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ORIGINAL ARTICLE

Downregulation of dopamine D_1 receptors and increased neuronal apoptosis upon ethanol and PTZ exposure in prenatal rat cortical and hippocampal neurons

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Abstract The objective of this study was to evaluate the effect of ethanol and pentylenetetrazol (PTZ) on the expression of dopamine receptors (D_1R) and to observe the apoptotic neurodegeneration in prenatal rat cortical and hippocampal neurons at gestational days (GD) 17.5. In the present study, ethanol (100 mM) and PTZ (15 mM) were exposed to the prenatal rat cortical and hippocampal neuronal cell cultures for 1 h. For mRNA RT-PCR and for protein Western blot analysis was done to elucidate D_1R, Bax, Bak, Bcl-2 and cleaved caspase-3 expression upon ethanol and PTZ exposure in neuronal cell cultures. Furthermore, ethanol and PTZ-induced apoptotic neurodegeneration was also observed using TUNEL staining and propidium iodide (PI) used as counter stain under confocal microscopy. The results of present study showed that ethanol and PTZ exposure significantly decreased D_1R expression and induced neuronal death by significantly increasing the expression of pro-apoptotic Bax, Bak and decreasing anti-apoptotic protein Bcl-2 leading to the apoptosis by increasing cleaved caspase-3 expression in cortical and hippocampal primary neuronal cell cultures. Our findings indicated that ethanol and PTZ exposure to the prenatal neurons showed not only downregulation of D_1R but also causes neuronal apoptosis in the developing rat brain. Further, this explains the possibility of higher risk of developmental disturbances and malformations during early developmental stage.

Keywords Ethanol · PTZ · D_1 receptors · Cleaved caspase-3 · Neurodegeneration

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Introduction

It is proved that multifaceted consequences have been observed in early development due to the use of ethanol during pregnancy [1] and most extensive outcome of prenatal ethanol exposure causes behavioral changes [2, 3]. Several clinical investigations showed that exposure of in utero ethanol exhibits hyperlocomotion particularly in boys, which diagnosed after few years of birth and this deficit may increase in severity during the adult stage [4–6]. Similarly, animal models studies also showed that upon prenatal ethanol the behavioral abnormality was observed in developing fetus after birth [7–9]. Moreover, the prenatal ethanol exposure also affects the pre- and postsynaptic DA receptors and DA receptor-mediated behaviors [10]. Although there is a substantial amount of data concerning the morphological and behavioral effects linked with prenatal ethanol exposure [11], still the mechanisms underlying developmental defects caused by maternal ethanol consumption remain unclear.

Dopamine (DA) a major neurotransmitter in the brain involved in the controlling abnormal neuronal excitability in the kindling and in neuroplasticity linked with this

phenomenon that alteration in the function of the dopaminergic system [12–14]. Previous studies on human and animal models suggested that DA plays an important role and any dysfunctions of the DA in cortical region may lead to impaired attention deficit/hyperactivity disorder [8, 15, 16].

Evidence indicates the importance of DA activation and behaviors for ethanol reinforcement and previously it was explained by a large number of groups [17], and the effects of DA are regulated by a family of G protein-coupled DA receptor subtypes, which include DA D_1–D_5 receptor and these are the most abundant in human cortex [18, 19]. Recently, we have studied the decreased $GABA_{B1}R$ expression and increased neuronal cell death in developing rat brain upon PTZ-induced seizure [20].

There are many potential mechanisms by which neurons may die after seizures and most important is the induction of apoptosis. We have also studied that maternal ethanol exposure and PTZ-induced seizure significantly decreased $GABA_{B1}R$ expression and increased neuronal cell death in prenatal rat brain may lead to the higher risk of developmental disturbances and malformations [21]. Moreover, ethanol exposure modulates the expression of $GABA_BR$, which may further lead to the modulation of DAD_1R activitiesthat might have important influence on the underlying cause of ethanol's effects [22].

It is well known that receptors undergo adaptive up- or downregulation following alterations in neuronal activity. The present study was designed to further characterize the role of D_1R upon exposure of ethanol and PTZ in prenatal rat cortical and hippocampal neurons. For this purpose, the expressional changes in D_1R were observed upon ethanol and PTZ exposure in prenatal rat neurons. Our results showed significant decreased D_1R expression and increased expression of pro-apoptotic Bax, Bak and decreased anti-apoptotic protein Bcl-2 expression, further leading to the apoptosis by significant increase in expression of cleaved caspase-3 in prenatal rat neuronal cell cultures. This neuronal apoptosis and downregulation of D_1R explain the possibility of higher risk of developmental disturbances and malformations during early developmental stage.

Methodology

Animal treatment

Female ($n = 10$) Sprague–Dawley rats 250 g were housed in a temperature-controlled environment with lights from 06:00 to 20:00 hours with food ad libitum. Timed pregnant [the day of insemination equals] to gestational days (GD) 0.5. After GD 17.5 pregnant female rat was killed by decapitation, after an i.v. injection of pentobarbital sodium (3 mg/100 g bw).

