American Journal of Pharmacology and Toxicology 6 (4): 124-129, 2011
ISSN 1557-4962

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

[1]Taherianfard, M., [2]B. Geramizadeh, [3]E. Evazi and [1]M. Sharifi
[1]Department of Physiology, School of Vet. Med., Shiraz University, Shiraz, Iran
[2]Transplant Research Center, Faculty of Medicine, Shiraz Mesdical University Shiraz, Iran
[3]Department of Development Biology, Faculty of Science,
Jahrom University, Jahrom, Iran

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 (Lovinger *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

Corresponding Author: Taherianfard, M., Depterment of Physiology, School of Vet. Med., Shiraz University, Shiraz, Iran
Fax: 0098-711-2286940 Tel: 0098-711-2286950

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 expression of NR2 subunits in the CNS (Fukaya *et al*., 2003). Zhou *et al*. (2009) suggests that the NR1 subunit of NMDA receptor plays an important role in the development of neuronal plasticity and central sensitization (Zhou *et al*., 2009).

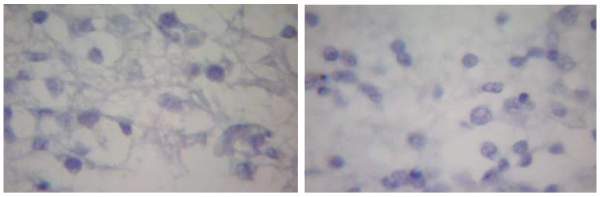
Therefore, the aim of the present study was to investigate whether chronic ethanol exposure altered NR1 subunit of NMDA receptor distribution in the developing chick brain on 10 and 15th day of egg incubation?

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 displaced by 10% ethanol during incubation.

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.



(a) (b)

Fig. 1: Brain cortex of negative control in 10^{th} and 15^{th} day of embryonic stage. Magnify is 40×10×70 (a) 15th day of embryonic stage (b) 10th day of embryonic stage

A microtome was used to cut the brains into 5 µm section and mounted in 25% L-lysine coated glass slides.

Immunohistochemical study: The slide-mounted section were dried and subsequently stored in a -20ºC freezer until used for antibody labeling. After removal from the freezer slide-mounted sections of chick brain were brought to room temperature, washed and the section outlined with a liquid-repellent slide marker pen (to retain reagents on the sections during the immunostaining procedure). Sections were incubated overnight with primary antibodies against NR1 (1/4000 dilution). The next day, sections were rinsed in PBS and incubated in secondary antibody (envision) and further washed in PBS. Finally the sections were rinsed in PBS three times for 10 min and reacted with cromogen (Dab) and following rinse in PBS, the sections were put in hemotoxilin for nucleus staining. Negative control sections were incubated with PBS in the absence of primary antibody and no immunoreactivity was detected. The Fig. 1 shows the cortex of brain in negative control. In this photograph there is no brown color due to reaction of primary antibody.

After preparing of digital image from slides, distribution of NR1 subunit of NMDA receptor of glutamate were analyzed by using Image Analyzer (version 1.33). This program determined the distribution of receptors according to three character, hue, saturation and intensity; and then showed the number that has reverse relation with the receptor distribution; meaning that higher receptor distribution represented by low number in the program.

Statistical analysis; statistical analysis were performed using SPSS (version 18). Student T test were used to determine the difference between control and experimental groups. Data reported as mean±SEM and the level of significant was considered $p<0.05$.

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).

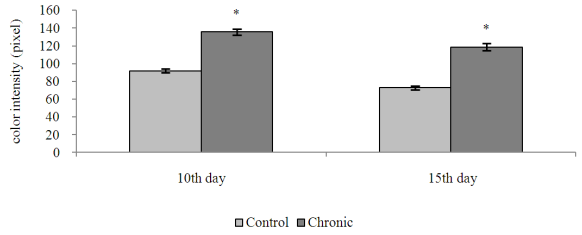


Fig. 2: Effect of prenatal exposure to ethanol on NR1 subunit of NMDA receptor on day 10 and 15 of embryonic stage of chick

