2006

Interaction Between Rax And Pkr Modulates The Effect Of Ethanol On Protein Synthesis And Survival Of Neurons

Gang Chen

Cuiling Ma

Kimberly A. Bower

Zunji Ke

Jia Luo

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 23, pp. 15909–15915, June 9, 2006

Interaction between RAX and PKR Modulates the Effect of Ethanol on Protein Synthesis and Survival of Neurons*

Received for publication, January 20, 2006, and in revised form, March 24, 2006 Published, JBC Papers in Press, March 29, 2006, DOI 10.1074/jbc.M600612200

Gang Chen[‡], Cuiling Ma[‡], Kimberly A. Bower[‡], Zunji Ke[§], and Jia Luo[‡§1]

From the [‡]Department of Microbiology, Immunology, and Cell Biology, West Virginia University School of Medicine, Robert C. Byrd Health Sciences Center, Morgantown, West Virginia 26506 and [§]Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

Ethanol exposure inhibits protein synthesis and causes cell death in the developing central nervous system. The double-stranded RNA (dsRNA)-activated protein kinase (PKR), a serine/threonine protein kinase, plays an important role in translational regulation and cell survival. PKR has been well known for its anti-viral response. Upon activation by viral infection or dsRNA, PKR phosphorylates its substrate, the α-subunit of eukaryotic translation initiation factor-2 (eIF2α) leading to inhibition of translation initiation. It has recently been shown that, in the absence of a virus or dsRNA, PKR can be activated by direct interactions with its protein activators, PACT, or its mouse homologue, RAX. We have demonstrated that exposure to ethanol increased the phosphorylation of PKR and eIF2α in the developing cerebellum. The effect of ethanol on PKR/eIF2α phosphorylation positively correlated to the expression of PACT/RAX in cultured neuronal cells. Using PKR inhibitors and PKR null mouse fibroblasts, we verified that ethanol-induced eIF2α phosphorylation was mediated by PKR. Overexpression of a wild-type RAX dramatically enhanced sensitivity to ethanol-induced PKR/eIF2α phosphorylation, as well as translational inhibition and cell death. In contrast, overexpression of a mutant (S18A) RAX inhibited ethanol-mediated PKR/eIF2α activation. Ethanol promoted PKR and RAX association in cells expressing wild-type RAX but not in cells expressing S18A RAX. S18A RAX functioned as a dominant negative protein and blocked ethanol-induced inhibition of protein synthesis and cell death. Our results suggest that the interactions between PKR and PACT/RAX modulate the effect of ethanol on protein synthesis and cell survival in the central nervous system.

Fetal alcohol syndrome is the most common non-hereditary cause of mental retardation (1). Prenatal exposure to alcohol disrupts many events of neuronal development, including neurogenesis, migration, cell survival, protein synthesis, axonal growth, and synaptogenesis (2–5). Neuronal death is a prominent pathologic effect of fetal alcohol exposure. This loss of neurons may underlie many of the behavioral deficits observed in fetal alcohol syndrome. The vulnerability of neurons to alcohol neurotoxicity differs among brain regions and changes with developmental stages (6, 7). The causes for ethanol-induced neuronal loss remain incompletely elucidated, as are the cellular and molecular mechanisms underlying the spatiotemporal window of susceptibility.

The double-stranded RNA (dsRNA)[2]-activated protein kinase (PKR) is a serine/threonine protein kinase ubiquitously expressed in mammalian cells (8, 9). PKR is initially identified as an interferon-induced protein that is activated in virus-infected cells by dsRNA produced during the virus life cycle (10, 11). PKR consists of two functionally distinct domains, an N-terminal dsRNA binding regulatory domain and a C-terminal catalytic domain. PKR is activated by dsRNA (11); interaction with dsRNA causes PKR to form homodimers and to autophosphorylate on multiple serine/threonine residues, including threonine 446 and 451 (12–14). PKR is a component of signal transduction pathways mediating many important cellular functions, such as survival, proliferation, differentiation, and stress responses (10, 12, 15, 16). In addition to dsRNA, PKR can be activated by cytokines, growth factors, serum deprivation, bacterial products, or physiochemical stress (12, 16, 17). Recent studies indicate that dsRNA-independent activation may be mediated by protein activators of PKR, PACT, and its mouse homologue, RAX (18–20). Following autophosphorylation, PKR catalyzes the phosphorylation of target substrates, the most well characterized being the α-subunit of eukaryotic initiation factor-2 (eIF2α) (12). Phosphorylated eIF2α sequesters eIF2B, a rate-limiting component of translation, leading to an inhibition of protein synthesis in the cells. In some cases, eIF2α phosphorylation leads to cell death (21).

In this study, we have demonstrated that ethanol promotes PKR/RAX interaction; this interaction in turn activates PKR and induces eIF2α phosphorylation. The levels of RAX expression determine the cellular sensitivity to ethanol. These findings provide important insight into the mechanisms of ethanol-induced damage to the CNS.

EXPERIMENTAL PROCEDURES

Materials—Sprague-Dawley rats were obtained from Hilltop Laboratory Inc. (Scottdale, PA). 2-Aminopurine (2-AP) was purchased from Sigma, and a selective PKR inhibitor was purchased from Calbiochem (La Jolla, CA; catalogue number 527450). All antibodies, except anti-PACT and anti-ATF4, were obtained from Cell Signaling Technology (Beverly, MA). The anti-PACT and anti-ATF4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Treatment—Human neuroblastoma cells (SH-SY5Y and SK-N-MC cells) and a human embryonic kidney cell line (HEK293) were obtained from the American Type Culture Collection. These cells were grown in minimum Eagle's medium containing 10% fetal bovine serum, 2 mM L-glutamine, and 25 μg/ml gentamycin at 37 °C with 5% CO_2. PKR+/+ and PKR−/− mouse embryonic fibroblasts (MEFs)

* This research was supported by Grants AA015407 and AA013984 from the National Institutes of Health and a grant from the Nature and Sciences Foundation of the Chinese Academy of Sciences (30470544). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Dept. of Microbiology, Immunology, and Cell Biology, West Virginia University School of Medicine, Morgantown, WV 26506. Tel.: 304-293-7208; Fax: 304-293-7823, E-mail: jluo@hsc.wvu.edu.

[2] The abbreviations used are: dsRNA, double-stranded RNA; CNS, central nervous system; eIF2α, eukaryotic initiation factor-2α; HA, hemagglutinin; MEF, mouse embryonic fibroblasts; PACT, PKR-activating protein; PERK, PKR-like endoplasmic reticular kinase; PKR, double-stranded RNA-activated protein kinase; RAX, PKR activator X; WT, wild-type; 2-AP, 2-aminopurine; IL, interleukin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; p-, phosphorylated.

were obtained from Dr. Antonis E. Koromilas (Lady Davis Institute for Medical Research, McGill University, Quebec, Canada) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 units/ml) at 37 °C with 5% CO_2. Cerebellar granule neurons (CGNs) were isolated from the cerebella of 7-day-old rat pups. CGNs were cultured for 48 h before exposing them to ethanol *in vitro*. The procedure for preparation and culture of CGNs has been previously described (22, 23).

