Audiogenic Seizures Potentiate Hippocampal Neuronal Loss in Ethanol-Dependent Rats

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Summary

Objective: Audiogenic seizure (AS) susceptibility is observed following withdrawal from chronic ethanol treatment in rodents. This is the first study to investigate and compare the effects of ethanol withdrawal on the hippocampal formation in AS appeared and non-appeared animals.

Material and Methods: Adult male Wistar rats (225-320 g) were used. Ethanol was given to rats in a modified liquid diet for twenty days. Daily ethanol consumption was in a range of 10.35±1.25 to 15.20±0.79 g/kg during the exposure to ethanol (7.2%). At the end of exposure to a 7.2% ethanol-containing liquid diet, ethanol was withdrawn and withdrawal signs were recorded or rated. Increased stereotyped behavior, wet dog shakes, agitation, tail-stiffness and abnormal posture and gait appeared during ethanol withdrawal in dependent rats. AS was also induced in 5 of 11 of ethanol dependent rats. Following audiogenic stimuli, rats were decapitated and their brains were removed. Neuron counts from pyramidal cell layers in CA1 and CA2-3 regions of the hippocampus were obtained from control rats and ethanol-dependent rats with and without audiogenic seizure.

Results: Significant neuronal losses were found in CA1 and CA2-3 regions of the hippocampal formation in the ethanol-dependent group. Neuronal loses in AS appeared group were significantly more than in AS non-appeared group.

Conclusions: ASs during ethanol withdrawal significantly potentiated neuronal degeneration in all subdivisions of the CA area of the hippocampal formation in rats. Thus, prevention of seizures in alcoholic individuals may be important for protection from excessive neuronal damage in the hippocampus.

Key words: Hippocampus; ethanol withdrawal; audiogenic seizure; ethanol; rat(s)

Odiyojenik Nöbetler Etanole Bağlı Şıçanlarda Hipokampal Nöron Kaybını Potansiyelize Ediyor

Özet

Amaç: Kronik etanole maruziyeti izleyen yoksunluk döneminde kemirgenlerde odiyojenik nöbet duyularlığı gözlenir. Sunulan çalışma alkol yoksunluğu döneminde odiyojenik nöbet geçiren ve geçirmeyen şıçanlarda etanol yoksunluğunun hipokampal yapısı üzerinde etkilerini inceleyen ve karşılaştıran ilk çalışmamızdır.

Gereç ve Yöntemler: Çalışmada erişkin erkek (225-320 g) Wistar şıçanlar kullanıldı. Etanol şıçanlara 20 gün süre ile modifiye edilmiş bir sİvî diyet içinde verildi. Bu sürecte şıçanlar ortalama olarak 10.35±1.25 ile 15.20±0.79 g/kg arasında etanol aldı. İçinde %7,2 oranında
etanol içeren sivi diyetten etanolün aniden çıkarılması ile çıkanlar etanol yoksunluğuna Sokuldu ve artmış stereotipik davranışlar, islak köpek silkinmesi, ajitasyon, kuryrug sertliği ve anormal postur ve duruş gibi yoksunluk belirtileri izlenerek değerlendirildi. Ayrıca 11 etanole bağlı şıçandan 5inde odiyojenik epileptik nöbetler görüldü. Odiyojenik stimülus sonrası nöbet geçiren ve geçirmeyen şıcanlar dekapite edilerek beyinleri çıkarıldı. Hem nöbet geçiren hem de geçirmeyen şıcanlarda hipokampal CA1 ve CA2-3 piramidal bölgelerinde nöron sayıları yapıldı.

**Bulgular:** İlgili bölgelerde etanol verilen ve yoksunluğa giren hayvanlarda kontrole göre anlamlı ölçüde nöronal kayıplar gözlandı. Hipokampal nöron kayıpları odiyojenik nöbet geçiren şıcanlara geçirmeyenlere göre istatistiksel olarak anlamlı ölçüde daha yüksekti.

**Sonuç:** Bulgularımız odiyojenik nöbet geçirmenin hipokampal yapının CA bölgesinde nöronal kaybını anlamlı ölçüde artırdığını işaret etmektedir. Alkoliklerde nöbetlerin önlenmesi hipokampusun daha fazla zarar görmemesinin engellenmesi bakımından önemli olabilir.

