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The Effects of Chronic Ethanol Administration on Amygdala Neuronal Firing and Ethanol Withdrawal Seizures

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Summary

Physical dependence on ethanol results in an ethanol withdrawal (ETX) syndrome including susceptibility to audiogenic seizures (AGS) in rodents after abrupt cessation of ethanol. Chronic ethanol administration and ETX induce functional changes of neurons in several brain regions, including the amygdala. Amygdala neurons are requisite elements of the neuronal network subserving AGS propagation during ETX induced by a subacute “binge” ethanol administration protocol. However, the effects of chronic ethanol administration on amygdala neuronal firing and ETX seizure behaviors are unknown. In the present study ethanol (5 g/kg) was administered intragastrically in Sprague-Dawley rats once daily for 28 days [chronic intermittent ethanol (CIE) protocol]. One week later the rats began receiving ethanol intragastrically 3 times daily for 4 days (binge protocol). Microwire electrodes were implanted prior to CIE or on the day after CIE ended day 29 to record extracellular action potentials in lateral amygdala (LAMG) neurons. The first dose of ethanol administered in the binge protocol following CIE treatment did not alter LAMG neuronal firing, which contrasts with firing suppression seen previously in the binge protocol alone. These data indicate that CIE induces neuroadaptive changes in the ETX network which reduce LAMG response to ethanol. LAMG neuronal responses to acoustic stimuli prior to AGS were significantly decreased during ETX as compared to those before ethanol treatment. LAMG neurons fired tonically throughout the tonic convulsions during AGS. CIE plus binge treatment resulted in a significantly greater mean seizure duration and a significantly elevated incidence of death than was seen previously with the binge protocol alone, indicating an elevated seizure severity following chronic ethanol administration.

Keywords

chronic intermittent ethanol; adaptive changes; ethanol withdrawal; audiogenic seizures; neuronal firing; amygdala; microwire

Introduction

Audiogenic seizures (AGS) can be induced by intense acoustic stimulation during ethanol withdrawal (ETX) after a “binge” ethanol administration protocol (Majchrowicz, 1975;

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Faingold and Riaz, 1994, 1995). The AGS in ETX rats are typically composed of a wild running episode, which is often followed by generalized clonic and/or tonic convulsions (Faingold and Riaz, 1994, 1995; Riaz and Faingold, 1994; Feng and Faingold, 2000; Feng et al., 2007). Previous studies demonstrated that several brainstem structures are involved in ETX seizure initiation and propagation (Faingold and Riaz, 1994, 1995; Faingold et al., 1998; Yang et al., 2003; Long et al., 2007). The amygdala is a limbic structure, which has been implicated in the ETX syndrome, based on metabolic, enzymatic, focal microinjection, immediate early gene, and behavioral evidence (Clemmesen et al., 1988; Rassnick et al., 1993; Knapp and Crews, 1999). We recently observed that amygdala neurons are also critically involved in the neuronal network subserving AGS propagation during ETX, since ETX seizures can be blocked by focal microinjection into amygdala, and amygdala neurons fire intensely during the later behavioral phases of AGS (Feng et al., 2007).

It has been well established that chronic ethanol administration leads to adaptive changes of neurotransmitter receptors in the mammalian brain (Faingold et al., 1998; Olsen et al., 2005; Weiner and Valenzuela, 2006). The experience of repeated withdrawals from chronic ethanol treatment lead to a kindling-like process (Gonzalez et al., 2001), and chronic ethanol treatment potentiates seizures evoked by several convulsants (Kokka et al., 1993; Becker et al., 1998). It has been shown that chronic ethanol administration leads to facilitated NMDA receptor function (Floyd et al., 2003; Roberto et al., 2004) and altered GABA_A receptor pharmacology and subunit expression (Floyd et al., 2004) in the amygdala. These data suggest that amygdala neurons may have different responses to chronic ethanol than those resulting from subacute ethanol administration. However, the effects of chronic ethanol treatment on the amygdala neuronal firing and the AGS inducible during ETX in behaving animals have not been investigated.