Primary cell culture and drug treatment

Cultures were prepared from the cortical and hippocampal areas from prenatal rats at GD 17.5 as previously explained by [21]. Briefly, pooled hippocampal and cortical tissues were treated with 0.25 % trypsin-EDTA for 20 min and dissociated by mechanical trituration in ice-cold calcium- and magnesium-free Hank's balanced salt solution (pH 7.4). After 3 days, cortical and hippocampal neuronal cells were treated with normal media as control and media contain ethanol 100, PTZ 15, 100 mM ethanol plus 15 mM PTZ in different groups and combinations. All drug-treated groups were incubated for 1 h in vitro culture.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR analysis was performed using cDNA from control and drug-treated neuronal culture. Total RNA was isolated with Trizol reagent (Life Technologies, Rockville, MD). First strand cDNA was transcribed from 2 μg of RNA using oligo (dt) 15, M-MLV reverse transcriptase (Promega), following the protocol provided by the company. A total of 4 μl of cDNA was used for PCR amplification in presence of 1 μl *Taq* DNA polymerase. Thermal cycling was performed under the following conditions: 94 °C for (5 min), 30 cycles at 94 °C (1 min) D_1R (53 °C, 28 cycles) for (1 min), and 72 °C for (1 min) followed by 72 °C (5 min) for the final extension. As a negative control GAPDH (58 °C, 25 cycle) was performed. PCR products were run on a 1 % agarose gel containing ethidium bromide and viewed under UV light. The primers used were the following: D_1R forward 5-ATTCTTCCCTGAACCCCATT-3, D_1R reverse: 5-GTGGAATGCTGTCC ACTGTG3′, GAPDH forward: 5-GCCATCAATGACCCCTTCATT-3, GAPDH reverse: 5-CGCCTGCTTCAC CACCTTCTT-3.

Western blotting

Western blot analysis was followed as previously [21]. Briefly, primary cultured cortical and hippocampal cells were homogenized in cell lysis buffer with protease inhibitor 100 mM PMSF. After ultra centrifugation (12,000g, 10 min X 2), the protein containing supernatant was separated. The protein content was measured by spectrophotometer at 595 nm using the Bio-Rad (Hercules, CA) protein assay and 30 μg proteins were applied per lane. Immunoreactions were carried out using a rabbit polyclonal IgG D_1R, cleaved caspase-3, Bax, Bcl-2 antibodies (1:1,000, 24 h, 48 °C, Santa Cruz, Cell signaling) or

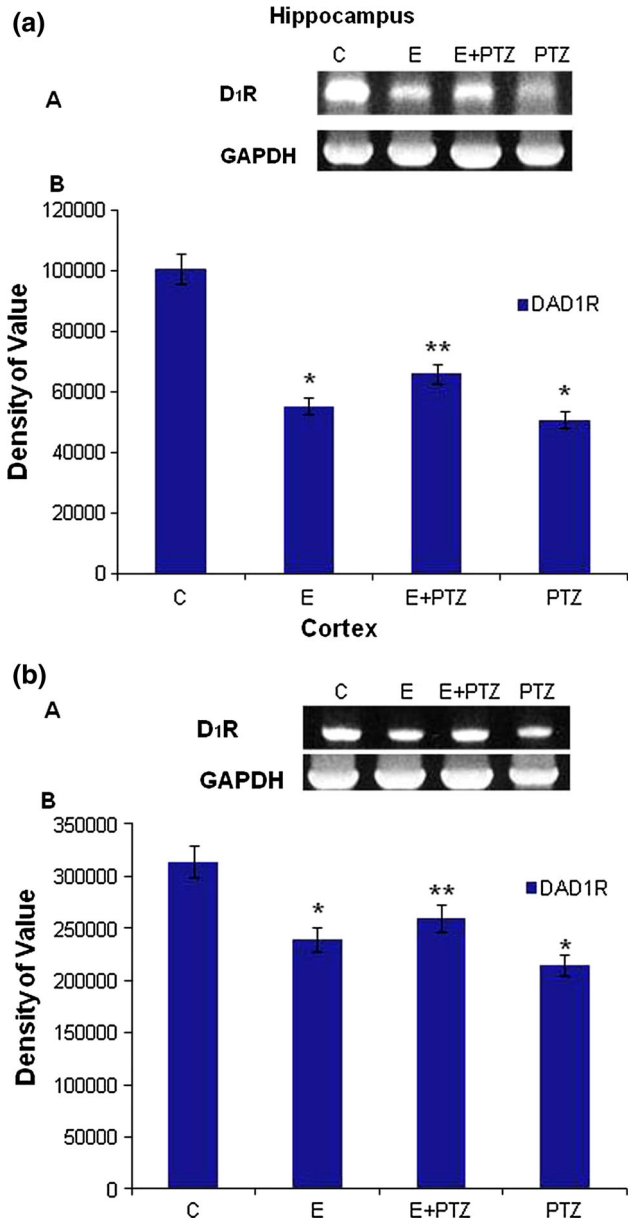

Fig. 1 RT-PCR analyses for the change in mRNA level of the D_1R in the primary cultured hippocampal (**a**) and cortical (**b**) neuronal cells. **B** Density values expressed as mean ± SEM ($n = 3$) of the corresponding mRNA of D_1R are presented. The density values on the *Y*-axis are expressed as arbitrary units (AU). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group