Ethanol Exposure Protocol—Due to the volatility of ethanol, a method utilizing sealed containers was used to maintain ethanol levels in the culture medium (24). With this method, ethanol concentrations in the culture medium could be accurately maintained. *In vivo* ethanol exposure was achieved through intragastric intubation as described by Green *et al.* (25). Briefly, two male and two female pups (postnatal day 9) of a given litter were given ethanol (6.72 g/kg/day) by two intragastric intubations separated by 2 h. The mean blood alcohol concentration produced by the paradigm is ~352 mg/dl. For controls, two male and two female pups of the same litter received the same intubation procedure without ethanol exposure (the sham-intubated group). Two paradigms of ethanol exposure were used. With a short exposure, ethanol was given on only postnatal day 9. With a paradigm of longer exposure, ethanol was delivered during postnatal days 6–9. Two hours after the last intubation, the pups were sacrificed and cerebella were dissected. Proteins were extracted and stored at −80 °C until further used. All animal protocols were approved by the Animal Care and Use Committee of West Virginia University and complied with the National Institutes of Health guidelines for animal care and use.

MTT Assay—The number of viable cells in culture was determined by the MTT assay as previously described (22).

Cell Transfection and Establishment of Stable Transfectants—Hemagglutinin (HA)-tagged RAX construct and S18A mutant (substitution of serine 18 to alanine) carried by vector pcDEF3 were generous gifts from Dr. Stratford May (University of Florida, Gainesville, FL) (26). Cell transfection was carried out with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Stable cell clones expressing exogenous RAX were screened by the treatment of G418 (600 μg/ml) for 3–4 weeks. Positive clones were verified by the expression of HA as well as the overexpression of RAX. The clones expressing the highest level of RAX were selected.

Protein Synthesis Assay—Protein synthesis was measured by the incorporation of ^{14}C-labeled amino acids (Amersham Biosciences; catalogue number CFB104). Briefly, cells were seeded in 24-well cell culture trays at the density of 5×10^5 cells/well. After ethanol treatment for the indicated time, 3 μCi of ^{14}C-labeled amino acid mix was added to the wells and incubated at 37 °C for 20 min, and then the medium was removed; the cells were lysed with 1 volume of cold radioimmune precipitation assay buffer (150 mM NaCl, 50 mM Tris, pH 8, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid sodium, 0.1% mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 3% aprotinin). Reactions were stopped by the addition of 1 volume of cold 20% trichloroacetic acid. The acid-insoluble fractions were collected on Whatman glass fiber filters (47 mM GF/A), and radioactivity was measured by a liquid scintillation counter.

Immunohistochemistry—Cerebella were sectioned parasagitally at a thickness of 12 μm using a cryostat and stored at −20 °C until further processing. The expression of RAX in the cerebella was determined by immunohistochemistry using a specific anti-PACT antibody (catalogue number 18768; Santa Cruz Biotechnology). The procedure for immunohistochemistry has been previously described (27). The specificity of this antibody has been confirmed by immunoblots. Negative controls were performed by omitting the primary antibody.

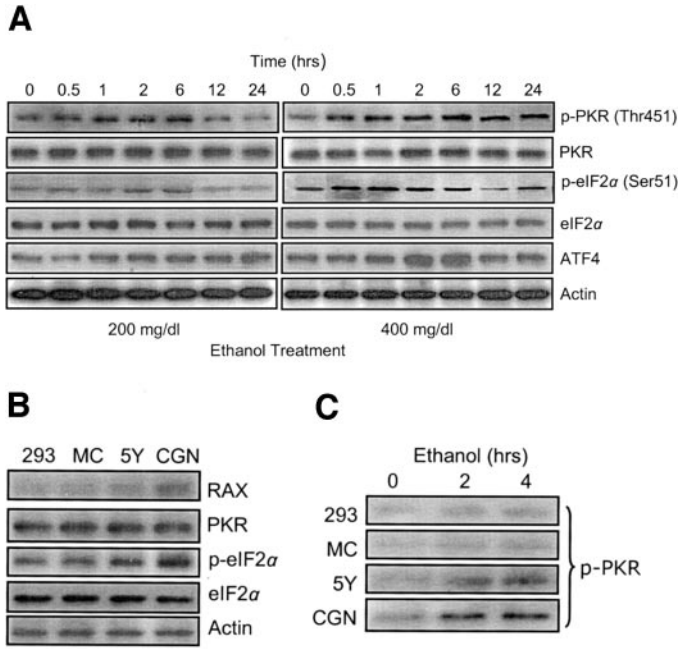

FIGURE 1. **Effect of ethanol on PKR and eIF2α phosphorylation *in vitro*.** *A*, SH-SY5Y cells cultured in serum-free medium were exposed to ethanol (0, 200, or 400 mg/dl) for 0.5–24 h. Cellular protein was extracted and analyzed by immunoblotting using antibodies directed against either phosphorylated PKR or eIF2α (*p-PKR* or *p-eIF2*α) as described under "Experimental Procedures." The expression of a downstream target of p-eIF2α (*ATF4*) was also examined. *B*, expression of PACT/Rax, PKR, eIF2α, and p-eIF2α in human embryonic kidney cell line HEK293 (*293*), human neuroblastoma SK-N-MC (*MC*), SH-SY5Y (*5Y*) cells, and cerebellar granule neurons (*CGNs*) was determined by immunoblotting. CGNs were isolated from the rat cerebella of 7-day-old rat pups. *C*, effect of ethanol (0 or 400 mg/dl) on PKR phosphorylation in human embryonic kidney cell line HEK293 (*293*), human neuroblastoma SK-N-MC (*MC*), SH-SY5Y (*5Y*) cells, and cerebellar granule neurons (*CGNs*) was determined by immunoblotting as described above. The experiment was replicated three times.

Immunoblotting and Immunoprecipitation—The procedure for immunoblotting has been previously described (22). Immunoprecipitation was performed as previously described (28). Briefly, an aliquot of cell lysate containing 200 μg of protein was incubated with either anti-PKR or anti-PACT antibody (1:50) overnight at 4 °C. Twenty microliters of protein A/G conjugated to agarose (Santa Cruz Biotechnology) were added to the lysate, and the mixture was incubated for 3 h at 4 °C. Immunoprecipitates were collected by centrifugation at 10,000 × *g* for 10 min. The pellet was washed three times with 0.5 ml of radioimmune precipitation assay buffer. Thereafter, the pellets were resuspended in 20 μl of 3× SDS sample buffer and analyzed for the expression of specific proteins by immunoblotting. The amount of protein expression was quantified with a densitometer using the software Optimas version 6.2 (Media Cybernetics, Silver Spring, MD).