**Anahtar Kelimeler:** Hipokampus; etanol yoksunluğu; odiyojenik nöbet; etanol; şıcan

**INTRODUCTION**

Ethanol abuse and dependence is one of the most common types of substance abuse and dependence worldwide. Alcoholism and some psychiatric illnesses, such as depression, anxiety and psychosis, are also often associated and are co-morbid in psychiatric patients. Thus alcoholism continues to be a major public health, social and medical problem in several societies. Long-term abuse of ethanol leads to the development of alcohol dependence. Ethanol withdrawal syndrome (EWS) precipitated by discontinuing chronic ethanol intake is the most important evidence indicating the presence of physical ethanol dependence in both humans and animals. It has been widely accepted that chronic consumption of ethanol and EWS also cause pathological alterations in the brain. During chronic ethanol exposure, various maladaptive homeostatic changes have been suggested to occur in neurons and neuro-humoral transmission. These maladaptive changes are often associated with the development of significant neuronal abnormalities and a reduced volume of many cortical and subcortical structures in brain. These alterations may also constitute the basis for cellular-level adaptations to ethanol and the development of ethanol-dependence. The hippocampal formation is particularly important among the brain regions affected by ethanol. Several previous reports demonstrated marked neuronal loss and degeneration in the hippocampal formation during chronic ethanol exposure and EWS. In particular, the cornu ammonis (CA) subdivisions of the hippocampus are fairly vulnerable to prolonged ethanol exposure in rodents. Although the harmful effects of ethanol and EWS are well-known, most studies have focused on long-term (more than a month) effects of ethanol on the hippocampal formation, and studies involving short-term effects of ethanol on the hippocampus are very limited.

Ethanol abuse and epileptic seizures are interrelated and have been linked because the time of Hippocrates. Patients with epilepsy who ingest moderate or heavy amounts of ethanol have an increased risk of seizures. Seizures may also occur during alcohol intoxication and withdrawal. One of the most epileptogenic regions of the rat brain is the hippocampus. Many of the specific pathologic alterations observed in the hippocampus following ethanol consumption are known to be epileptogenic, including the loss of pyramidal cells in the CA subdivisions of the hippocampus and alterations in circuitry. Bonthius et al. also...
suggested that the reduced seizure threshold in ethanol-exposed rats is due to hippocampal pathology.

Audiogenic seizure (AS) susceptibility is observed following withdrawal from chronic ethanol treatment in rodents. The AS response is considered to be the most reliable and easily quantifiable behavioral symptom of EWS in rats (27,46). Interestingly ASs do not appear in all ethanol-dependent rats; the observed incidence of ASs during ethanol withdrawal varies from 50% to 75% in rats (10,23,30,44,46,47,49). The cellular mechanisms underlying AS vulnerability during ethanol withdrawal are not completely understood. Furthermore, there are no published studies investigating comparing the effects of ethanol and ethanol withdrawal on the hippocampal formation in AS appeared and non-appeared animals.

We hypothesized that an investigation and comparison of pathological changes in the CA subdivisions of the hippocampal formation during ethanol withdrawal in rats both with and without audiogenic seizure could provide a valuable contribution towards understanding the cellular mechanisms underlying AS susceptibility in ethanol-dependent rats. Thus, in the present study, we focused on the relationship between ASs, other EWS symptoms and neuronal losses in the hippocampal formation of ethanol-dependent rats.

**MATERIAL AND METHODS**

**Animals and Laboratory**

All procedures in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health (USA) and the local ethical committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Adult male Wistar rats were used and weighed 225-320 g at the beginning of the experiments. They were housed in a quiet and temperature- and humidity-controlled room (22±3 °C and 65±5%, respectively) in which a 12-h light/dark cycle was maintained (07:00 – 19:00 h light). Exposure to ethanol and all behavioral experiments involved in ethanol withdrawal syndrome were carried out in separate, isolated laboratories that have the same environmental conditions with the colony room.

