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The Influence of a Chronic Adolescent Nicotine Exposure on Ethanol Withdrawal Severity During Adulthood in C3H Mice

Hugh H. Riley, Ph.D., André W. Zalud, Ph.D., and Jaime L. Diaz-Granados, Ph.D.

Department of Psychology and Neuroscience, Baylor University

Abstract

Animal and human studies have shown tolerance, consumption, relapse, and behavioral interactions between ethanol and nicotine, but little is understood about their interaction, especially as it relates to ethanol withdrawal in adulthood for subjects who have an adolescent history of using these drugs. This study investigated nicotine's influence on ethanol withdrawal seizures in two different age groups of male C3H mice. Adolescent and adult male C3H mice, beginning at postnatal day (PD) 28 or 70, respectively, were subjected to a 7 day chronic exposure to ethanol only, ethanol plus nicotine, nicotine only, or vehicle treatment. Six weeks later, all groups were subjected to chronic exposure to ethanol vapors and the severity of their ethanol withdrawal seizures was assessed by handling-induced convulsions (HIC). An adolescent exposure to chronic nicotine resulted in an exacerbation of ethanol withdrawal seizures in adulthood. Given this, adolescence may contain a neurophysiological critical period that is sensitive to nicotine and which may result in an altered response to ethanol dependency in adulthood. These findings have serious implications for the long-term consequences following co-abuse of these drugs during adolescence.

Keywords

Adolescence; Mice; Ethanol Withdrawal; Nicotine

INTRODUCTION

Ethanol and nicotine are commonly abused drugs with major social, health, and economic consequences, and they also pose significant problems for our adolescent population. According to the 2005 National Survey on Drug Use and Health, 41% of 8th graders and 75% of 12th graders have tried ethanol, with 31% of 12th graders reporting binge ethanol use, which is defined as drinking five or more drinks on the same occasion within the past 30 days, and 11% reporting heavy ethanol use, which is defined as drinking five or more drinks during one occasion on five or more days in the past 30 days. With respect to smoking, 63% of new smokers are under age 18, with 25% reporting being current smokers (Substance Abuse and Mental Health Services Administration, 2006).

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Corresponding Author: J.L. Diaz-Granados, Ph.D. One Bear Place #97334 Waco, TX 76798 254-710-2961 (Office) 254-710-3033 (Fax) Jim_Diaz-Granados@baylor.edu.

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Although ethanol and nicotine are commonly abused drugs in both adult and adolescent populations, the adolescent period is characterized by numerous and dynamic central nervous system (CNS) changes such as synapse overproduction and elimination, maturation of numerous neurochemical systems, continuing myelination, and dramatic increases in acetylcholinesterase expression (Giedd, 2004; Huttenlocher, 1984; Lenroot and Giedd, 2006; Rakic et al., 1986; Robertson et al., 1991), and studies have also shown that numerous neurotransmitter systems are affected or permanently altered by exposure to ethanol, nicotine, or both during this developmental period (Acheson et al., 1999; Sircar and Sircar, 2006; Lopez et al., 2001; McGehee et al., 1995; Adriani et al., 2004; Mansvelder et al., 2002). Therefore, given the use of these drugs by adolescents and the multiple neuromodulatory systems that experience significant changes during this developmental period and which are influenced by these drugs, in this study we hypothesized that a chronic adolescent exposure to nicotine and/or ethanol would alter the animal's response to ethanol withdrawal in adulthood. The implications of this research are important in showing that the adolescent period is vulnerable to drug exposure, which can leave the mature organism with an altered response to ethanol withdrawal.

MATERIALS AND METHODS

Subjects

Male C3H mice (N = 220), obtained from Charles River Laboratories (Raleigh, NC), were randomly assigned to the treatment conditions. Subjects were housed three to five animals in a Plexiglas cage that was lined with wood shavings and equipped with a wire lid. Animals were maintained under a 12-hour dark/light cycle in an Institutional Animal Care and Use Committee approved animal facility with ad libitum access to rodent lab chow and tap water. Throughout the experiment, all procedures and care of the animals were conducted in accordance with institutional guidelines and NIH policies (National Research Council, Guide for the Care and Use of Laboratory Animals. National Academy Press, 1996).

Protocol & Treatment Groups

The experimental protocol consisted of two separate phases of drug administration, with phase I beginning at a specific postnatal day (PD) age and phase II beginning six weeks later. At the start of phase I, which persisted daily for seven consecutive days, subjects were either adolescents at PD28 (n = 155) or adults at PD70 (n = 65). During this phase, the subject's weight was measured daily. In phase II, all subjects were first weighed and then exposed to chronic ethanol via continuous inhalation and the severity of their ethanol withdrawal seizures was measured by Handling Induced Convulsions (HIC), and the HIC scoring criteria is shown in Table 1. Immediately after Phase II, the subjects were again weighed and the severity of ethanol withdrawal seizures was measured by HIC (Becker et al., 1997). HIC testing began one hour after the subjects had been removed from the ethanol inhalation chamber and continued hourly for the next 10 hours. A final HIC test was conducted on the following day, 24 hours after their chamber removal. For each phase of drug administration, the changes in the subjects' weights were recorded as the percent change that had occurred during that phase.