Statistical Analysis—Differences among treatment groups were tested using analysis of variance. Differences in which $p = <0.05$ were considered statistically significant. In cases where significant differences were detected, specific post hoc comparisons between treatment groups were examined with Student-Newman-Keuls tests.

RESULTS

Ethanol Induces Phosphorylation of PKR and eIF2α—The effect of ethanol on the phosphorylation of PKR and eIF2α was first examined in SH-SY5Y cells. Ethanol (200 and 400 mg/dl) modestly increased phosphorylation of PKR and eIF2α; at 400 mg/dl, it caused a more sustained phosphorylation of PKR and eIF2α (Fig. 1*A*). As a result, the expression

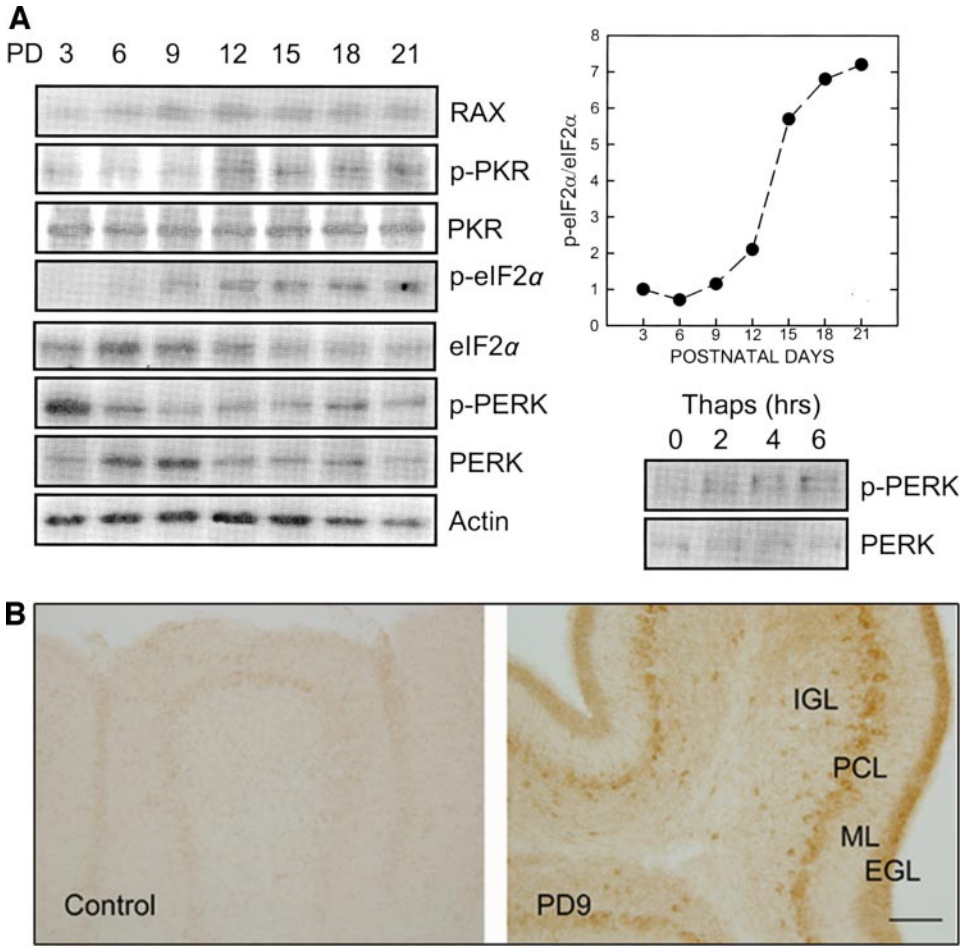

FIGURE 2. **Expression of RAX in the developing cerebellum.** *A*, cerebella were isolated from rat pups aged from postnatal days (*PD*s) 3 to 21. The expression of RAX, PKR, eIF2α, and PERK was determined by immunoblotting. The ratio of p-eIF2α/eIF2α is given at the *upper panel* on the *right*. A positive control for phosphorylated PERK (SH-SY5Y cells treated with thapsigargin) is shown at the *lower panel* on the *right*. The experiment was replicated three times. *B*, localization of RAX was determined by immunohistochemistry. Negative controls were performed by omitting the primary antibody. *EGL*, external granule layer; *ML*, molecular layer; *PCL*, Purkinje cell layer; *IGL*, internal granule layer. A representative image of the cerebellum from a postnatal day 9 rat pup is shown. *Scale bar* = 150 μM.

of a downstream effector of p-eIF2α, ATF4, was up-regulated by ethanol exposure. We examined the expression of PACT/RAX in neuronal cells (SH-SY5Y and SK-N-MC neuroblastoma cells and primary CGNs) and a non-neuronal cell line (embryonic kidney cell line HEK293). Consistent with previous findings showing the expression of PACT/RAX as generally low in mammalian cells (18, 19, 29), these cells expressed PACT with low abundance. Among these cells, CGNs isolated from rats of postnatal day 7 exhibited the highest expression of RAX (Fig. 1*B*). Interestingly, CGNs (the cells with the highest PACT/RAX expression) were also most sensitive to ethanol-stimulated PKR phosphorylation (Fig. 1, *B* and *C*).

The developing rat cerebellum has been extensively used for investigating ethanol-induced neurotoxicity. Cerebellum of postnatal days 4–10 represents a window of vulnerability to ethanol exposure (30–32). We examined the expression of RAX, PKR, and eIF2α in the developing cerebellum. A rapid increase in RAX expression was observed during postnatal days 6–9 (Fig. 2*A*). RAX expression decreased slightly from postnatal day 12 and remained stable thereafter. The phosphorylated form of PKR and eIF2α increased dramatically from postnatal day 9 and continued to remain at a high level. We also examined the expression of another eIF2α kinase, PKR-like endoplasmic reticular kinase (PERK). Interestingly, the level of phosphorylated PERK was relatively low during postnatal days 9–12. Immunohistochemical analysis demonstrated a strong RAX staining in the external granule layer and Purkinje cell layer in the cerebellum of postnatal day 9 (Fig. 2*B*). Some RAX-positive cells were scattered in the internal granule layer. To determine whether *in vivo* exposure to ethanol altered PKR/eIF2α activity, we delivered ethanol to rat pups through intragastric intubation. As shown in Fig. 3,