**Chronic Exposure to Ethanol**

Ethanol was given in the modified liquid diet to individually housed rats as previously described (46). The modified liquid diet with or without ethanol was available ad libitum. No extra chow or water was supplied. The composition of the modified liquid diet with ethanol was: cow's milk 925-975 ml (Sütaş, Bursa, Turkey), ethanol 25-75 ml (96.5% ethyl alcohol; Tekel, Turkish State Monopoly), vitamin A 5000 IU (Aksu Farma, Turkey) and sucrose (46). This mixture always supplied 1000.7 kcal/L, which was adjusted by changing the sucrose concentration according to ethanol and milk concentrations. The contribution of ethanol to the total energy is approximately 40% in the modified liquid diet. Although the liquid diet does not include any fiber or bulk, it is nutritionally replete and valid for chronic ethanol experiments (46).

At the beginning of the study, rats were given the modified liquid diet without ethanol for 3 days. The liquid diet containing 2.4% ethanol was then administered for 3 days. The ethanol concentration was increased to 4.8% for the next 4 days and, finally, to 7.2% for the following 14 days. Liquid diet was freshly prepared every morning and presented at the same time of the day (09:30 h). The weight of each rat was recorded every day, and daily ethanol intake was measured and expressed as g per kg per day. Control rats were pair fed with an isocaloric liquid diet containing sucrose as a caloric substitute for ethanol.
Evaluation of Ethanol Withdrawal Syndrome

At the end of exposure to the 7.2% ethanol-containing liquid diet, ethanol was withdrawn and replaced with an isocaloric ethanol-free diet at 09:30 h. At 6 h following ethanol withdrawal, rats were observed for 5 min, and withdrawal signs that included agitation, abnormal posture and gait, tail-stiffness, stereotyped behavior and wet dog shakes were recorded or rated.

Grooming, sniffing, head weaving, gnawing and chewing were observed as the major stereotypic behaviors during ethanol withdrawal in the study. The total number of stereotyped behaviors and wet dog shakes in a 5 min observation period was calculated and expressed as the mean ± S.E.M. Agitation, tail stiffness, and abnormal posture and gait were scored using the rating scale as previously described.(47)

After 6 h of withdrawal testing, rats were exposed to an audiogenic stimulus (100 dB) for 60 s in a separate and sound-proof place in the laboratory. The incidence and latency of ASs were recorded. Control rats receiving a liquid diet containing no ethanol were also evaluated for ethanol withdrawal signs and subjected to audiogenic stimuli in parallel to ethanol-dependent groups.

Stereological Cell Counts

Following the audiogenic stimuli, rats were decapitated and their brains were removed by craniotomy and immediately frozen in a cryostat chamber (Shandon AS620) at -35 °C. The frozen brains were cut in horizontal planes at a thickness of 60 µM at -15 °C. Sections were collected using systematic random sampling, and stained with haematoxylin-eosin. Microscopic images from pyramidal cell layers in the CA1 and CA2-3 regions of the hippocampus were obtained by using a microscope (Olympus CX31; 100x oil objective; Numerical Aperture= 1.25). The images were captured by a video camera (Exwave HAD Color Video Camera SSC-DC88P) and displayed on a monitor (Sony LCD LMD-2010). Stepping on microscopic images was performed as previously described(1). The thickness of the sections was measured using a microcator (Heidenhain MT 12). The total number of pyramidal neurons in the pyramidal cell layer of both the CA1 and CA2-CA3 regions of the hippocampus was estimated using an optical fractionator, an unbiased stereological method, as described in previous studies(52,55). The CA1 and CA2-CA3 regions of the hippocampus were delineated according to the rat brain atlas of Paxinos and Watson(34).

The hippocampus is divided into two major components: the “region superior” (CA1) and the “region inferior” (CA3). The pyramidal cell layer is the principal cell layer of the hippocampus, but the neuronal cell bodies and nuclei of the pyramidal cells of CA3 are larger than those of CA1. The CA2 region was considered as belonging to the CA3 region because the boundaries between these two fields of the hippocampus are not discrete in conventionally stained sections(21).