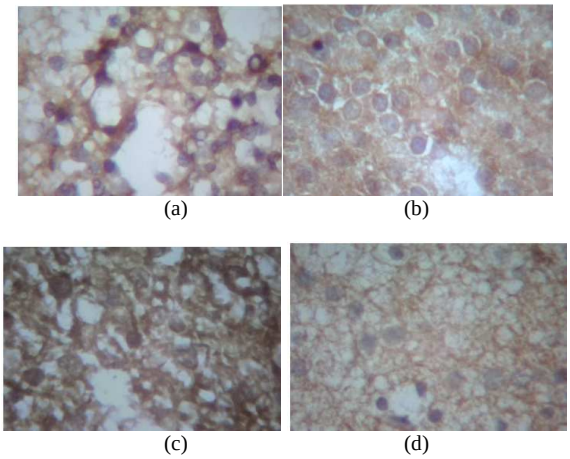


Fig. 3: Comparison of brain cortex of control group and chronic group on 10 and 15th of embryonic stage. Magnify is 40×10×70 (a)10th day of embryonic stage of control group (b) 10th day of embryonic stage of chronic group (c) 15th day of embryonic stage of control group (d)15th day of embryonic stage of chronic group

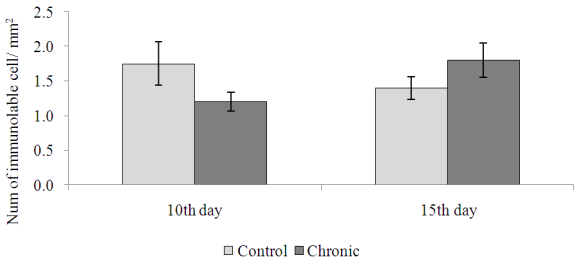


Fig. 4: Of prenatal exposure to ethanol on number of labled cell by NR1 subunit of NMDA receptor on day 10 and 15 of embryonic stage of chick

On the other hand in comparison between day 10 and 15 of control and ethanol exposure chick, the NR1 subunit of NMDA receptor distribution was significantly ($p<0.05$) higher on 15th day of embryonic stage than on 10th day of embryonic stage (Fig. 3 and 2).

In comparison to immunohistolabelled cell number in 1 mm^2 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

REFERENCES

Allgaier, C., 2002. Ethanol sensitivity of NMDA receptors. Neurochem Int., 41: 377-382. DOI: 10.1016/S0197-0186(02)00046-3

Bennet, G.D., K. Moser, T. Chaudoin and T.H. Rosenquist, 2006. The expression of the NR1-subunit of the NMDA receptor during mouse and early chicken development. Reprod Toxicol, 22: 536-541. DOI: 10.1016/j.reprotox.2006.03.010

Carpenter-Hyland, E.P., J.J. Woodward and L.J. Chandler, 2004. Chronic ethanol induces synaptic but not extrasynaptic targeting of NMDA receptors. J. Neurosci, 24: 7859-7868. DOI: 10.1523/JNEUROSCI.1902-04.2004

Chang, L.R., J.P. Liu, N. Zhang, Y.J. Wang and X.L. Gao *et al*., 2009. Different expression of NR2B and PSD-95 in rat hippocampal subregions during postnatal development. Microsc Res. Tech., 72: 517-524. DOI: 10.1002/jemt.20708

Cristovao, A.J., C.R. Oliveira and C.M. Carvalho, 2002. Expression of functional N-methyl-D-aspartate receptors during development of chick embryo retina cells: *In vitro* versus *in vivo* studies. Brain Res. Mol. Brain Res., 99: 125-133. PMID: 11978403

Criswell, H.E., Z. Ming, B.L. Griffith and G.R. Breese. 2003. Comparison of effect of ethanol onn-methyl-d-aspartate- and gaba-gated currents from acutely dissociated neurons: Absence of regional differences in sensitivity to ethanol. J. Pharmacol Exp. Ther., 304: 192-199. DOI: 10.1124/jpet.102.041590

Escher, T., S.B. Call, C.D. Blaha and G. Mittleman. 2011. Behavioral effects of aminoadamantane class NMDA receptor antagonists on schedule-induced alcohol and self-administration of water in mice. Psychopharmacology, 187: 424-434. DOI: 10.1007/s00213-006-0465-5

