Prenatal Exposure to Chronic
Ethanol on N Methyl D Aspartate Receptor Distribution

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Abstract: Problem statement: Effect of chronic exposure to ethanol on NR1 subunit of the NMDA receptor distribution in chick embryo brain chronic ethanol intake leads to the development of dependence on and tolerance to ethanol in both human and animals. The glutamate system plays a major role in mediating ethanol effects on brain and behavior. The aim of the present study was to investigate whether chronic ethanol exposure altered NR1 subunit of N-Methyl-D-Aspartate (NMDA) receptor distribution in the developing chick brain on 10 and 15th day of egg incubation.

Approach: Forty fertilized eggs divided in 2 groups, control and chronic. In the chronic group the eggs were chronically exposed to ethanol 10% during 15 day of embryonic period. Immunohistochemical procedure was used for NR1 subunit of NMDA receptor distribution determination. Image analyzer program were used for color analyzing of immunohistochemistry slides. Data were analyzed statistically by Student T test. The level of significance was assumed to be (p<0.05).

Results: Immunohistological finding of these experiments indicated that chronic exposure to ethanol significantly (p<0.05) decreased density of NR1 subunit of NMDA receptors in cerebral cortex of chick brain on 10 and 15th day of embryonic stage. But the number of immunolable cell per mm$^2$ of cerebral cortex did not show any difference.

Conclusion/Recommendation: Our results indicated that chronic exposure to ethanol decreased NR1 subunit of NMDA receptor distribution in cerebral cortex of chick embryo on 10 and 15th day of embryonic stage. But did not have any effect on number of immunolable cell per mm$^2$ of cerebral cortex.

Key words: Phosphate Buffer (PB), results indicated, incubated overnight, electrophysiological studies, higher expression found, immunohistochemical procedure

INTRODUCTION

Chronic and excessive alcohol consumption can lead to changes in neuronal structure and function in several brain regions, including the frontal cortex. Study of postmortem tissue from alcoholics have reported decreased neuronal density in superior prefrontal cortex (Harper, 2009). Several studies have proposed that NMDA receptors are important in neuronal out growth (Prithviraj and Inglis, 2009), in the control of protein synthesis at developing synapse (Martin et al., 2000) and in neuronal plasticity of dendritic spines (Fischer et al., 2000). Convincing evidence has been shown that NMDA receptors constitute an important site of action of ethanol (Hicklina et al., 2011). Ethanol sensitivity of NMDA receptor was determined, at least in part, by the subunit composition of the receptor (Lovinger, 2002). In electrophysiological studies, prenatal ethanol exposure has been shown to reduce spontaneous and prolonged latency synaptic events that were attributable to NMDA receptors (Powrozeka and Zhoub, 2005). There are several reports regarding inhibition of glutamate receptors by ethanol in several brain regions (Lovingier et al., 1989; Tabakoff and Hoffman, 1996). Cristovao et al. (2002) determined that NMDA receptors were functionally active since the early embryonic stages, this occurs before synapse formation in the embryo. The NMDA receptor in the chick embryo retina is mainly NR1 and regulated by NR2A and NR2C (Cristovao et al., 2002), the same results were seen in rat retina (Grunder et al., 2000).

Chronic ethanol treatment causes upregulation in the levels of NMDA receptors subunit (Kumari and
Ticku, 1998). This upregulation in the levels of NMDA receptor subunit was reversed after 48 h of ethanol withdrawal, which indicated the reversibility of the changes (Okumoto et al., 2005). Kalluri et al. (1998) showed that chronic ethanol treatment enhanced the polypeptide levels of NR1, NR2 subunits in the rat cerebral cortex and hippocampus (Kalluri et al., 1998).

The NMDA receptor is a ligand-gated ion channel complex that belongs to the family of glutamate receptors (Waxman and Lynch, 2005). This receptor is thought to consist of a pentameric assembly of two NR1 subunits and three NR2 subunits (Luque et al., 2003). Evidence has shown that NMDA receptor subunit expression is developmentally regulated during brain maturation (Allgaier, 2002; Magnusson et al., 2002; Law et al., 2003; Chang et al., 2009). Bennet et al. (2006) represented that NR1 subunit of NMDA receptor expression were present in mid and late phase of embryonic stage of chick (Bennet et al., 2006). In the CNS, NR1 subunit of NMDA receptor is expressed in every brain region that has been examined, with higher expression found in the cortex, cerebellum, hippocampus, basal ganglia, thalamus, hypothalamus and olfactory bulb (Monyer et al., 1994). Evidence suggest that the NR1 subunit is required for normal development of neuronal plasticity and central sensitization (Zhou et al., 2009).

Therefore, the aim of the present study was to investigate whether chronic ethanol exposure altered NR1 subunit of NMDA receptor distribution in the developing chick brain on 10 and 15th day of egg incubation?

**MATERIALS AND METHODS**

**Material:** NR1 antibody was obtained from Tocris company (UK), envision (second antibody) and Dab were obtained from Dako company (Denmark).