Statistical Analyses

Total numbers of hippocampal pyramidal neurons are given as the mean ± standard error of mean (S.E.M.). The cell counts of the different groups were compared by one-way analysis of variance (ANOVA) test followed by Tukey's test for post hoc comparisons. The behavioral data including stereotyped behaviors, wet dog shakes, agitation, tail-stiffness, and abnormal posture and gait in different groups were expressed as median values, and they were analysed by the Kruskall-Wallis test followed by the Mann-Whitney-U test for post-hoc comparisons. Correlation between the neuron numbers of CA1 and CA2-CA3 regions of hippocampus and behavioral signs analysed using a Spearman's correlation.
The levels of significance were set at a level of \( p < 0.05 \).

**RESULTS**

**Ethanol Consumption and Body Weight Changes**

The rats' daily ethanol consumption ranged from 10.35 ± 1.25 to 15.20 ± 0.79 g/kg during the exposure to ethanol (7.2%). No significant differences between the ethanol-ingesting groups were observed.

The body weights of controls rats were 253.1 ± 10.8 g and 256.3 ± 13.4 g at the beginning of the study, respectively. The body weights of ethanol-treated rats were 232.4 ± 4.20 g prior to ethanol consumption, and 240 ± 3.15 g prior to ethanol withdrawal. We observed some increases in body weight of rats fed by liquid diet with or without ethanol during the study, but these changes were not statistically significant \((p > 0.05)\).

**Behavioral Changes during Ethanol Withdrawal**

ASs occurred at 6 h following ethanol withdrawal, with an incidence of 45% (5/11) and latency of 18.25 ± 5.12 sec in the ethanol-dependent control group. Ethanol withdrawn rats were assigned into two groups depending on whether they had a seizure. Additional behavioral and histological analyses were performed on each of the three groups: the isocaloric diet control group, the AS ethanol withdrawal group and the no AS ethanol withdrawal group.

Other behavioral signs of ethanol withdrawal syndrome such as stereotypical behaviors and wet dog shakes were significantly different among the groups \((KW= 6.466 \text{ and } 6.595, \ p< 0.05, \ \text{respectively})\). Post-hoc analyses revealed that ethanol withdrawn seized and non-seized rats had significantly more stereotypical behaviors and wet dog shakes compared to the control group \((p= 0.004 \text{ and } p= 0.002, \ \text{respectively}; \ \text{Mann-Whitney U test})\) (Fig. 1A and B). Agitation, tail stiffness, and abnormal posture and gait were also significantly different among the groups \((KW= 10.349, 9.429 \text{ and } 11.664 \ p<0.05, \ \text{respectively})\). Post-hoc analyses indicated that both seized and non-seized ethanol withdrawn rats had a higher incidence of these three ethanol withdrawal symptoms \((p<0.05; \ \text{Mann-Whitney U test})\) (Fig. 1C-E). However, there was no significant difference in the intensity of behavioral signs of EWS between seized and non-seized rats \((p > 0.05)\) (Fig. 1A-E).

**Neuron Numbers and Correlation Analysis**

The total number of pyramidal neurons in the CA1 region was 117081±3443, 100454±2592 and 84504±1415 in the control, no AS and AS ethanol withdrawal groups, respectively. The total number of pyramidal neurons in the CA2-CA3 region was 207171±5208, 176109±4405, 143054±2996 in control, no AS and AS ethanol withdrawal groups, respectively.

The numbers of neurons in both the CA1 and CA2-CA3 fields of the hippocampus were significantly different among the groups [\(F(2,14)=49.442 \text{ and } 70.773; \ p<0.0001, \ \text{respectively}\)]. Post-hoc analyses revealed significant reductions in the number of hippocampal neurons in both seized and non-seized ethanol withdrawn rats compared to the control group \((p<0.001, \ \text{Tukey's test})\) (Fig. 2A-B). Furthermore, the AS ethanol withdrawal group of rats had significantly fewer hippocampal neurons in CA1 and CA2-CA3 compared to the no AS ethanol withdrawn group \((p<0.05, \ \text{Mann-Whitney U test})\) (Fig. 2A-B). Figures 3 and 4 show representative images of pyramidal neuron loss analyses comparing the hippocampal regions (CA1 and CA2-CA3, respectively) studied in all groups.