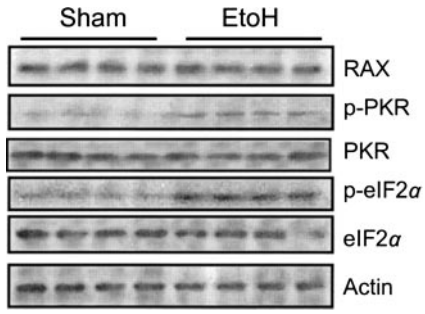

FIGURE 3. **Effect of ethanol on the developing cerebellum.** Four rat pups (9-day-old) of a given litter were exposed to ethanol (*EtoH*) through intragastric intubation as described under "Experimental Procedures." As a control, four rat pups received the same intubation procedure without ethanol exposure (the *Sham*-intubated group). Two hours after the second intubation (4 h following initial ethanol exposure), the pups were sacrificed and cerebella were dissected. The expression of RAX and phosphorylated as well as total PKR and eIF2α was determined by immunoblotting. Each *lane* represents a sample collected from an individual pup. The experiment was replicated three times.

ethanol exposure increased the phosphorylation of PKR and eIF2α in the cerebella of postnatal day 9 rats (Fig. 3). Ethanol did not affect the expression of RAX, PKR, and eIF2α. Similar results were observed following longer exposure to ethanol (postnatal days 6–9) (data not shown).

In addition to PKR, other eIF2α kinases can also phosphorylate eIF2α; these include PERK, heme-regulated inhibitor and GCN2 family members (16). We therefore sought to determine whether ethanol-induced eIF2α phosphorylation was mediated by PKR. As shown in Fig. 4*A*, two selective inhibitors of PKR (2-AP and PKR-I) effectively blocked etha-

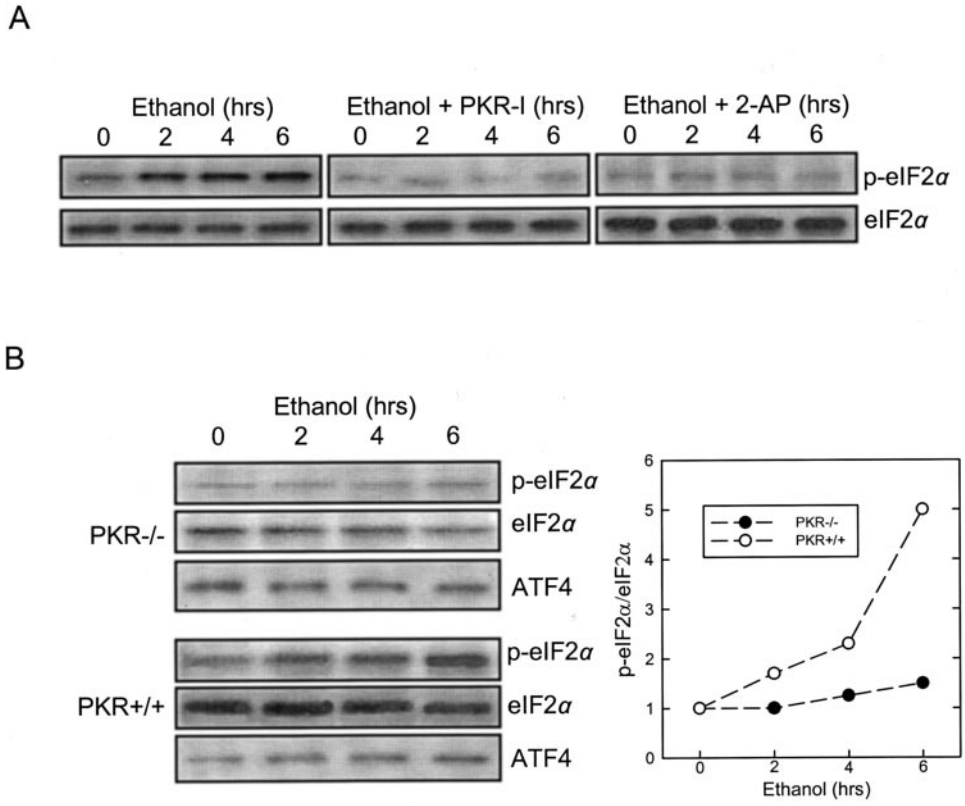

FIGURE 4. **PKR mediates ethanol-induced phosphorylation of eIF2α.** *A*, SH-SY5Y cells were pretreated with PKR inhibitors, 2-AP (10 mM), or PKR-I (500 nM) for 30 min and then exposed to ethanol (0 or 400 mg/dl) for 2–6 h. The expression of p-eIF2α was determined by immunoblotting. *B*, PKR+/+ and PKR−/− MEFs were exposed to ethanol (0 or 400 mg/dl) for 2–6 h. The expression of p-eIF2α was determined by immunoblotting. The expression of a downstream target of p-eIF2α (*ATF4*) was also examined. The ratio of p-eIF2α/eIF2α is shown at the *panel* on the *right*. The experiment was replicated three times.

nol-induced eIF2α phosphorylation in SH-SY5Y cells, suggesting that PKR was involved in ethanol-induced eIF2α phosphorylation. The observation was further validated by an experiment using PKR knockout MEFs. As shown in Fig. 4*B*, ethanol promoted eIF2α phosphorylation in PKR+/+ cells, but not in PKR−/− MEF cells. Consistently, ethanol increased ATF4 expression only in PKR+/+ cells (Fig. 4*B*).

Overexpression of PACT/RAX Enhances Sensitivity to Ethanol—The correlation between PACT/RAX expression and ethanol-induced PKR/eIF2α phosphorylation led us to postulate that PACT/RAX played a role in the action of ethanol. To determine whether a high expression of PACT/RAX facilitated ethanol-induced PKR/eIF2α phosphorylation, we artificially increased the levels of PACT/RAX expression by transfecting SK-N-MC cells with either a wild-type (WT) or a mutant (S18A) RAX cDNA. It has been previously shown that phosphorylation of RAX at serine 18 plays an essential role in PKR activation. Overexpression of RAX was verified by immunoblotting using anti-HA and anti-PACT antibodies (Fig. 5*A*). As shown in Fig. 5*B*, overexpression of WT RAX in SK-N-MC cells dramatically enhanced ethanol-stimulated PKR and eIF2α phosphorylation, compared with vector-transfected cells. In contrast, overexpression of S18A RAX inhibited ethanol-induced PKR/eIF2α phosphorylation. We then established stable transfectants overexpressing WT or S18A RAX and examined the effect of ethanol on PKR and eIF2α phosphorylation; similar results were obtained (Fig. 5*C*). The observation was not limited to neuronal cells; overexpression of RAX also sensitized HEK293 cells to ethanol-induced PKR/eIF2α phosphorylation (Fig. 5*D*). These findings validated that a high expression of PACT/RAX facilitated ethanol-mediated PKR/eIF2α phosphorylation.