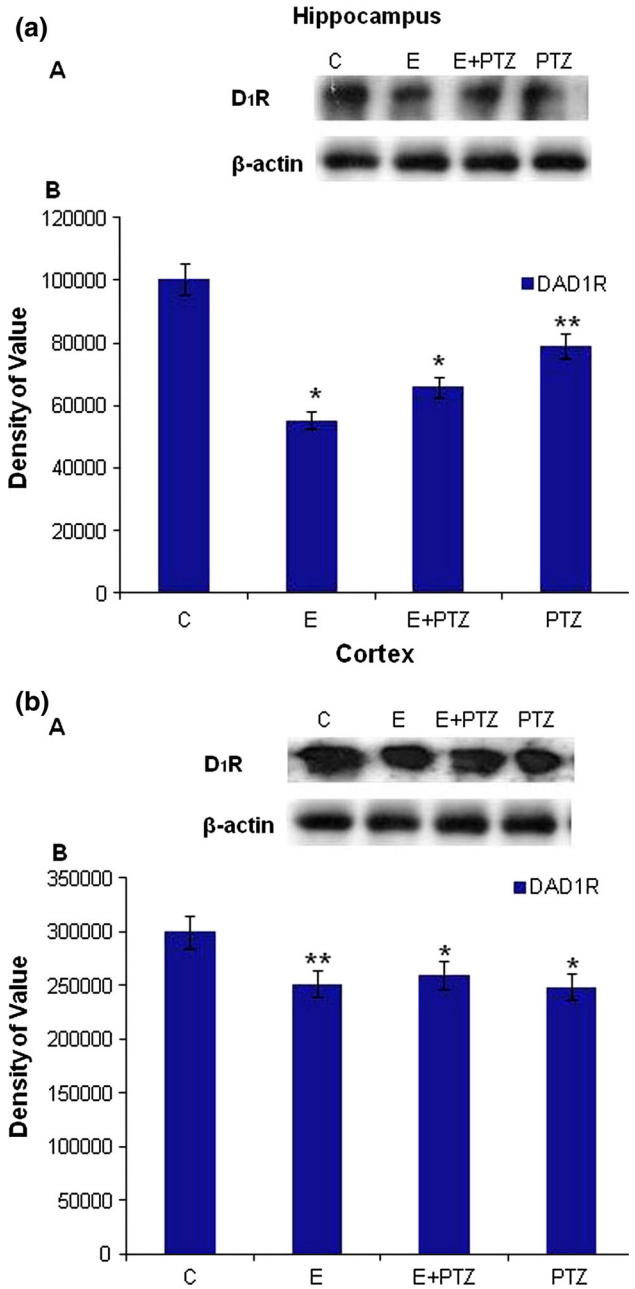

Fig. 2 Western blot analyses of the D_1R protein in the primary cultured hippocampal (**a**) and cortical (**b**) neuronal cells. **B** Density values expressed as mean ± SEM ($n = 3$) of the corresponding mRNA of D_1R are presented. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group

rabbit-derived anti-rat D_1R, cleaved caspase-3, Bax, Bak, Bcl-2 antibodies (1:1,000, Abcam Limited, UK). Following rinses, horseradish peroxidase conjugated goat anti-rabbit IgG-HRP (1:10,000, Bio-Rad) was added for 2 h at room temperature. Proteins were detected by chemiluminescence using an ECL-detecting reagent according to their protocol. Western blots were analyzed by densitometry using the computer-based Sigma Gel (SPSS, Chicago) system. Density values were expressed as mean ± SEM. In every case, the acceptance level for statistical significance was $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group.

TUNEL staining for neuronal cell death

In situ detection of apoptotic cell death was performed using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) on hippocampal

neuronal cell culture as previously done [23]. TUNEL staining was performed according to supplier's recommendations using kit (Genescript, NJ, USA). Propidium iodide (PI) was as a counter stain for 10 min at room temperature. TUNEL-positive (green) and PI-positive (red) staining patterns were acquired by the use of a confocal laser scanning microscope (Fluoview FV 1000, Olympus, Japan). TUNEL-positive cells in the different regions of each section were counted by observers blinded to the treatment conditions.