Fischer, M., S. Kaech, U. Wanger, H. Brinkhaus and A. Matus, 2000. Glutamate receptors regulate actin-based plasticity in dendritic spines. Nat Neurosci, 3: 887-894. DOI: 10.1038/78791

Fukaya, M., A. Kato, C. Lovett, S. Tonegawa and M. Watanabe, 2003. Retention of NMDA receptor NR2 subunits in the lumen of endoplasmic reticulum in targeted NR1 Knockout mice. Proc. Natl. Acad. Sci. USA., 100: 4855-4860. DOI: 10.1073/pnas.0830996100

Grunder, T., K. Kohler, A. Kaletta and E. Guenther, 2000. The distribution and developmental regulation of NMDA receptor subunit proteins in the outer and inner retina of the rat. J. Neurobiol, 44: 333-342. DOI: 10.1002/1097-4695(20000905)44:3<333::AID-NEU4>3.0.CO;2-S

Harper, C., 2009. The neuropathology of alcohol-related brain damage. Alcohol Alcoholism, 44: 136-140. DOI: 10.1093/alcalc/agn102

Hicklina, T.R., P.H. Wub, R.A. Radcliffed, R.K. Freunde and S.M. Goebel-Goodya *et al*., 2011. Alcohol inhibition of the NMDA receptor function, long-term potentiation and fear learning requires striatal-enriched protein tyrosine phosphatase. PNAS., 108: 6650-6655 DOI: 10.1073/pnas.1017856108

Kalev-Zylinska, M.L. and M.J. During, 2007. Paradoxical facilitatory effect of low-dose alcohol consumption on memory mediated by NMDA receptors. J. Neurosci., 27: 10456-10467. DOI: 10.1523/JNEUROSCI.2789-07.2007

Kalluri, H.S.G., A.K. Mehta and M.K. Ticku, 1998. Up-regulation of NMDA receptor subunits in rat brain following chronic ethanol treatment. Mol. Brain Res., 58: 221-224. DOI: 10.1016/S0169-328X(98)00112-0

Kumari, M. and M.K. Ticku, 1998. Ethanol and regulation of the NMDA receptor subunits in fetal cortical neurons. J. Neurochem., 70: 1467-1473. DOI: 10.1046/j.1471-4159.1998.70041467.x

Law, A.J., C.S. Weickert, M.J. Webster, M.M. Herman and J.E. Kleinman *et al*., 2003. Expression of NMDA receptor NR1, NR2A and NR2B subunit mRNAs during development of the human hippocampal formation. Eur. J. Neurosci, 18: 1197-1205. DOI: 10.1046/j.1460-9568.2003.02850.x

Lovinger, D.M., 2002. NMDA receptors lose their inhibitions. Nat Neurosci., 5: 614-616. PMID: 12085089

Lovinger, D.M., G. White and F.F. Weight, 1989 Ethanol inhibits NMDA-activated ion current in hippocampal neurons. Science, 243: 1721-1724. DOI: 10.1126/science.2467382

Luque, J.M., Z. Bleuel, P. Malherbe and J.G. Richards, 2003. Alternatively spliced isoforms of the N-methyl-d-aspartate receptor subunit 1 are differentially distributed within the rat spinal cord. Neuroscience, 63: 629-635. DOI: 10.1016/0306-4522(94)90510-X

Magnusson, K.R., S.E. Nelson and A.B. Young, 2002. Age-related changes in the protein expression of subunits of the NMDA receptor. Brain Res. Mol. Brain Res., 99: 40-45. DOI: 10.1016/S0169-328X(01)00344-8

Marshall, A.G., M.M. McCarthy, K.M. Brishnehan, V. Rao and L.M. Batia *et al*., 2009. Effect of gestational ethanol exposure on parvalbumin and calretinin expressing hippocampal neurons in a chick model of fetal alcohol syndrome. Alcohol, 43: 147-161. DOI: 10.1016/j.alcohol.2008.12.004

Martin, K.C., M. Barad and E.R. Kandel, 2000. Local protein synthesis and its role in synapse-specific plasticity. Curr. Opinion Neurobiol., 10: 587-592. DOI: 10.1016/S0959-4388(00)00128-8

Monyer, H., N. Burnashev, D.J. Laurie, B. Sakmann and P.H. Seeburg, 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron, 12: 529-540. DOI: 10.1016/0896-6273(94)90210-0