Ethanol Promotes the Interaction between PKR and PACT/RAX—We sought to determine whether ethanol promoted the interaction between PKR and PACT/RAX. We examined the effect of ethanol on the association between PKR and PACT/RAX in SK-N-MC cells stably transfected with WT RAX, S18A RAX, or an empty vector. As shown in Fig. 6, the association between RAX and PKR was demonstrated by their co-immunoprecipitation. In cells overexpressing WT RAX, ethanol promoted the association between PKR and PACT/RAX. Although PKR and S18A RAX were co-immunoprecipitated, ethanol failed to enhance the association between PKR and PACT/RAX in cells overexpressing S18A RAX (Fig. 6, *bottom panel*). No co-immunoprecipitation of PKR and PACT/RAX was detected in the cells expressing vector only. This was probably because of the low endogenous level of PACT/RAX in SK-N-MC cells. We also observed that ethanol enhanced PKR and PACT/RAX association in HEK293 cells overexpressing WT RAX (data not shown). In all cases, however, ethanol did not promote PKR binding to RAX in cells overexpressing S18A RAX.

Overexpression of PACT/RAX Enhances Ethanol-induced Protein Synthesis Inhibition and Cell Death—The activity of eIF2α regulates protein synthesis. Since we showed that eIF2α phosphorylation was enhanced by ethanol in a PACT/RAX-dependent manner, we sought to determine whether ethanol-inhibited protein synthesis also depended on the status of PACT/RAX expression. As shown in Fig. 7, ethanol exposure (400 mg/dl, 6 h) decreased the rate of protein synthesis by 32% in vector-transfected SK-N-MC cells; however, it inhibited the rate of protein synthesis by 63% in cells overexpressing WT RAX. Ethanol only modestly (16%) affected protein synthesis in cells transfected with S18A RAX. A similar result was observed following exposure to ethanol for 12 h (Fig. 7). During this period of exposure (6–12 h), ethanol did not significantly affect cell number (data not shown). Therefore, the decrease in the rate of protein synthesis did not result from an alteration in cell number.

We further determined whether PACT/RAX modulated the effect of ethanol on cell survival. As shown in Fig. 8, ethanol (400 mg/dl, 48 h) decreased the number of SK-N-MC cells transfected with an empty vector by ~16%. On the other hand, in SK-N-MC cells stably transfected with WT RAX, ethanol-induced cell loss was increased to 33%. Ethanol failed to alter

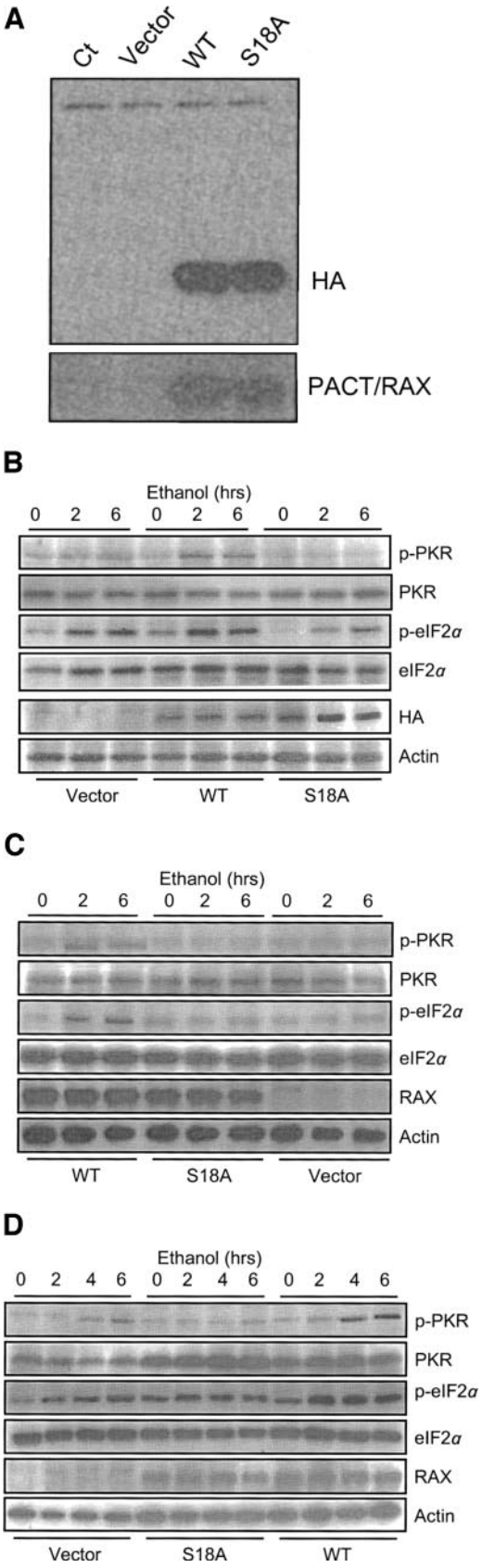

FIGURE 5. **Effect of ethanol on the phosphorylation of PKR and eIF2α in SK-N-MC cells overexpressing wild-type (*WT*) and mutant (*S18A*) RAX.** *A*, SK-N-MC cells were transiently transfected with either an empty vector or a vector carrying a wild-type RAX (*WT*) or a mutant (*S18A*) RAX cDNA that was tagged with a HA sequence. The expression of exogenous RAX was determined by immunoblotting using an anti-HA antibody, and overexpression of RAX was verified by immunoblotting using an anti-PACT antibody. *Ct*, control. *B*, forty-eight hours after the transfection with RAX (*WT* or *S18A*), SK-N-MC cells were exposed to ethanol (0 or 400 mg/dl) for 2–6 h. The phosphorylation of PKR and eIF2α was determined as described above. *C*, SK-N-MC cells that stably expressed WT or S18A RAX were established as described under "Experimental Procedures." These cells were exposed to ethanol (0 or 400 mg/dl) for 2–6 h. The phosphorylation of PKR and eIF2α was determined as described above. *D*, HEK293 cells were transiently transfected with either WT or S18A RAX and then exposed to ethanol (0 or 400 mg/dl) for 2–6 h. The phosphorylation of PKR and eIF2α was determined as described above. The experiment was replicated three times.