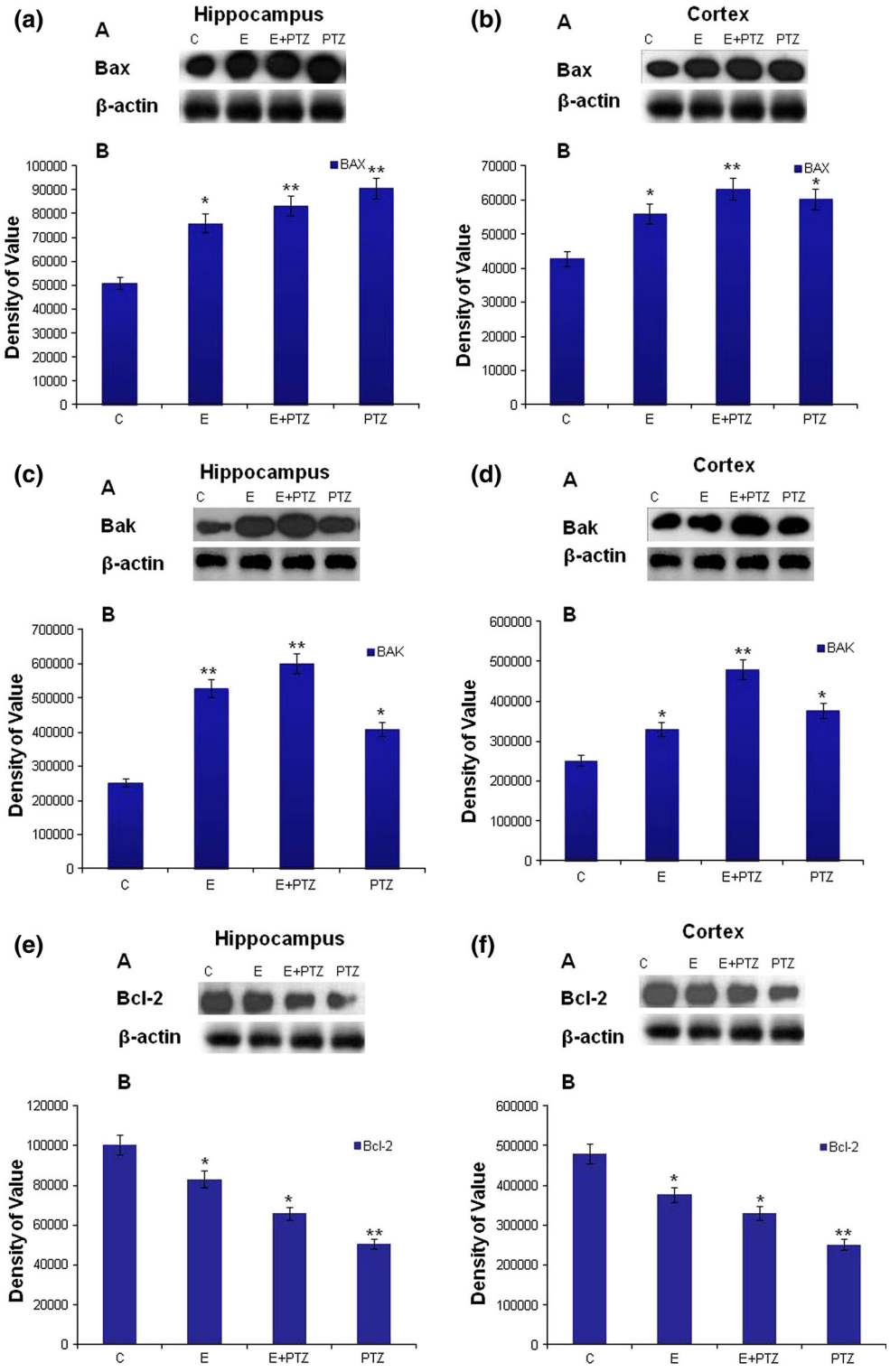

Fig. 3 Western blot analyses of the Bax, Bak and Bcl-2 in the primary cultured hippocampal (**a**, **c**, **e**) and cortical (**b**, **d**, **f**) neuronal cells. β-actin is taken as loading control in each case. **A** Immunoblots of Bax, Bak and Bcl-2 of hippocampal and cortical neuronal cells under different treatment conditions. The immunoblots were labeled with an anti-Bax and Bcl-2 antibodies. **B** Density values were expressed as mean ± SEM ($n = 4$). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group

Data analysis and statistics

Western blots were scanned and analyzed by densitometry using a computer based on the Sigma Gel System (SPSS Inc., Chicago, IL, USA). Density values were expressed as mean ± SEM. Comparisons between treated groups and controls were done by Student's t test to determine the significance of differences between relevant treatment groups. In every case, the acceptance level for statistical significance was $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group.