A coefficient of error (CE) value less than 10% is in the acceptable range\(^{16}\). All estimated CE values in our study were below 0.05%. These findings indicate that the total number of pyramidal neurons that
we estimated in the present study were reliable.

There were no significant correlations between the neuron numbers of each CA1 and CA2-CA3 regions of hippocampus and behavioral parameters including stereotypical behavior, wet dog shakes, agitation, tail stiffness, abnormal posture and gait (p>0.05).

**Figure 1:** Effects of ethanol withdrawal syndrome (EWS) on stereotypical behaviors (A), wet dog shakes (B), agitation (C), tail-stiffness (D) and abnormal posture and gait (E) in rats with (S+) or without (S-) an audiogenic seizure [n=6 for control and (S-) groups, n=5 for (S+) group; *p<0.05 significantly different from control group, Mann-Whitney U test].

**Figure 2:** Effects of ethanol withdrawal syndrome (EWS) on neuron counts in hippocampal areas CA1 (A) and CA2-CA3 (B) in rats with (S+) or without (S-) an audiogenic seizure [n=6 for control and (S-) groups, n=5 for (S+) group; *p<0.001 significantly different from control group, # p<0.001 significantly different from non-seized (S-) group, Tukey's test].
DISCUSSION

Consistent with our previous findings,\textsuperscript{(10,39,45)} the present study confirmed that daily ethanol consumption, which ranged from 10 to 15 g/kg for 21 consecutive days, produced physical dependence in rats. Majchrowicz\textsuperscript{27} also showed that dependence and signs of ethanol withdrawal could be produced in rats with a 4-day intragastric administration of 9-15 g/kg of ethanol per
day. We observed several signs of ethanol withdrawal, such as stereotypical behavior, wet dog shakes, agitation, tail stiffness, abnormal posture and gait, and audiogenic seizures. In our study, the dose of ethanol and administration period were adequate for development of physical dependence to ethanol in rats.

Excessive ethanol consumption can lead to the impairment of cognitive functions and structural brain changes. Patterns of damage appear to relate to lifetime ethanol consumption but are also equally important to other associated health problems, such as Wernicke-Korsakoff syndrome and some types of dementia, particularly as it relates to impairments in learning and memory\(^\text{(18)}\). Although ethanol acts as a general depressant in the central nervous system, it also differentially affects specific areas of the brain. In a novel experimental paradigm, Riley and Walker\(^\text{(37)}\) described marked neuronal loss hippocampal formation of mice after 5 months of ethanol exposure using a nutritionally controlled model. The hippocampal formation is essential for learning, memory and cognitive functions. Thus, ethanol consumption impairs these important tasks in humans and animals. Memory impairments due to ethanol have been associated with disruption of the hippocampal function, particularly affecting gamma-aminobutyric acid (GABA) and N-methyl-D-aspartate (NMDA) neurotransmission, which negatively impacts long-term potentiation (LTP)\(^\text{(Rose and Grant, 2010)}\). The molecular basis of LTP is associated with learning and memory\(^\text{(3)}\). In particular, damage to hippocampal CA1 cells adversely affects memory formation\(^\text{(56)}\), and this disruption has been linked to dose-dependent levels of ethanol consumption\(^\text{(54)}\). In addition to chronic ethanol consumption, impaired LTP and cognitive functions are also due to the repeated experience of withdrawal from ethanol\(^\text{(18)}\).

In the present study, although we did not identify apoptotic or necrotic tissue in hippocampal formation, some significant reductions of neuron counts in this area were observed and this statement was interpreted as a loss of normal neurons. Thus, the present data may provide further evidence that chronic ethanol exposure and withdrawal produce neurodegeneration of the hippocampus. Continuous exposure to ethanol for 20 days followed by withdrawal caused some significant reductions of neurons in both the CA1 and CA2-3 subdivisions of the hippocampal formation in rats. This finding is in accord with previous reports indicating that long-term (more than a month) chronic exposure to ethanol and EWS results in serious structural changes and damage in the hippocampus of rats\(^\text{(2,25,26,28,29,32)}\). Many previous studies showed that neuronal losses in the hippocampus during EWS were observed after at least 2 or more months of chronic ethanol drinking. Here, we demonstrate that EWS following a shorter chronic ethanol exposure can cause significant neuronal reductions in the hippocampal formation of rats. Our observations also imply that the hippocampus may be very susceptible to the destructive effects of ethanol at earlier time points. In the present study, we used a valid liquid diet for chronic ethanol administration. Because we observed some increases in body weight of rats fed by ethanol containing liquid diet, the changes in hippocampal formation cannot be related to malnutrition or dietary aversion.