We used fertilized white leghorn eggs obtained from a local hatchery, incubated at 37.8°C and relative humidity of 80-90%. In the control group humidity were induced by water, but in experimental group water were removed from incubator in 10th day of embryonic stage and another half of eggs were removed on 15th day of embryonic stage. Embryo were removed from the eggs and brain were removed and washed by normal saline and fixed for 24 h in 10% formaldehyde in 0.1M Phosphate Buffer (PB, PH 7.4), then brain were post-fixed in 4% formaldehyde in 0.1M phosphate buffer (PB, PH 7.4). Paraffin embed were done by Tissue processor and paraffin block were prepared.

**Tissue preparation:** In both groups half of eggs were removed from incubator in 10th day of embryonic stage and another half of eggs were removed on 15th day of embryonic stage. embryo were removed from the eggs and brain were removed and washed by normal saline and fixed for 24 h in 10% formaldehyde in 0.1M Phosphate Buffer (PB, PH 7.4), then brain were post-fixed in 4% formaldehyde in 0.1M phosphate buffer (PB, PH 7.4). Paraffin embed were done by Tissue processor and paraffin block were prepared.

**RESULTS**

Figure 2 shows that exposure to ethanol lead to significantly (p<0.05) decrease in NR1 subunit of NMDA receptor of glutamate distribution in brain cortex of chick on day 10 and 15 of embryonic stage (Fig. 3).
In comparison to immunohistolabelled cell number in 1 mm² of cerebral cortex there was no significant difference between control and chronic groups on day 10 and 15 of embryonic stage (Fig. 4).

**DISCUSSION**

In the present study chronic exposure of eggs to ethanol lead to decrease NR1 subunit of NMDA receptor in cerebral cortex on 10 and 15th day of embryonic stage. Kotlinska reported that NMDA receptors are involved in the development of ethanol dependence (Escher et al., 2011). Chronic exposure of neurons to ethanol results in up-regulation of NMDA receptor function and enhanced glutamate-mediated excitatory (Carpenter-Hyland et al., 2004; Roberto et al., 2006). However, other research suggests that this up-regulation may be subunit dependent (Nagy, 2008). Both NMDA and non NMDA (AMPA and Kainate) glutamate receptor are inhibited by ethanol in several brain regions (Kumari and Ticku, 1998; Criswell et al., 2003; Tsai and Coyle, 1998; Ron, 2004).

Ridge et al. (2008) reported that the expression of NR1, NR2A and NR2B subunit mRNA in superior frontal and primary motor cortex tissue was significantly lower than in the corresponding areas in control (Ridge et al., 2008). Wang et al. (2011) illustrate that upregulation of NR2B-NMDAR activity by ethanol is selective and that ethanol’s effect on NMDA receptor activity is gradual and cumulative (Wang et al., 2011). Roberto et al. (2006) chronic ethanol treatment also significantly increased mRNA levels of NR1 and NR2B NMDAR subunits compared to control rats. At 1 week withdrawal, mRNA levels for NR1 and NR2B subunits were significantly decreased. These data indicate that CET induces reversible neuroadaptations in synaptic function, gene expression and protein composition of NMDAR at CeA synapses (Roberto et al., 2006).

Kalev-Zylinska and During (2007) hypothesized that chronic moderate alcohol intake leads to improve memory via adaptive responses in the expression of NMDA receptors and downstream signaling. The moderate ethanol intake improved memory, increased NR1 expression and changed some aspects of neurotrophin signaling. NR1 knock-down prevented ethanol facilitatory effects, whereas hippocampal NR1 overexpression mimicked the effect of chronic low-dose ethanol intake on memory. In contrast, high-dose ethanol reduced neurogenesis, inhibited NR2B expression and impaired visual memory. In conclusion, adaptive changes in hippocampal NMDA receptor expression may contribute to the positive effects of ethanol on cognition (Kalev-Zylinska and During, 2007).
The first time Zhao et al. (2006), report that immunoreactivity of anti-NR1 is widely distributed throughout the central nervous system in AGS and is similar to other species (Zhao et al., 2006).

The results of Sircar and Sircar (2006) indicate that ethanol exposure during the adolescent period produces brain region-specific alterations in NR1 activity. These changes are different from those reported in literature for ethanol administration during the perinatal period or adulthood. Together, these data suggest that adolescence represents a unique stage in brain development in its long-term sensitivity to ethanol (Sircar and Sircar, 2006).

The results of Rani et al. (2005) show that chronic intermittent ethanol regimen has less pronounced effects on GABA(A) receptor expression, but increases NR2B expression more dramatically than chronic ethanol treatment in cultured cortical neurons. These differential effects on subunit expression may result in altered receptor structure and function as a result of ethanol exposure (Rani et al., 2005). Chronic ethanol consumption increases NR2 expression persists for at least 2 week (Obara et al., 2009).

In the present study exposure to chronic ethanol does not have any effect on the number of cell/mm2 of cerebral cortex of chick brain. Marshall et al. (2009) showed that prenatal ethanol exposure in chicks reduced the mean number per section of hippocampal neurons without alteration in hippocampal volume or nuclear size of these neurons (Marshall et al., 2009).

CONCLUSION

Our results indicated that:

- Exposure to chronic ethanol lead to reduction in NR1 subunit of NMDA receptor in cerebral cortex of chick in 10 and 15th day of embryonic stages
- Exposure to chronic ethanol has no significant effect on number of NR1 subunit of NMDA receptor in cerebral cortex of chick in 10 and 15th day of embryonic stages

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