Results

Ethanol and PTZ exposure modulates D_1R expression in the prenatal rat cortical and hippocampal neurons

To study the effect of ethanol and PTZ on the expression of D_1R in hippocampal and cortical cells, primary cultured neuronal cells were divided into four groups and treated as control, ethanol, PTZ, ethanol plus PTZ. RT-PCR analysis was used to examine the D_1R mRNA expression level at early developmental stage. The results showed that D_1R expression at mRNA level in hippocampal neurons was decreased significantly upon ethanol, PTZ and ethanol plus PTZ treated as compared to control group (Fig. 1a, b). Further, Western blot analysis was done to confirm whether the changes in D_1R are similar as resulted at mRNA level. The results showed similar expression that upon exposure of ethanol, PTZ and ethanol plus PTZ on neuronal cells significantly decreased D_1R protein expression in hippocampal as well as cortical neurons (Fig. 2a, b). Thus, the decreased protein level of D_1R expression was as a result of decrease in mRNA level upon exposure of ethanol, PTZ ethanol plus PTZ as compared to the control groups (Fig. 2a, b).

Pro-apoptotic Bax, Bak and anti-apoptotic Bcl-2 proteins expression in neuronal cell cultures upon ethanol and PTZ exposure

In this study, we observed the change in pro-apoptotic Bax, Bak and anti-apoptotic Bcl-2 protein expression upon ethanol, PTZ and ethanol plus PTZ in primary cultures of hippocampal and cortical neurons using Western blot analysis. Our results showed that ethanol, PTZ and ethanol plus PTZ exposure significantly increased the expression of pro-apoptotic protein Bax and Bak (Fig. 3a–d) and significant reduction in the level of the anti-apoptotic protein Bcl-2 in neuronal cell cultures as shown in Fig. 3e and f. Moreover, the significant increase in Bax, Bak and decrease in Bcl-2 expression suggested that ethanol, PTZ and ethanol plus PTZ exposure in neuronal cell cultures have a significant deleterious effect which further leads to the apoptosis.

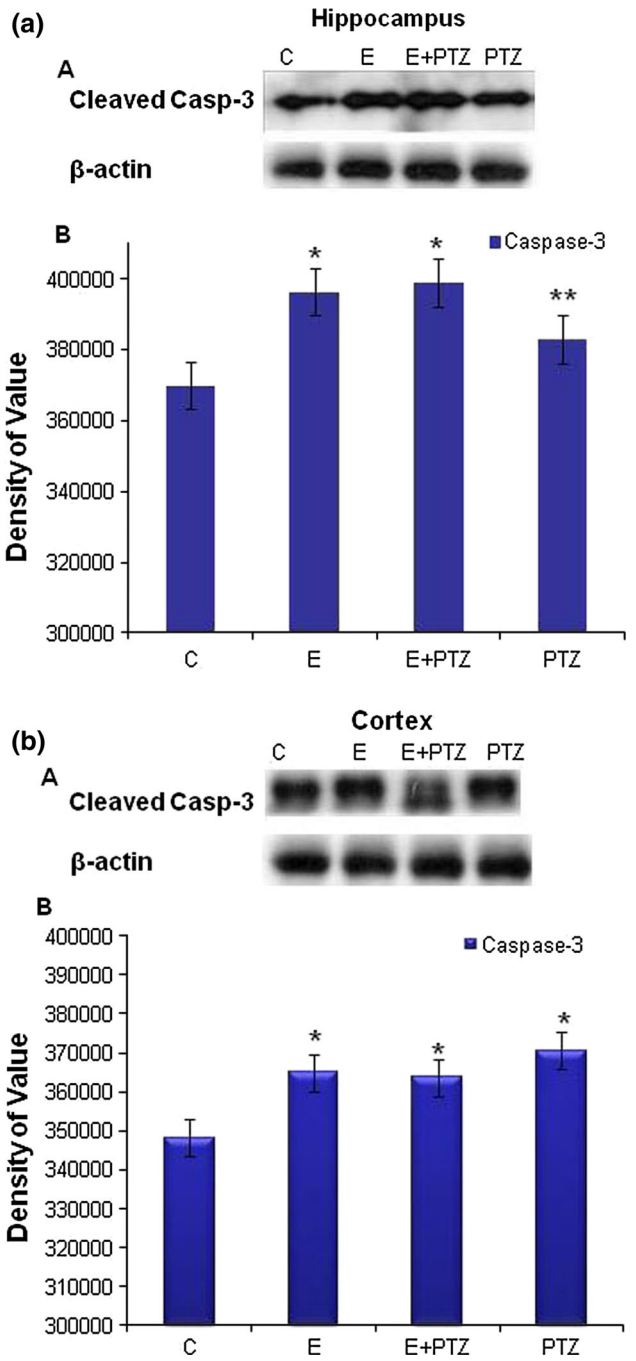

Fig. 4 Western blot analyses of the caspase-3 in the primary cultured hippocampal (**a**) and cortical (**b**) neuronal cells. β-actin is taken as loading control in each case. **A** Immunoblots of caspase-3 of hippocampal and cortical neuronal cells under different treatment conditions. The immunoblots were labeled with an anti-caspase-3 antibody. **B** Density values were expressed as mean ± SEM ($n = 4$). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group