Nagy, J., 2008. Alcohol related changes in regulation of NMDA receptor functions. Curr. Neuropharmacol., 6: 39-54. DOI: 10.2174/157015908783769662

Obara, I., R.L. Bell, S.P. Goulding, C.M. Reyes and L.A. Larson *et al*., 2009. Differential effects of chronic ethanol consumption and withdrawal on homer/glutamate receptor expression in subregions of the accumbens and amygdala of P rats. Alcohol. Clin. Exp. Res., 33: 1924-1934. DOI: 10.1111/j.1530-0277.2009.01030.x

Okumoto, S., L.L. Looger, K.D. Micheva, R.J. Reimer and S.J. Smith *et al*., 2005. Detection of glutamate release from neurons by genetically encoded surface-displayed FRET nanosensors. PNAS, 102: 8740-8745. DOI: 10.1073/pnas.0503274102

Powrozeka, T.A. and F.C. Zhoub, 2005. Effects of prenatal alcohol exposure on the development of the vibrissal somatosensory cortical barrel network. Dev. Brain Res., 155: 135-146. DOI: 10.1016/j.devbrainres.2005.01.003

Prithviraj, R. and F.M. Inglis, 2009. Expression of the N-methyl-d-aspartate receptor subunit NR3B regulates dendrite morphogenesis in spinal motor neurons. Neuroscience, 155: 145-153. DOI: 10.1016/j.neuroscience.2008.03.089

Rani, C.S., M. Qiang and M.K. Ticku, 2005. Potential role of cAMP response element-binding protein in ethanol-induced N-methyl-D-aspartate receptor 2B subunit gene transcription in fetal mouse cortical cells. Mol. Pharmacol., 67: 2126-2136. DOI: 10.1124/mol.104.007872

Ridge, J.P., A.M. Ho, D.J. Innes and P.R. Dodd, 2008. The expression of NMDA receptor subunit mRNA in human chronic alcoholics. Ann. N. Y. Acad. Sci., 1139: 10-19. DOI: 10.1196/annals.1432.053

Roberto, M., M. Bajo, E. Crawford, S.G. Madamba and G.R. Siggins, 2006. Chronic ethanol exposure and protracted abstinence alter NMDA receptors in central amygdala. Neuropsychopharmacology, 31: 988-996. DOI: 10.1038/sj.npp.1300840\

Ron, D., 2004. Signaling cascades regulating NMDA receptor sensitivity to ethanol. Neuroscientist, 10: 325-336. PMID: 15271260

Sircar, R. and D. Sircar, 2006. Repeated ethanol treatment in adolescent rats alters cortical NMDA receptor. Alcohol, 39: 51-58. DOI: 10.1016/j.alcohol.2006.07.002

Tabakoff, B. and P.L. Hoffman, 1996. Alcohol addiction: An enigma among us. Neuron, 16: 909-912. DOI: 10.1016/S0896-6273(00)80113-0

Tsai, G. and J.T. Coyle, 1998. The role of glutamatergic neurotransmission in the pathophysiology of alcoholism. Annu. Rev. Med., 49: 173-184. DOI: 10.1146/annurev.med.49.1.173

Wang, J., M.F. Lanfranco, S.L. Gibb and D. Ron, 2011. Ethanol-mediated long-lasting adaptations of the NR2B-containing NMDA receptors in the dorsomedial striatum. Channels (Austin), 5: 205-209. PMID: 21289476

Waxman, E.A. and D.R. Lynch, 2005. N-methyl-D-aspartate receptor subtypes: Multiple roles in excitotoxicity and neurological disease. Neuroscientist, 11: 37-49. DOI: 10.1177/1073858404269012

Zhao, H.W., S.L. Christian, M.R. Castillo, A. Bult-Ito and K.L. Drew, 2006. Distribution of NMDA receptor subunit NR1 in arctic ground squirrel central nervous system. J. Chem. Neuroanat, 32: 196-207. PMID: 17097266

Zhou, Q., D.D. Price, R.M. Caudle and G.N. Verne, 2009. Spinal NMDA NR1 subunit expression following transient TNBS colitis. Brain Res., 1279: 109-120. DOI: 10.1016/j.brainres.2009.04.038