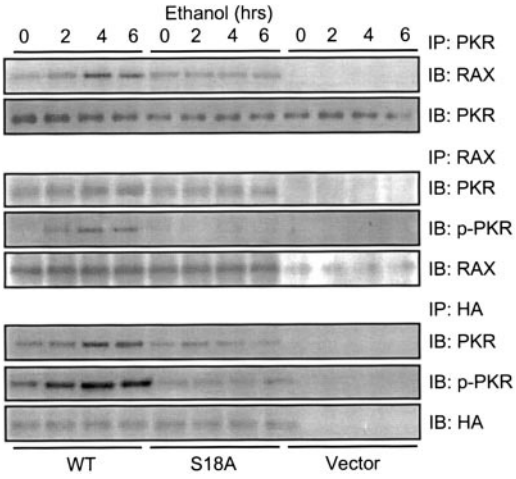

FIGURE 6. **Ethanol promotes an association between RAX and PKR.** SK-N-MC cells that stably overexpressed either WT or S18A RAX were exposed to ethanol (0 or 400 mg/dl) for 2–6 h, and cell lysates were collected. *Top panel*, two hundred μg of cell lysates for each treatment group were immunoprecipitated (*IP*) with an anti-PKR antibody and probed with either an anti-RAX or PKR antibody. *Middle panel*, cell lysates for each treatment group were immunoprecipitated with an anti-PACT antibody and probed with antibodies directed against p-PKR, PKR, or PACT. *Bottom panel*, cell lysates for each treatment group were immunoprecipitated with an anti-HA antibody and probed with antibodies directed against p-PKR, PKR, or HA. The experiment was replicated three times. *IB*, immunoblot.

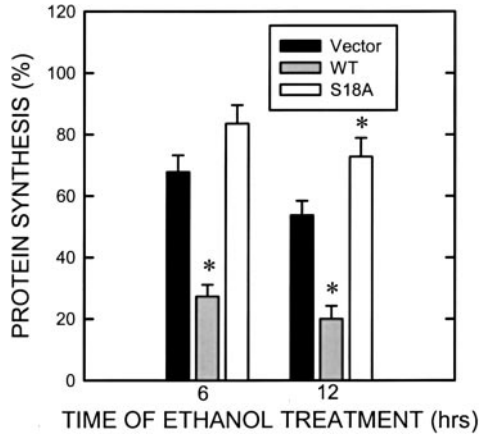

FIGURE 7. **Effect of ethanol on protein synthesis.** SK-N-MC cells that stably overexpressed either WT or S18A RAX were exposed to ethanol (0 or 400 mg/dl) for 6–12 h. The rate of protein synthesis was determined as described under "Experimental Procedures." The cells stably transfected with an empty vector were used as a control. The experiment was replicated three times. The *asterisk* denotes significant difference from controls (transfected with an empty vector).

the number of cells overexpressing S18A RAX. Because these cells were maintained in serum-free medium and did not proliferate, the decrease in cell number must have resulted from ethanol-induced cell death. Furthermore, pretreatment of 2-AP was sufficient to block ethanol-induced cell loss (Fig. 8). The protective effect of PKR inhibition was observed using another selective PKR inhibitor (data not shown). This indicated that the effect of ethanol was mediated by PKR.

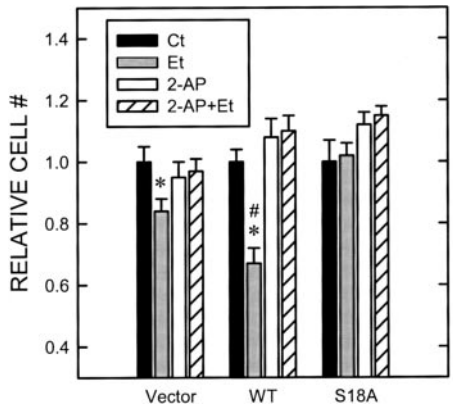

FIGURE 8. **Effect of ethanol on cell survival.** SK-N-MC cells that stably overexpressed either W T or S18A RAX were pretreated with a PKR inhibitor, 2-AP (10 mM), for 30 min and then exposed to ethanol (*Et*) (0 or 400 mg/dl) for 48 h. The number of viable cells was determined by MTT assay as described under "Experimental Procedures." The experiment was replicated three times. The *asterisk* denotes significant difference from controls (transfected with an empty vector) (*Ct*). # denotes significant difference from empty vector-transfected and ethanol-exposed cells.

DISCUSSION

Ethanol-mediated PKR/RAX Interaction—PKR is a ubiquitously expressed serine/threonine kinase. Traditionally, PKR has been studied in the context of the host anti-viral response (15, 16). PKR contains two dsRNA binding domains and is activated by viral dsRNA, resulting in the phosphorylation of a physiological substrate of PKR, eIF2α, and consequent translation inhibition. In addition to its role in translation, PKR has been implicated as a signal integrator in transcriptional control pathways (16). For example, the activity of transcription factors, such as NFκB, p53, STAT1, and STAT3, is regulated by PKR (16). PKR is also an essential mediator of signaling by both cytokines and growth factors (16). PKR participates in the signaling of the stress-activated protein kinase (p38) and c-Jun NH_2-terminal kinase (JNK) in response to extracellular signals acting through cell surface receptors (16). PKR can be activated under many circumstances that lack apparent sources of dsRNA. This dsRNA-independent activation is believed to be mediated by the PKR-activating protein PACT and its mouse homologue RAX (18, 19, 26). PACT and RAX are the only known cellular activators for PKR so far; they are 98% identical in amino acid sequence and contain three conserved dsRNA binding motifs. It has been demonstrated that PACT/RAX can efficiently activate PKR *in vitro*, a cell-free system (18, 19). However, *in vivo*, PACT/RAX-mediated activation is dependent on stress applications to the cells (18, 20, 26, 33). Our study indicates that PACT/RAX is an important mediator of the action of ethanol. Although ethanol only induces a modest activation of PKR/eIF2α in cells expressing low PACT/RAX, a high expression of PACT/RAX dramatically enhances ethanol-induced PKR/eIF2α phosphorylation. Ethanol apparently does not affect the expression of PACT/RAX; it promotes the association between PACT/RAX and PKR. Ethanol enhances co-immunoprecipitation of RAX and PKR in cells overexpressing PACT/RAX. In control cells, however, we do not observe this enhancement. This is probably because of the low abundance of PACT/RAX in these cells. Similar to ours, several studies show that other stress signals caused by serum starvation, arsenite, thapsigargin, peroxide, and IL-3 deprivation can promote PACT/RAX and PKR association, which leads to PKR activation and eIF2α phosphorylation (18, 20, 26).

Phosphorylation of RAX at serine 18 seems critical for its effect on PKR activation. Bennett *et al.* (26) demonstrate that IL-3 deprivation enhances RAX/PKR association and RAX phosphorylation at serine 18 in hematopoietic cells. Overexpression of the non-phosphorylatable mutant of RAX, S18A RAX, inhibits IL-3 deprivation-induced PKR activation. Furthermore, their results suggest that RAX/PKR association precedes both RAX phosphorylation and PKR activation. Based on these findings, Bennett *et al.* (26) propose a model RAX/PKR interaction. In this model, PACT/RAX first associates with PKR and is then phosphorylated at serine 18; phosphorylation of PACT/RAX at serine 18 may induce a conformational change in PACT/RAX that allows its C-terminal dsRNA binding domain to interact and activate PKR. The S18A RAX, although still able to associate with PKR, fails to activate PKR following stress. Our study using neuronal cells supports the conclusion that phosphorylation of RAX in serine 18 is critical for PKR activation in response to ethanol; overexpression of S18A RAX inhibits ethanol-induced PKR/eIF2α phosphorylation. S18A RAX functions as a "dominant negative protein" and blocks ethanol-mediated alteration. S18A RAX and PKR are co-immunoprecipitated in the absence of ethanol, indicating that RAX lacking phosphorylation at serine 18 is still able to bind PKR (Fig. 6). However, our results reveal that ethanol cannot effectively promote the association between S18A RAX and PKR. Therefore, phosphorylation at serine 18 is important for RAX/PKR association in response to ethanol exposure.