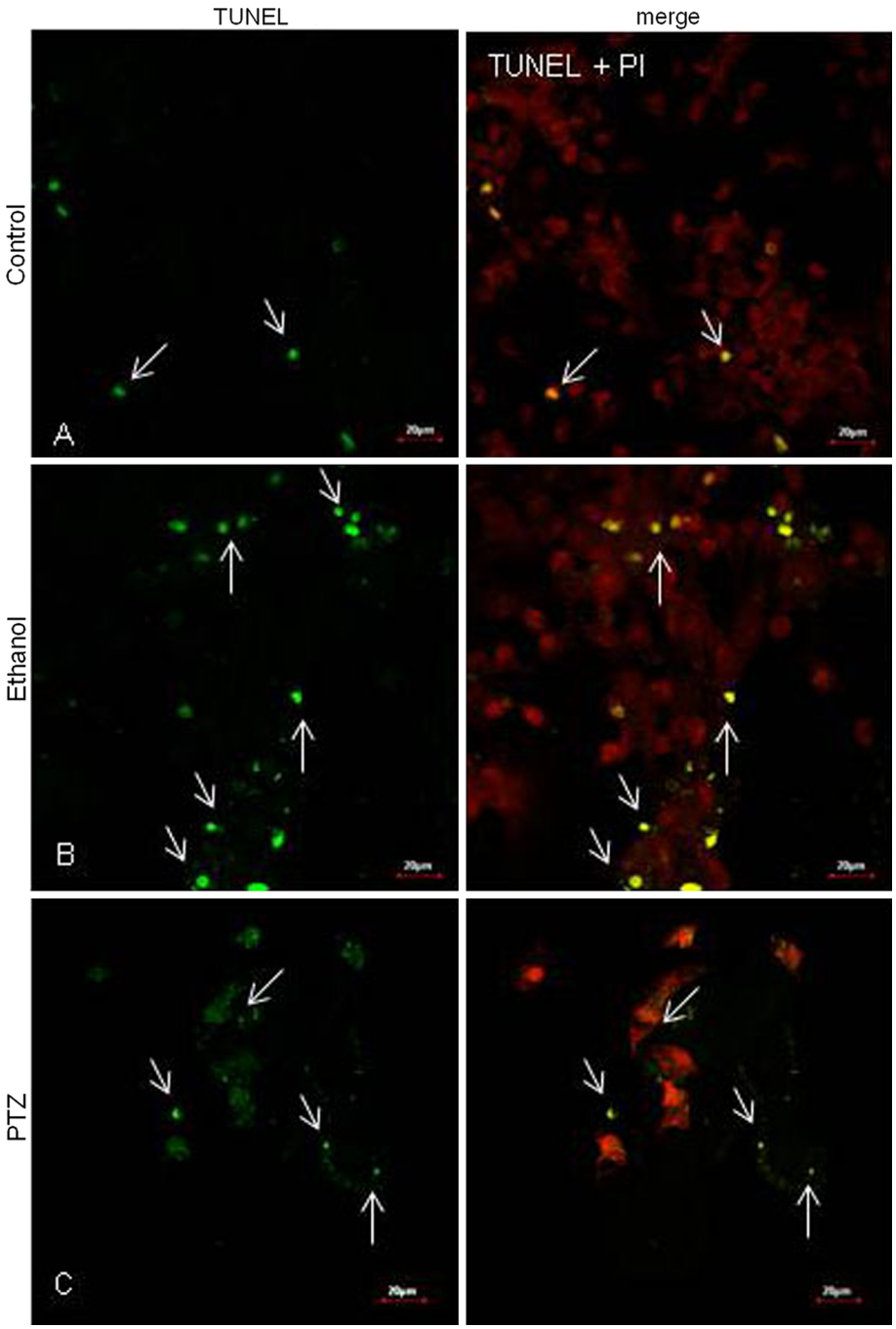

Fig. 5 Representative photomicrographs of TUNEL staining showed apoptotic neuronal cells death upon ethanol administration along with PTZ. The *arrows* indicate ethanol-induced TUNEL stained apoptotic dead neurons counterstained with PI in the hippocampal neuronal cells. ×40 objective field, *scale bar* 20 μm

Caspase-3 protein expression increased in neuronal cell culture upon ethanol and PTZ exposure

Caspase activity is a main feature of an apoptotic pathway. To study the effect of ethanol, PTZ and ethanol plus PTZ on neuronal cells at early developmental stage, Western blot analysis was done. Our results showed that upon exposure of ethanol, PTZ and ethanol plus PTZ on primary neuronal cell culture the expression of cleaved caspase-3 was increased significantly as compared to the control group. The results suggested that ethanol, PTZ and ethanol plus PTZ exposure on cortical and hippocampal cells at early stage lead to apoptotic neuronal cell death on both part of the brain (Fig. 4a, b).

