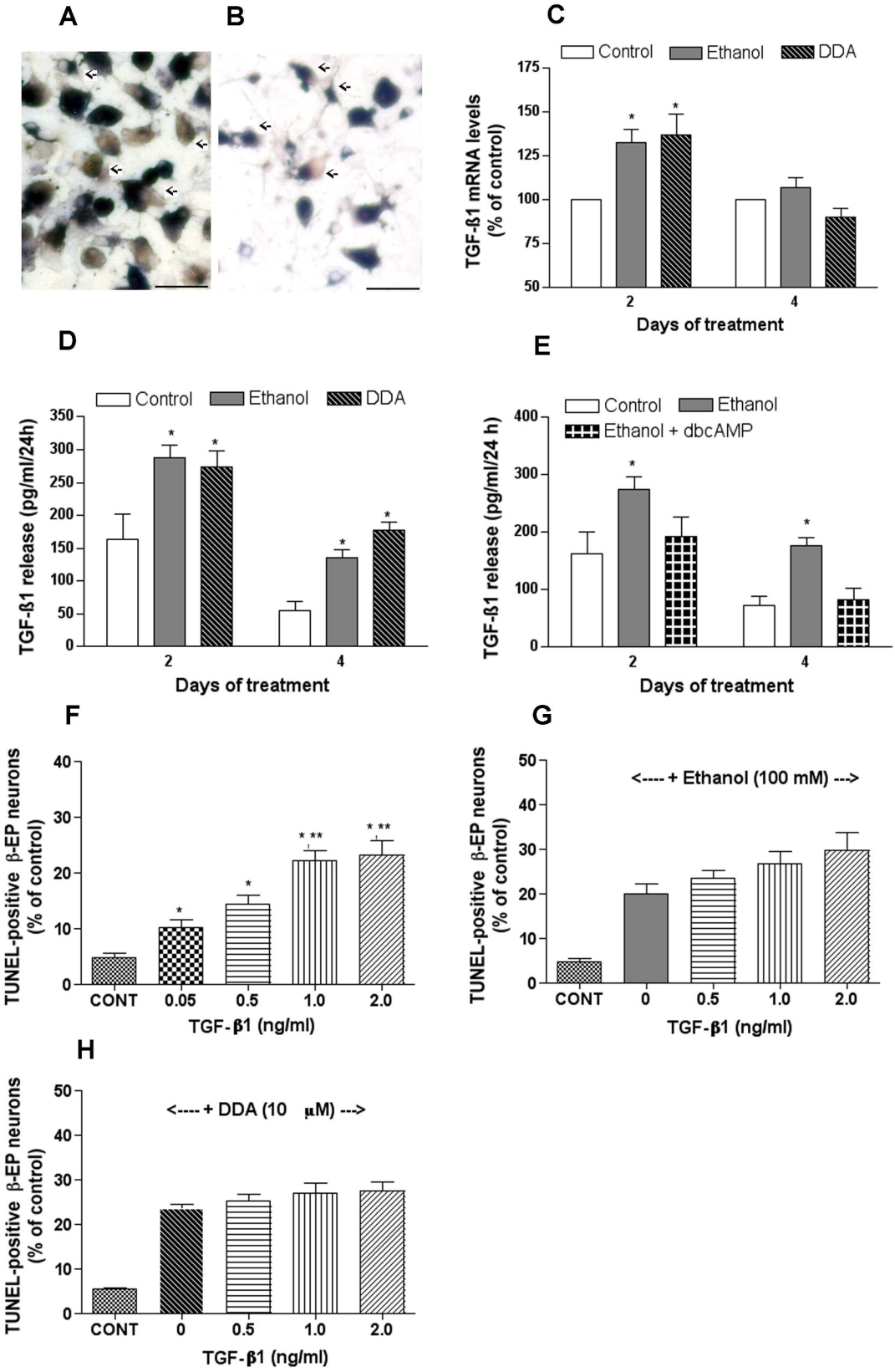

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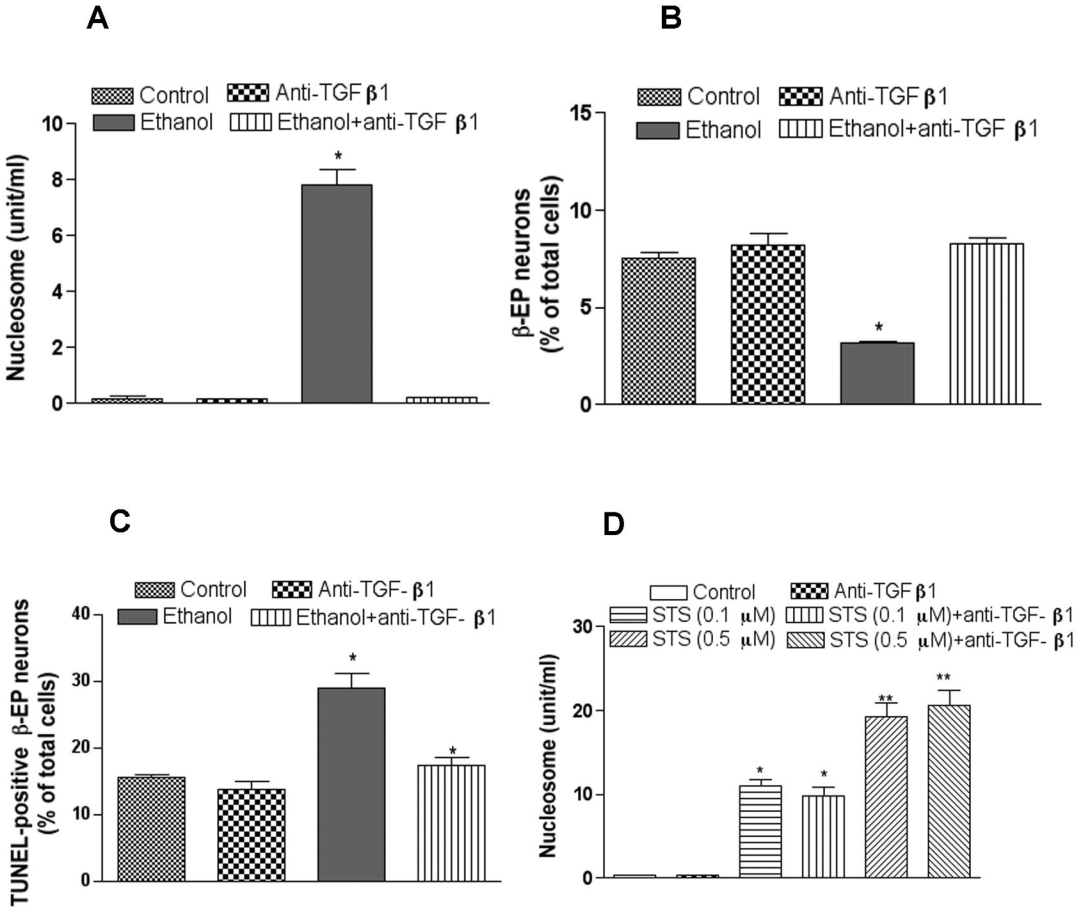

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