We also observed that AS activity caused a significant reduction in the numbers of hippocampal CA1-CA3 pyramidal cells compared with both control and AS non-appeared animals. Although we could not see any significant alterations in the intensity of other behavioral signs (i.e., increased stereotypical behaviors and wet dog shakes, agitation, tail-stiffness or abnormal posture or gait) of EWS between AS appeared and non-appeared dependent rats, neuronal losses in hippocampal
formations were significantly elevated in seized rats compared to the non-seized group. First, this finding indicates that there is no relationship between the intensity of the behavioral signs of ethanol withdrawal and a predisposition to AS in ethanol-dependent rats. Second, and more important, AS activity is associated with more damage to the hippocampus during ethanol withdrawal. On the other hand, audiogenic stimulus itself may induce seizures result in hippocampal neuronal loss. Therefore, we suggest that the prevention of epileptic activity in ethanol dependents may be important for protection of the hippocampus from further damage.

An interesting observation in the present study is that there was no significant difference in the intensity of behavioral signs of EWS between seized and non-seized rats. All ethanol-dependent animals exhibited similar intensities of EWS symptoms during ethanol withdrawal. This finding indicates that the strength of the behavioral symptoms of EWS does not affect susceptibility of rats to ASs.

Pawlak et al. (33) showed that long-term ethanol exposure causes enduring damage to the CA1 and CA2 subdivisions of hippocampus in mice. Furthermore, the cell loss in the CA1 region occurs during the withdrawal period, but not during ethanol drinking. Previously, it was suggested that ethanol withdrawal is a sufficient trigger for this degenerative process. (25,35) Lukayanov et al. (25) also observed that chronic ethanol treatment produced marked neuronal loss in the CA1 and CA3 hippocampal areas and that this effect was more profound in withdrawn animals. Thus, these authors suggested that withdrawal from ethanol consumption aggravated the ethanol-induced degenerative processes in the hippocampal formation. Our findings are in accord with the previous results because we found prominent neuronal degeneration in all CA (CA1-3) fields of the hippocampus.

Furthermore, we also expanded this information with our data, observing higher levels of neuronal degeneration in AS rats during ethanol withdrawal. In the present study, AS rats had significantly increased neuronal loss in CA subdivisions of the hippocampal formation compared to no AS ethanol withdrawn rats. Once again, we would like to emphasize that AS activity seems to be a more detrimental sign of EWS in terms of hippocampal damage during ethanol withdrawal. Alcoholic individuals that are susceptible to seizure may have a greater risk of hippocampal destruction and related dysfunctional problems (i.e., learning and memory deficits). However, in our study, we did not test behaviors relating to cognitive functions of rats. Evaluation of the effects of ASs on some cognitive functions and comparing the data with the results in non-seized rats during ethanol withdrawal would enhance our findings. In contrast, Lukayanov et al. (25) observed unimpaired water maze performance in ethanol withdrawn rats despite considerable cell loss in both the CA1 and CA3 subdivisions of the hippocampus after 13 months of ethanol intake. Although twenty days ethanol intake does not seem to be enough time to detect the effects of excessive hippocampal damage on cognitive functions, evaluation of the effects of AS activity on cognitive functions in ethanol withdrawn rats remains important. Further studies are necessary to understand if the hippocampal neuronal losses in seized ethanol-dependent rats also impair cognitive functions.