The present study used chronic single unit recording (microwire) techniques to examine the neuronal firing patterns and responses to acoustic stimuli in the lateral nucleus of the amygdala (LAMG). The animals in the present study were subjected to chronic intermittent ethanol (CIE) administration once daily for 28 days. Microwire chronic electrodes were implanted either before or after CIE. One week after CIE the rats were treated with the 4-day “binge” ethanol protocol (Majchrowicz, 1975). LAMG neuronal responses were evaluated during CIE, one hr after the first dose of the binge ethanol, and during ETX in behaving animals. LAMG neuronal firing in response to acoustic stimuli and during AGS and its relationship with each behavioral component of ETX seizures were examined. An abstract on these data has been presented previously (Feng and Faingold, 2001).

Methods

Male Sprague-Dawley rats, weighing 300–400 g, were used in this study. The animals were housed in a temperature-controlled vivarium under a 12-hr light/dark cycle with water and food available *ad libitum*. The experimental protocol was approved by the Laboratory Animal Care and Use Committee of Southern Illinois University School of Medicine. All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to *in vivo* techniques.

Ethanol (95%) was diluted with water to form a 30% (v/v) ethanol solution, in which 5 ml of low-iron infant formula (Similac, Abbott Laboratories, Columbus, OH) was added to supplement nutrition. The rats were intubated with 5 g/kg ethanol once daily for 28 days. The animals had free access to food and water during chronic intermittent ethanol (CIE) administration.

Microwire electrodes were implanted under anesthesia [ketamine/xylazine (85/3 mg/kg, i.p.)] prior to CIE or one day after CIE into the lateral nucleus of the amygdale (LAMG) at the following stereotaxic coordinates (−3.3 mm posterior to bregma, 5.3 mm medial/lateral, 7.6 mm vertical below the surface of the skull) (Paxinos and Watson, 1998). The microwire electrode assembly was composed of an amphenol connector (Dale Electronics, Yankton, SD), six 13- μ m diameter insulated tungsten wires (California Fine Wire Company, Grover City, CA) and a harpoon. The microwires were bundled together using polyethylene glycol, which melted inside the brain, allowing the microwires a small degree of migration towards LAMG cell bodies (Faingold and Anderson, 1991). The animals were given tetracycline (1 g/liter) in the drinking water to minimize post-operative infection during the 7-day recovery period (no ethanol solution was given during recovery).

One week after the surgery, the neuronal firing in the LAMG was recorded in a plexiglass cylinder (diameter: 44 cm) inside a sound-attenuating chamber. The acoustic stimulus was given in the form of tone bursts [12 kHz, 100 ms duration, 5 ms rise-fall, 50–100 dB SPL (re: 0.0002 dyne/cm²), stimulus repetition rate of 0.5–4 Hz]. This involved 50 stimulus presentations at each of 4 stimulus intensities over a ~10 min period, and no evidence of tolerance or sensitivity was observed during the recordings. The neuronal firing patterns were recorded digitally. Ethanol dependence was induced by a subacute “binge” ethanol protocol (Majchrowicz, 1975) (see below). LAMG neuronal firing was recorded prior to and one hr after the first dose of ethanol administration by this protocol and during ETX. LAMG neuronal firing simultaneous with seizure behaviors during ETX were videotaped with split-screen video techniques.

Ethanol dependence was induced in rats by intragastric intubation of ethanol solution (Majchrowicz, 1975). Initially, each animal was given a dose of 5 g/kg of ethanol in the form of ethanol solution (30% v/v), and each subsequent dose was based on the behavior to maintain the intoxication of the rats at the mild-to-moderate ataxic state (Majchrowicz, 1975). The rats were given the ethanol solution every 8 hrs for 4 days and ethanol was withdrawn at the end of the fourth day.