TUNEL staining showed that ethanol and PTZ exposure causes neuronal cell death

DNA break is one of the main features of apoptosis and the visualization of which is possible by TUNEL staining. TUNEL staining was used to observe the DNA damage upon ethanol and PTZ treatment that is one of the

hallmarks of apoptosis. The neuronal cell deaths were analyzed and results showed that ethanol and PTZ treatment significantly increased the total number of TUNEL-positive cells within the hippocampal neuronal cell culture (Fig. 5b, c) as compared to the control group.

Discussion

In the present study, we have observed the D_1R expression and apoptotic neuronal cell death in cortical and hippocampal prenatal rat brain upon ethanol and PTZ exposure. Our results showed that ethanol and PTZ treatment significantly induce the downregulation of D_1R and increased in neuronal cell death in both part of the prenatal rat brain. Prenatal rat primary neuronal cell culture was observed using RT-PCR analysis for mRNA and Western blot analysis was done for the detection of D_1R protein expression, pro apoptotic proteins Bax, Bak, anti-apoptotic Bcl-2 and cleaved caspase-3 expression while, TUNEL and PI was used for detection of neurodegeneration in the prenatal brain.

Previously, it was reported that density of D_1- and D_2-like receptors was decreased in the hippocampal CA3, dorsal caudate-putamen and striatal regions of the brains of epileptic rats [24].

It was reported that ethanol significantly decreased dopaminergic D_1 as well as D_2 receptors and further biochemical and behavioral studies showed that in utero ethanol exposure produces a long-lasting effect in the development of electrophysiological and pharmacological characteristics of midbrain DA systems, in adulthood [25, 26].

Further it was also reported that prenatal exposure to 3 g/kg ethanol causes a significant decrease in the number of DA D2 binding sites within the dorsal and ventral striatum [27]. Earlier findings indicated that prenatal ethanol exposure may predominantly produce diminished reactivity of the D_2 but not D_1 subtypes of DA receptors or an opposite outcome [28]. Others [29] reported that prenatal ethanol exposure did not alter DA concentration or turnover and produced a transient increase in D_1 but not D_2 receptor binding, in mice. Furthermore, we have recently shown [30] that ethanol administered for 1 week to rats produces decreases in D_1 and D_2 receptor densities and no changes in dissociation constants. Reduced dopamine levels and D_2 receptor numbers have been shown in brains of alcohol-preferring animals, in genetic models of alcoholism [31].

In the present study, we have observed that ethanol and PTZ exposure down regulate the D_1R expression and induced apoptotic neuronal death in prenatal rat brain. Therefore, our results suggested that exposure of ethanol and PTZ in primary neuronal cell culture leads to significant decrease in the expression of D_1R and further leads to apoptotic neuronal cell death by significantly increasing the expression of pro apoptotic Bax, Bak and significantly decreasing the anti-apoptotic protein Bcl-2 leading to the increase in the expression of cleaved caspase-3 protein. These results further explain the possibility of higher risk of developmental disturbances and malformations during early developmental stage.

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Conflict of interest There is no conflict of interest regarding this article.

References

1. Riley EP, McGee CL (2005) Fetal alcohol spectrum disorders: an overview with emphasis on changes in brain and behavior. Exp Biol Med 230:357–365
2. Olney JW (2002) New insights and new issues in developmental neurotoxicology. Neurotoxicology 23:659–668
3. Guerri C (1998) Neuroanatomical and neurophysiological mechanisms involved in central nervous system dysfunctions induced by prenatal alcohol exposure. Alcohol Clin Exp Res 22:304–312
4. Coles CD, Platzman KA, Raskind-Hood CL, Brown RT, Falek A, Smith IE (1997) A comparison of children affected by prenatal alcohol exposure and attention deficit, hyperactivity disorder. Alcohol Clin Exp Res 21:150–161
5. O'Leary CM (2004) Fetal alcohol syndrome: diagnosis, epidemiology, and developmental outcomes. J Paediatr Child Health 40:2–7
6. O'Malley KD, Nanson J (2002) Clinical implications of a link between fetal alcohol spectrum disorder and attention-deficit hyperactivity disorder. Can J Psychiatry 47:349–354
7. Abel EL, Reddy PP (1997) Prenatal high saturated fat diet modifies behavioral effects of prenatal alcohol exposure in rats. Alcohol 14:25–29
8. Hausknecht KA, Acheson A, Farrar AM, Kieres AK, Shen RY, Richards JB et al (2005) Prenatal alcohol exposure causes attention deficits in male rats. Behav Neurosci 119:302–310
9. Barbier E, Houchi H, Warnault V, Pierrefiche O, Daoust M, Naassila M (2009) Effects of prenatal and postnatal maternal ethanol on offspring response to alcohol and psychostimulants in long evans rats. Neuroscience 161:427–440
10. Shen RY, Hannigan JH, Chiodo LA (1995) The effects of chronic amphetamine treatment on prenatal ethanol-induced changes in dopamine receptor function: electrophysiological findings. J Pharmacol Exp Ther 274:1054–1060
11. Goodlett CR, Horn KH (2001) Mechanisms of alcohol-induced damage to the developing nervous system. Alcohol Res Health 25:175–184
12. Becker A, Grecksch G, Thiemann W, Hollt V (2000) Pentylenetetrazol-kindling modulates stimulated dopamine release in the nucleus accumbens of rats. Pharmacol Biochem Behav 66: 425–428
13. Bozzi Y, Vallone D, Borrelli E (2000) Neuroprotective role of dopamine against hippocampal cell death. J Neurosci 20: 8643–8649