Ethanol stimulates eIF2α phosphorylation in a PACT/RAX-dependent manner. In addition to PKR, other eIF2α kinases also phosphorylate eIF2α. These include PERK, heme-regulated inhibitor, and GCN2 family members (16). Using selective PKR inhibitors, we have demonstrated that PKR mediates ethanol-induced eIF2α phosphorylation. The argument is further validated by the study using PKR$-/-$ MEFs; ethanol fails to stimulate eIF2α phosphorylation in PKR$-/-$ MEFs.

Protein Synthesis and Cell Survival—It has been shown that ethanol inhibits protein synthesis (34, 35). However, the underlying mechanisms remain incompletely elucidated. Lang *et al.* (36) demonstrate that ethanol feeding decreases hepatic eIF2B activity and increases eIF2α phosphorylation *in vivo*. In the reticulocyte cell-free system, ethanol inhibits peptide chain initiation through increased phosphorylation of eIF2α (37, 38). Our results support the conclusion that eIF2α is involved; we have identified that PKR, an eIF2α kinase, is a target of ethanol. We have further revealed that the status of PACT/RAX expression in a given cell determines sensitivity to ethanol-induced translational inhibition. The importance of PACT/RAX status for translational regulation has been demonstrated in response to other stress signals. For example, overexpression of PACT/RAX is shown to potentiate translational inhibition caused by ceramide exposure or IL-3 deprivation (26, 33). Our study provides an insight into the mechanisms of ethanol-induced translational inhibition.

Ethanol exposure induces the death of certain types of cells under some circumstances (5, 23, 39, 40). Our results indicate that the PKR/eIF2α pathway plays a role in ethanol-mediated cell death. Both PKR and eIF2α have been reported to have involvement in apoptotic mechanisms (15, 17). Our study shows that the status of PACT/RAX expression is important in determining susceptibility to ethanol. Overexpression of WT RAX potentiates ethanol-mediated PKR activation and subsequent cell death. In contrast, S18A RAX has a dominant negative effect and alleviates ethanol-induced cell death. Thus, phosphorylation of RAX at serine 18 is necessary for PKR activation and subsequent cell death caused by ethanol exposure. Overexpression of PACT in NIH-3T3 and HeLa cells induces apoptosis and also potentiates cell death induced by other stress signals caused by serum-starvation, arsenite, and peroxide (20). However, our study using neuronal cells as well as other reports using leukemia cells indicate that overexpression of RAX is not sufficient to cause cell death (18, 26). Instead, high expression of RAX enhances cell death caused by ethanol, ceramide, and IL-3 depri-

vation. Therefore, whether or not high expression of PACT/RAX induces cell death is cell type-dependent.

Activation of PKR causes cell death through either transcriptional or translational mechanisms (15). A number of cell signaling mechanisms involving FADD, NFκB, p38, and JNK have been implicated in PKR-regulated cell death (15, 16). On the other hand, inhibition of translation may induce apoptosis or enhance the cell death caused by different stimuli (21, 41). Although phosphorylation of eIF2α causes a general block in protein synthesis, translation of mRNAs encoding pro-apoptotic functions, such as GADD153, has been suggested under these conditions (15, 42). It remains to be determined which downstream signaling components of PKR/eIF2α are responsible for ethanol-induced cell death.

Implication in Ethanol-induced Damage to the Developing CNS—It appears that the extent of ethanol-induced PKR/eIF2α phosphorylation correlates to the expression level of PACT/RAX in a given cell (Fig. 1). Overexpression of PACT/RAX potentiates the effect of ethanol on PKR/eIF2α phosphorylation as well as protein synthesis and cell survival. PACT and RAX are generally less abundant and ubiquitously expressed; unlike PKR, PACT and RAX are not regulated by interferons or dsRNA (18, 19, 29).

The cerebellum is one of the CNS regions most susceptible to ethanol; rodent cerebellum of an early postnatal period, which is equivalent to the third trimester in humans, is extensively used for modeling ethanol-induced CNS damage (43). Developmental ethanol exposure causes a significant reduction in volume, weight, and protein contents of the cerebellum (32, 44–47). In particular, ethanol exposure during postnatal days 4–10 results in a significant loss of two major neuronal populations of the cerebellum, Purkinje cells and granule neurons (6, 32, 48). We have demonstrated here that RAX is developmentally regulated in the cerebellum; a rapid increase is observed during postnatal days 6–9, which falls in the temporal window of ethanol vulnerability. A strong expression of RAX is localized in the Purkinje cell layer and external granule layer. Ethanol exposure during this susceptible window activates the PKR/eIF2α pathway in the cerebellum, suggesting that the PACT/RAX/PKR/eIF2α pathway may modulate the effect of ethanol on the CNS. To fully evaluate the role of the PACT/RAX/PKR/eIF2α system in ethanol neurotoxicity, a systematical study of the spatiotemporal expression of PACT/RAX in the CNS is necessary.

PKR has recently emerged as a potential mediator of neurodegeneration (49). For example, β-amyloid (Aβ) peptide activates PKR/eIF2α and induces the death of neurons in culture (50, 51). Activated PKR has been shown to associate with Aβ plaques (49). PKR is suggested to be involved in the extrastriatal degeneration in Parkinson disease and Huntington disease; accumulation of phosphorylated PKR is observed in the nucleus of neurons affected by these diseases (52). PKR preferentially binds to mutant huntingtin RNA transcripts, and activated PKR is found in the brain of Huntington disease (53). Therefore, alteration in the PACT/RAX/PKR system may not only underlie ethanol neurotoxicity but also be involved in the CNS damages caused by other insults.

Acknowledgments—We thank Dr. Charles Goodlett (Indiana University-Purdue University at Indianapolis, Indianapolis, Indiana) for his assistance in the intubation procedure on rat pups. We also thank Dr. Stratford May (University of Florida, Gainesville, FL) for the RAX constructs, Dr. Antonis E. Koromilas (McGill University, Quebec, Canada) for PKR−/− MEFs, and Mohammed Odetallah for help in the quantification of immunoblots.