In order to examine the effect of ethanol on LAMG neuronal firing during the 4-week CIE treatment period, one group of rats was implanted with microwire electrodes, and the LAMG neuronal firing was recorded each week thereafter.

The data collected in the recordings were analyzed off-line. Single unit responses with a ratio of ≥ 3 to 1 to background activity were discriminated using the Digitizer Program (Plexon Inc., Dallas, TX) and the Offline Waveform Sorter (Plexon Inc.). One single unit was recorded from each animal. The resulting data were analyzed using poststimulus time histograms (PSTHs) (400 ms scan length, 1 ms bin width, 50 presentations) using the NEX program (Plexon/NEX Technologies, Dallas, TX).

The comparisons of LAMG neuronal firing in mean number of action potentials per PSTH after CIE treatment, one hr after the first dose of binge ethanol and during ETX were evaluated using ANOVA of repeated measures with Tukey’s test as post-hoc analysis. The comparison of the LAMG neuronal firing weekly during the 4-week CIE treatment was carried out using the paired Student *t* test. Seizure severity differences between the CIE+binge ethanol vs. binge ethanol alone observed in a previous study (Feng et al., 2007) were compared using the Student *t* test. Statistical comparisons of fatality data during ETX between the present study and previous studies involved the use of the Chi-Square test.

At the end of the experiments, the rats were deeply anesthetized with pentobarbital (100 mg/kg, i.p.), and the microwire electrode tips were marked with lesions by passage of DC current (10 μ A, 30 sec). The rats were perfused transcardially with normal saline and then 10%

formalin. The brains were removed and preserved in formalin. Sections of brain (40 μ m) were sliced on a freezing microtome. The sections were stained with Neutral Red, and the sites of electrode tips were verified microscopically.

Results

Single unit extracellular action potentials were recorded from a total of 17 neurons in the LAMG from two groups of rats subjected to CIE. One group of 8 rats had microwires implanted prior to CIE, and the second group of 9 rats were implanted after CIE. Histological examination revealed that the microwire electrode locations were confined to the LAMG as shown in Fig. 1.

Neuronal firing in the 8 animals that were implanted prior to CIE was recorded at weekly intervals during the 28-day CIE administration period. Mean LAMG neuronal responses (action potentials per PSTH) in response to acoustic stimuli at the weekly intervals of CIE administration were evaluated but did not exhibit any statistically significant change (data not shown). No consistent weight changes were observed during the CIE treatment period. A typical example of neuronal firing during the 28-day treatment period is presented in Fig. 2 and shows little consistent change in firing recorded from the same animal and electrode site.

One hr after the first dose of ethanol in the 4-day binge protocol single unit activity in all 17 rats was evaluated, and neuronal responses were also examined beginning 20 hr after ETX when most rats became susceptible to AGS. The mean number of action potentials per PSTH in response to increasing acoustic stimulus intensity at the 0.5 Hz repetition rate was not significantly altered one hr after the first dose of ethanol in the binge protocol as compared with that after the 4-week CIE treatment (ANOVA of repeated measures) ($n=17$) (Fig. 3). The binge ethanol protocol for the 9 rats in the second group began one week after surgery to allow for recovery. To be consistent with this time course, the binge protocol for the 8 rats in the first group began one week after CIE treatment. No ethanol was administered during this 7 day period in either rat group. There were no consistent differences between the two groups of rats in any parameter evaluated, and the data from both groups were subsequently pooled.