14. Turski WA, Cavalheiro EA, Ikonomidou C, Bortolotto ZA, Klockgether T, Turski L (1990) Dopamine control of seizure propagation: intranigral dopamine D1 agonist SKF-38393 enhances susceptibility to seizures. Synapse 5:113–119
15. Davids E, Zhang K, Tarazi FI, Baldessarini RJ (2003) Animal models of attention-deficit hyperactivity disorder. Brain Res Brain Res Rev 42:1–21
16. Denney CB (2001) Stimulant effects in attention deficit hyperactivity disorder: theoretical and empirical issues. J Clin Child Psychol 30:98–109
17. Tupala E, Hall H, Halonen P, Tiihonen J (2004) Cortical dopamine D_2 receptors in type 1 and 2 alcoholics measured with human whole hemisphere autoradiography. Synapse 54:129–137
18. Hall H, Sedvall G, Magnusson O, Kopp J, Halldin C, Farde L (1994) Distribution of D1- and D2-dopamine receptors, and dopamine and its metabolites in the human brain. Neuropsychopharmacology 11:245–256
19. Hartman DS, Lanau F (1997) Diversity of dopamine receptors: new molecular and pharmacological developments. Pol J Pharmacol 49:191–199
20. Naseer MI, Ullah I, Al-Qahtani MH, Karim S, Ullah N, Ansari SA et al (2013) Decreased GABABR expression and increased neuronal cell death in developing rat brain after PTZ-induced seizure. Neurol Sci 34:497–503
21. Naseer MI, Shupeng L, Kim MO (2009) Maternal epileptic seizure induced by pentylenetetrazol: apoptotic neurodegeneration and decreased GABAB1 receptor expression in prenatal rat brain. Mol Brain 2:20
22. Lee HY, Naha N, Li SP, Jo MJ, Naseer MI, Park MS et al (2008) In vivo and in vitro ethanol exposure in prenatal rat brain: GABA(B) receptor modulation on dopamine D(1) receptor and protein kinase A. Synapse 62:534–543
23. Ullah N, Naseer MI, Ullah I, Kim TH, Lee HY, Kim MO (2013) Neuroprotective profile of pyruvate against ethanol-induced neurodegeneration in developing mice brain. Neurol Sci 15:15
24. Birioukova LM, Midzyanovskaya IS, Lensu S, Tuomisto L, van Luijtelaar G (2005) Distribution of D1-like and D2-like dopamine receptors in the brain of genetic epileptic WAG/Rij rats. Epilepsy Res 63:89–96
25. Shen RY, Chiodo LA (1993) The effects of in utero ethanol administration on the electrophysiological activity of rat nigrostriatal dopaminergic neurons. Brain Res 624:216–222
26. Shen RY, Hannigan JH, Kapatos G (1999) Prenatal ethanol reduces the activity of adult midbrain dopamine neurons. Alcohol Clin Exp Res 23:1801–1807
27. Randall S, Hannigan JH (1999) In utero alcohol and postnatal methylphenidate: locomotion and dopamine receptors. Neurotoxicol Teratol 21:587–593
28. Brus R, Felinska W, Rykaczewska M, Kostrzewa RM, Szkilnik R, Plech A (1995) Prenatal ethanol diminishes reactivity of presumed dopamine D3 receptors in rats. Pol J Pharmacol 47:109–114
29. Boggan WO, Xu W, Shepherd CL, Middaugh LD (1996) Effects of prenatal ethanol exposure on dopamine systems in C57BL/6 J mice. Neurotoxicol Teratol 18:41–48
30. Vasconcelos SM, Macedo DS, Lima LO, Sousa FC, Fonteles MM, Viana GS (2003) Effect of one-week ethanol treatment on monoamine levels and dopaminergic receptors in rat striatum. Braz J Med Biol Res 36:503–509
31. Noble EP (1996) Alcoholism and the dopaminergic system: a review. Addict Biol 1:333–348