REFERENCES

1. May, P. A., and Gossage, J. P. (2001) *Alcohol Res. Health* **25,** 159–167
2. Diamond, I., and Gordon, A. S. (1997) *Physiol. Rev.* **77,** 1–20
3. Goodlett, C. R., and Horn, K. H. (2001) *Alcohol Res. Health* **25,** 175–184
4. Luo, J., and Miller, M. W. (1998) *Brain Res. Brain Res. Rev.* **27,** 157–167
5. Olney, J. W. (2004) *Addict. Biol.* **9,** 137–149
6. Maier, S. E., Miller, J. A., Blackwell, J. M., and West, J. R. (1999) *Alcohol Clin. Exp. Res.* **23,** 726–734
7. Maier, S. E., and West, J. R. (2001) *Alcohol* **23,** 49–57
8. Kaufman, R. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11693–11695
9. Levin, D., and London, I. M. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 1121–1125
10. Tan, S. L., and Katze, M. G. (1999) *J. Interferon Cytokine Res.* **19,** 543–554
11. Ung, T. L., Cao, C., Lu, J., Ozato, K., and Dever, T. E. (2001) *EMBO J.* **20,** 3728–3737
12. Williams, B. R. (1999) *Oncogene* **18,** 6112–6120
13. Wu, S., and Kaufman, R. J. (1997) *J. Biol. Chem.* **272,** 1291–1296
14. Zhang, F., Romano, P. R., Nagamura-Inoue, T., Tian, B., Dever, T. E., Mathews, M. B., Ozato, K., and Hinnebusch, A. G. (2001) *J. Biol. Chem.* **276,** 24946–24958
15. Barber, G. N. (2005) *Cell Death Differ.* **12,** 563–570
16. Williams, B. R. (2001) *Science's STKE* 2001, RE2
17. Gil, J., and Esteban, M. (2000) *Apoptosis* **5,** 107–114
18. Ito, T., Yang, M., and May, W. S. (1999) *J. Biol. Chem.* **274,** 15427–15432
19. Patel, R. C., and Sen, G. C. (1998) *EMBO J.* **17,** 4379–4390
20. Patel, C. V., Handy, I., Goldsmith, T., and Patel, R. C. (2000) *J. Biol. Chem.* **275,** 37993–37998
21. Saelens, X., Kalai, M., and Vandenabeele, P. (2001) *J. Biol. Chem.* **276,** 41620–41628
22. Chen, G., Bower, K. A., Ma, C., Fang, S., Thiele, C. J., and Luo J. (2004) *FASEB J.* **18,** 1162–1164
23. Li, Z., Lin, H., Zhu, Y., Wang, M., and Luo J. (2001) *Brain Res. Dev. Brain Res.* **132,** 47–58
24. Luo, J., and Miller, M. W. (1997) *Alcohol Clin. Exp. Res.* **21,** 1186–1194
25. Green, J. T., Tran, T., Steinmetz, J. E., and Goodlett, C. R. (2002) *Brain Res.* **956,** 302–311
26. Bennett, R. L., Blalock, W. L., and May, W. S. (2004) *J. Biol. Chem.* **279,** 42687–42693
27. Ayoub, A. E., Cai, T. Q., Kaplan, R. A., and Luo, J. (2005) *J. Comp. Neurol.* **481,** 403–415
28. Luo, J., and Miller, M. W. (1999) *J. Neurosci.* **19,** 10014–10025
29. Peters, G. A., Hartmann, R., Qin, J., and Sen, G. C. (2001) *Mol. Cell. Biol.* **21,** 1908–1920
30. Bonthius, D. J., and West, J. R. (1990) *Alcohol Clin. Exp. Res.* **14,** 107–118
31. Marcussen, B. L., Goodlett, C. R., Mahoney, J. C., and West, J. R. (1994) *Alcohol* **11,** 147–156
32. Pierce, D. R., Goodlett, C. R., and West, J. R. (1989) *Teratology* **40,** 113–126
33. Ruvolo, P. P., Gao, F., Blalock, W. L., Deng, X., and May, W. S. (2001) *J. Biol. Chem.* **276,** 11754–11758
34. Bonner, A. B., Dalwai, S., Marway, J. S., and Preedy, V. R. (2003) *Metabolism* **52,** 389–396
35. Lang, C. H., Kimball, S. R., Frost, R. A., and Vary, T. C. (2001) *Int. J. Biochem. Cell Biol.* **33,** 457–473
36. Lang, C. H., Wu, D., Frost, R. A., Jefferson, L. S., Vary, T. C., and Kimball, S. R. (1999) *Am. J. Physiol.* **277,** E805–E814
37. Cox, S., and Proud, C. G. (1988) *Biochem. Pharmacol.* **37,** 2045–2049
38. Wu, J. M. (1981) *J. Biol. Chem.* **256,** 4164–4167
39. Luo, J., West, J. R., and Pantazis, N. J. (1997) *Alcohol Clin. Exp. Res.* **21,** 1108–1120
40. Luo, J., West, J. R., Cook, R. T., and Pantazis, N. J. (1999) *Alcohol Clin. Exp. Res.* **23,** 644–656
41. Polunovsky, V. A., Wendt, C. H., Ingbar, D. H., Peterson, M. S., and Bitterman, P. B. (1994) *Exp. Cell Res.* **214,** 584–594
42. Balachandran, S., Kim, C. N., Yeh, W. C., Mak, T. W., Bhalla, K., and Barber, G. N. (1998) *EMBO J.* **17,** 6888–6902
43. West, J. R. (1993) *J. Nutr.* **123,** 382–385
44. Bauer-Moffett, C., and Altman, J. (1977) *Brain Res.* **119,** 249–268
45. Borges, S., and Lewis, P. D. (1983) *Neuropathol. Appl. Neurobiol.* **9,** 53–60
46. Miller, M. W. (1996) *Brain Res.* **734,** 286–294
47. Nathaniel, E. J., Nathaniel, D. R., Mohamed, S. A., Nahnybida, L., and Nathaniel, L. (1986) *Exp. Neurol.* **93,** 610–620
48. Napper, R. M., and West, J. R. (1995) *J. Comp. Neurol.* **362,** 283–292
49. Peel, A. L. (2004) *J. Neuropathol. Exp. Neurol.* **63,** 97–105
50. Chang, R. C., Suen, K. C., Ma, C. H., Elyaman, W., Ng, H. K., and Hugon, J. (2002) *J. Neurochem.* **83,** 1215–1225
51. Suen, K. C., Yu, M. S., So, K. F., Chang, R. C., and Hugon, J. (2003) *J. Biol. Chem.* **278,** 49819–49827
52. Bando, Y., Onuki, R., Katayama, T., Manabe, T., Kudo, T., Taira, K., and Tohyama, M. (2005) *Neurochem. Int.* **46,** 11–18
53. Peel, A. L., Rao, R. V., Cottrell, B. A., Hayden, M. R., Ellerby, L. M., and Bredesen, D. E. (2001) *Hum. Mol. Genet.* **10,** 1531–1538