For the acoustic stimulation intensities used (57–80 dB), overall ANOVA analysis indicated that significant changes in neuronal response were observed after CIE treatment, one hr after the first dose of binge ethanol and during ETX (57 dB, $F_{(2,32)}=4.79$, $p<0.05$; 62 dB, $F_{(2,32)}=16.68$, $p<0.01$; 68 dB, $F_{(2,32)}=25.78$, $p<0.01$; 80 dB, $F_{(2,32)}=37.19$, $p<0.01$) (ANOVA of repeated measures). Post hoc analysis found that for intensities from 62–80 dB, LAMG neuronal responses to acoustic stimuli were significantly decreased during ETX, following the binge protocol as compared with either the responses after CIE treatment or the responses one hr after the first dose of ethanol in the binge protocol ($p<0.01$) in both groups of rats ($n=17$) (Fig. 3). A significant difference in neuronal responses was observed for the 57 dB intensity between one hr after the first dose of binge ethanol and during ETX ($p<0.05$). Previous studies from our lab and others indicate that blood alcohol levels at these time points after ETX were negligible (Faingold and Riaz, 1995).

Figure 4 illustrates a typical example of the pattern of LAMG neuronal response change in rats that had received CIE administration, as compared to one hr after the first dose of binge ethanol and during ETX. This LAMG neuron exhibited a short-latency onset response to acoustic stimulus after CIE administration that was typically seen in these neurons (Fig. 4A). The neuronal response one hr after the first dose of binge ethanol was not noticeably different from that after CIE administration (Fig. 4B). However, the neuronal firing was greatly suppressed during ETX prior to AGS (Fig. 4C).

Of the 17 rats, 11 animals exhibited AGS during ETX. AGS were triggered by 12 kHz tone bursts at 100 dB SPL given at repetition rates up to 4 Hz. Wild running was observed in 9 rats. Eight of these rats exhibited tonus and 9 rats underwent clonus. Six rats exhibited continuous (duration ≥ 60 sec) jumping and bouncing behavior. The mean duration of clonus [45.4 ± 10.9 (SEM) sec] induced by CIE+binge ethanol was significantly greater ($p < 0.05$, Student *t* test) than that seen in a previous study with binge ethanol alone (12.7 ± 3.2 sec) (Feng et al., 2007). The mean duration of wild running and tonus was 16.2 ± 8.3 sec and 10.3 ± 1.5 sec in the present study, which was not significantly different from the previous study with binge ethanol alone (Feng et al., 2007). Seven rats died as a result of ETX seizures, mainly following the continuous jumping and bouncing behavior. The incidence of death that occurred with the CIE+binge ethanol protocol was significantly increased ($p < 0.05$, Chi-square test) as compared with the previous binge ethanol alone study in which none of the 7 rats died from ETX seizures (Feng et al., 2007). The seizure incidence and the percentage of wild running, tonus and clonus were not significantly different between the present study and previous binge ethanol alone study.

LAMG neuronal firing and simultaneous behavior were recorded with split-screen video prior to and during each component of AGS during ETX ($n=11$). Typical examples of ETX seizure behaviors and corresponding LAMG neuronal responses are shown in Fig. 5. Prior to binge ethanol treatment, LAMG neurons exhibited an onset response, as illustrated by the trace in Fig. 5A. During ETX, the neuronal response was completely suppressed (e.g., Fig. 5B). The AGS during ETX was triggered by the 4 Hz stimulus repetition rate. The acoustic stimulation ceased as the AGS began. LAMG neurons exhibited tonic firing during AGS in the tonic behavioral phase as shown by the example in Fig. 5D. During wild running, clonus and jumping and bouncing behaviors, the LAMG neuronal firing was suppressed (Fig. 5C, E, and F). These firing patterns are consistent with a previous study using binge ethanol alone, indicating that LAMG neurons are critically involved in tonic seizures but not in the other behavioral components of ETX seizures (Feng et al., 2007).