Bonthius et al. (4) reported that daily exposure to ethanol resulted in a dose-dependent decrease in the seizure threshold and in the selective loss of CA1 pyramidal cells in rat postnatal pups. In this study, reduction in the seizure threshold was found to be significantly correlated with neuronal losses in the CA1 area. While CA1 the pyramidal cell population was reduced in number following postnatal
ethanol exposure, the numbers of CA3 pyramidal cells and dentate granule cells were unaffected. Tran and Kely(43) also suggested that the CA1 area of the hippocampus is highly susceptible to ethanol exposure that occurs during gestation or the early neonatal period, while areas CA3 and dentate gyrus are more resistant to ethanol-induced insult during all periods of hippocampal development. In the present study, we observed marked reductions in the number of neurons in all subdivisions of the CA area of the hippocampal formation. Our results imply that the CA subdivisions of the hippocampal formation may be more sensitive to the effects of ethanol in the adult. Discrepancies between the studies may be due to using differences in animal models and methodology.

There are two possibilities to explain the mechanism of neuronal loss in the hippocampus during ethanol withdrawal. First, NMDA-nitric oxide (NO) induced neurotoxicity may responsible for these neuronal losses. Chronic ethanol treatment of cortical cultures led to hypersensitive NMDA receptors that remained inhibited in the presence of ethanol, but ethanol removal led to NMDA-mediated NO production and neurotoxicity(11,12). In addition, Prendergast et al. (36) suggested that withdrawal from chronic ethanol treatment and pathological activation of NMDA-type glutamate receptors produced neurodegeneration in the hippocampus. In a comprehensive review, Uzbay and Oglesby(48) hypothesized a model for the operation of the NO system during ethanol withdrawal in the CNS. According to this model, glutamate released from the presynaptic nerve terminal acts upon NMDA receptors during ethanol withdrawal. Glutamate activates NMDA receptors, through which Ca²⁺ enters and, via calmodulin (CaM), activates the nitric oxide synthase (NOS) enzyme. The NO that is produced diffuses back to the presynaptic neuron where it enhances the release of glutamate via guanylate cyclase (GC) and cGMP(48). In a previous study from our laboratory, we observed significantly high cGMP levels in the hippocampus, but not cerebral cortex and striatum, in ethanol-dependent rats during ethanol withdrawal(50). Furthermore, both NMDA receptor antagonists(30) and NOS inhibitors(47) potently inhibited ethanol withdrawal-induced ASs in rats. Thus, ethanol-induced neurotoxicity in the hippocampal formation may relate to glutamate and/or NO excitotoxicity, which occurred primarily during ethanol withdrawal.

Second, GABAergic alterations could provide a contribution to the neurodegenerative effects of ethanol or ethanol withdrawal in the hippocampal formation. Repeated exposure to ethanol alters GABA-A receptor subunit composition within the CA1 area of the hippocampus in rats(22). Reduction of GABAergic inhibition in CA1 pyramidal cells induced by these changes to GABA receptors could make the CA1 pyramidal cells hyperexcitable and could transform CA1 into a focus of seizure onset(4). The GABAergic contribution to seizures may also support neurodegenerative processes in the hippocampus.

Alterations in some transcription factors such as the cAMP-response-element-binding-protein (CREB) and brain-derived neurotrophic factor (BDNF) during ethanol intake and/or withdrawal may also play a role in hippocampal degeneration(12,13). Further studies are needed to understand the mechanism.

In conclusion, although audiogenic stimuli itself may induce seizures result in hippocampal neuronal loss, our results suggest that ASs during ethanol withdrawal significantly potentiate neuronal degeneration in all subdivisions of CA areas in the hippocampal formation in rats. Thus, the prevention of seizures in alcoholic individuals during ethanol withdrawal may be important for
protection from excessive neuronal damage in the hippocampus.

**Acknowledgments**

This study has been supported by The Scientific and Technological Research Council of Turkey (TUBITAK) (Project No: 105S387, SBAG-3194).

The authors would like to thank Professor Esat Adiguzel from Pamukkale University, Denizli, Turkey for his valuable comments and support of the manuscript.

Language of the manuscript has been revised and corrected by American Journal Experts.

**Conflict of interest**

None of the authors has any conflicts of interest to declare.

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**Received by:** 22 July 2014  
**Revised by:** 25 September 2014  
**Accepted:** 02 October 2014

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