Discussion

The present study found that the first dose of binge ethanol administered one week after CIE treatment did not suppress LAMG neuronal responses. This finding is in contrast with previous studies using the binge ethanol alone protocol, in which the neuronal responses were significantly suppressed one hr after the first dose of binge ethanol administration in several brainstem structures (Faingold and Riaz, 1994; Chakravarty and Faingold, 1998; Faingold et al., 1998; Yang et al., 2003) and in LAMG (Feng et al., 2007). No ethanol was administered during the week between the last dose of CIE and the first dose of the binge ethanol administration. Therefore, this dichotomy between the effects of the CIE+binge ethanol protocol and the binge ethanol protocol alone, which also involved multiple intragastric intubations, suggests that LAMG neurons underwent long-lasting adaptive changes as a result of CIE administration.

Chronic ethanol administration has been observed to lead to supersensitivity of NMDA receptors (Nelson et al., 2005; Hendricson et al., 2007). One mechanism for the change of NMDA receptor sensitivity has been proposed to involve alterations of NMDA receptor subunits (Sheela Rani and Ticku, 2006), since relatively little change of NMDA receptor density was observed after chronic ethanol treatment (Tremwel et al., 1994; Rudolph et al., 1997; Freund and Anderson, 1999). It has been observed that chronic ethanol treatment facilitated NMDA receptor function in the amygdala (Floyd et al., 2003; Roberto et al., 2004). Thus, the tolerance of LAMG neuronal firing to the first dose of binge ethanol observed in the present study may be related to adaptive changes of NMDA receptors induced by CIE administration. It has been observed that chronic ethanol treatment also reduces the action of

GABA (Kang et al., 1996; Faingold et al., 1998), which may involve GABA_A receptor subunit alterations in the brain including the amygdala (Devaud et al., 1995; Grobin et al., 2000; Floyd et al., 2004; Liang et al., 2006). Therefore, a decrease in GABA_A receptor function may also contribute to the tolerance of LAMG neuronal firing to ethanol following CIE administration.

LAMG neuronal firing was significantly suppressed during ETX in the present study. This finding is consistent with a previous study in the amygdala and in another part of the ETX seizure network following the binge ethanol protocol (Feng et al., 2007; Yang et al., 2001). However, these findings contrast with increases in firing seen during ETX in several brainstem structures in the ETX network (Faingold and Riaz, 1994, 1995; Chakravarty and Faingold, 1998; Faingold et al., 1998). ETX-induced firing increases may be due, in part, to enhanced NMDA receptor-mediated excitation and decreased GABA receptor-mediated inhibition observed during ETX (N'Gouemo et al., 1996; Hu and Ticku, 1997; Faingold et al., 1998; Thomas et al., 1998; Olsen et al., 2005). These data indicate that ETX may exert different influences on neuronal firing in different parts of the network for AGS of ETX. Several other neurotransmitter alterations occur in the amygdala during ETX, including changes in cholinergic function (Girod et al., 2000; Arendt et al., 1988), which could also contribute to the present findings. ETX resulted in a region-specific reduction of local cerebral glucose consumption in forebrain structures including the amygdala (Clemmesen et al., 1988), which may be related to the suppression of amygdala neuronal firing seen in the present study. These changes may overshadow the adaptive changes of glutamatergic and GABAergic functions and contribute to the suppression of LAMG neuronal firing during ETX.

The present study found that tonic LAMG neuronal firing occurred during tonic convulsions of ETX seizures, but not during the other behavioral phases of AGS, which is consistent with our previous report that amygdala neurons are critically involved in ETX seizure propagation rather than initiation (Feng et al., 2007). These data suggest that the role of LAMG neurons in ETX seizures was not altered by CIE. However, the present study observed that the seizure duration and the incidence of death during ETX were significantly increased after CIE+binge ethanol treatment as compared with binge ethanol alone, suggesting that other structures may be responsible for the seizure severity increase after CIE treatment. Further experiments are needed to explore this possibility.

Chronic ethanol treatment has been proposed to result in a kindling-like process (Gonzalez et al., 2001). ETX after chronic ethanol or CIE treatment enhanced the neuronal GABA_A receptor sensitivity to convulsant drugs (Hu and Ticku, 1997; Kokka et al., 1993; Becker et al., 1998). Facilitation of electric kindling of the amygdala by chronic ethanol treatment was also observed (Carrington et al., 1984), suggesting that chronic ethanol administration results in synaptic changes in the amygdala that are similar to those that occur during electric kindling (Ripley et al., 2002). The present study demonstrated that CIE treatment results in long-lasting adaptive neuronal changes in the amygdala and increases ETX seizure severity, further supporting the notion that chronic ethanol treatment produces a kindling-like process in the brain.

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Figure 1.

This drawing shows recording sites of the microwire electrode assemblies in the lateral nucleus of amygdala (LAMG), according to the atlas of Paxinos and Watson (1998). (AST, amygdalostriatal transition area; BLA, basolateral nucleus of amygdala; BMA, basomedial nucleus of amygdala; CA, central nucleus of amygdala; CPU, caudate putamen; DE, dorsal endopiriform nucleus; ec, external capsule; ot, optic tract; st, stria terminalis).

Figure 2.

A typical example of LAMG neuronal firing during the chronic intermittent ethanol (CIE) treatment period. LAMG neuronal firing was recorded once a week from the same site in the same animal during the 28-day CIE treatment period, and CIE treatment did not consistently affect LAMG neuronal firing. Panels A–D represent poststimulus time histograms (PSTHs). The insets E–H are examples of the corresponding digital oscilloscope traces. AP amplitude: E–H=200 μ V. Acoustic stimulus parameters: 12 kHz tone burst, 100 ms duration, 5 ms rise-fall, 80 dB SPL, 0.5 Hz rate, 50 presentations.

Figure 3.

The mean LAMG neuronal responses after CIE treatment (squares), one hr after the first dose of binge ethanol treatment (circles) and during ETX (triangles). The mean (error bars represent SEM) number of action potentials per PSTH was not significantly changed one hr after the first dose of binge ethanol as compared with that after 28-day CIE administration. LAMG neuronal firing was significantly decreased during ETX in all rats ($n=17$), which includes both the first group of 8 rats and the second group with 9 rats subjected to binge ethanol. Note: Intensities > 80 dB were not utilized in these trials to avoid triggering seizures. ** Significantly different from the responses after 28-day CIE treatment and one hr after the first dose of binge ethanol at $p<0.01$ (ANOVA of repeated measures).

Figure 4.

A typical example of LAMG neuronal firing from the same animal and electrode after CIE treatment, one hr after the first dose of binge ethanol administration and during ETX. Panels A–C are PSTHs. The insets (D–F) are corresponding digital oscilloscope traces. The LAMG neuron exhibited an onset firing pattern after 28-day CIE administration (A, D). One hr after the first dose of binge ethanol administration, the neuronal firing was not greatly affected (B, E). The neuronal firing was almost completely blocked during ETX (C, F). Action potential amplitude in D: 250 μ V. Acoustic stimulus parameters: 12 kHz tone burst, 100 ms duration, 5 ms rise-fall, 80 dB SPL, 0.5 Hz rate, 50 presentations. (N = number of extracellular action potentials per PSTH).

Figure 5.

Typical examples of each behavioral component of AGS during ETX and the corresponding LAMG neuronal firing recorded from the same behaving rat and electrode. After CIE treatment, the LAMG neuron exhibited an onset response to the acoustic stimulus (A). During ETX, just prior to the initiation of AGS, the LAMG neuronal response to the acoustic stimulus was depressed (B). AGS was triggered by 12 kHz tone bursts (100 dB SPL at the 4 Hz repetition rate). Acoustic stimulation ceased when the AGS began. LAMG neuronal firing continued to be suppressed during the wild running (n=9) (C), clonus (n=9) (E) and jumping/bouncing behavior (F). LAMG neurons consistently exhibited tonic firing during tonic seizures (n=8) (D). Action potential amplitude in A: 250 μ V. (Drawings based on Naritoku et al., 1992).