In phase I, there were four treatment conditions: ethanol only (EO) (n = 65), nicotine only (NO) (n = 53), ethanol plus nicotine (EN) (n = 50), and controls (CO) (n = 52) who received physiological saline only. Each subject received their appropriate drug via a morning intraperitoneal (IP) injection at 10:00 am and an afternoon subcutaneous (SC) injection at 2:00 pm. More specifically, the ethanol only (EO) group received 1.8 g/kg ethanol plus 1 mmol/kg pyrazole, an alcohol dehydrogenase inhibitor, for the morning injection and physiological saline for their afternoon injection. Pyrazole was used to extend BEC stability during this phase. The nicotine only (NO) group received physiological saline and 1 mmol/kg of pyrazole for the

morning injection and 1.0 mg/kg (-)nicotine (hydrogen tartrate salt) for their afternoon injection. The ethanol plus nicotine (EN) group received 1.8 g/kg ethanol plus 1 mmol/kg pyrazole for the morning injection and 1.0 mg/kg (-)nicotine (hydrogen tartrate salt) for their afternoon injection. Controls (CO) received physiological saline and 1.0 mmol/kg pyrazole for their morning injection and physiological saline only for their afternoon injections. All of the morning injections were administered at an injection volume of .02 ml/g body weight; the afternoon injections were administered at an injection volume of .01 ml/g body weight. All injections were delivered using 1.0 ml syringes equipped with a 26-gauge needle.

In phase II, all groups were subjected to 64 hours of continuous ethanol inhalation in a clear Plexiglas inhalation chamber that measured 61 × 38 × 61 cm. Before chamber entry however, and in order to establish an initial blood ethanol concentration (BEC), each subject received an IP injection of 1.8 g/kg ethanol (12% v/v using 95% ethanol) and 1 mmol/kg pyrazole (.02 ml/g body weight). Isotonic saline was used as the vehicle for all injections. In all cases, in order to control for any possible influence of pyrazole on handling-induced convulsions (Crabbe et al., 1981), pyrazole injections were equated among all groups. To deliver ethanol vapor to the inhalation chamber, pure American Chemical Society grade ethanol (Pharmco, Brookfield, CT) was delivered to a volatilizing flask at a rate of 200 µl/min and the volatilized ethanol vapor was then mixed with air and the resultant ethanol/air mixture was delivered to the ethanol inhalation chamber at a rate of 7 liters per minute. In prior research by our lab, this consistently results in an approximate ethanol chamber concentration of 11-13 mg of ethanol per liter of air and will maintain a BEC range from 0.15% to 0.20%. At the end of phase II, the subjects were removed from the ethanol inhalation chamber and the severity of their ethanol withdrawal seizures was measured hourly by HIC testing (Becker et al., 1997).

To ensure that the BEC was uniform across all treatment groups and thus could not influence the HIC scores, blood samples from each subject were collected at the end of phase II. This was accomplished by drawing 10 µl of blood from the retro orbital sinus of each subject, which was then placed on ice and diluted 50:1 with 3.4% (volume/volume) perchloric acid and subjected to 8000 × g centrifugation at 4° C for 15 minutes. The supernatant was then used in an enzymatic assay modified after the Calbiochem-Behring method (La Jolla, CA). The assay is based on the conversion of alcohol to acetaldehyde by alcohol dehydrogenase in the presence of the cofactor nicotinamide adenine dinucleotide (NAD). NAD is reduced stoichiometrically during the reaction to NADH that is detectable by UV spectrophotometry at 340 nm. BEC is expressed as grams per deciliter (g/dl) plasma.

Statistical Analyses

For each age cohort and treatment condition, the statistical analyses included an examination of BEC, weight changes, and ethanol withdrawal severity. For BEC and the weight changes that had occurred during phase I and II, the analyses were conducted as a one-way analysis of variance (ANOVA). For severity of ethanol withdrawal, the HIC scores were used to calculate the area under the curve (AUC) and a two-way (age × phase I treatment) ANOVA was utilized to determine if age, phase I treatment, or their interaction had a significant influence on ethanol withdrawal severity. Additionally, a repeated measure ANOVA was carried out for all treatment groups in both age cohorts to identify any possible differences in the withdrawal patterns over time. Post hoc comparisons, when appropriate, were conducted using Tukey's Honest Significant Difference.

RESULTS

As previously stated, BEC was monitored at the end of phase II, and a significant effect of treatment was found for BEC for the PD28 subjects, $F(3, 155) = 3.72$, $p < 0.05$, with post hoc analysis showing that the EO group differed only from controls. There was also a significant

effect for weight change during phase I, $F(3, 155) = 23.68, p < 0.01$, with post hoc analysis revealing that the NO and CO groups were different from the EO and EN groups. As expected, all groups lost weight during phase II, but there were no significant group differences.

For PD70 subjects, treatment effect on BEC was not significant. However, a significant effect was found for weight change during phase I, $F(3, 65) = 26.0, p < 0.05$, with post hoc analysis showing that the NO and CO groups were different from the EO and EN groups, and the EN group was different from the EO group. Weight change for this age cohort during phase II was not significant. Table 2 shows the specific by treatment group BEC values and percent of weight change that occurred during each phase of drug administration for both the PD28 and PD70 cohorts.

For the 10-hour AUC values, the two-way ANOVA was significant, $F(7, 220) = 7.36, p < 0.01$, as was age, $F(1, 220) = 5.71, p < 0.05$; phase I treatment (i.e., adolescent exposure to nicotine, ethanol, nicotine and ethanol, or vehicle), $F(3, 220) = 4.07, p < 0.01$; and their interaction, $F(7, 220) = 3.99, p < 0.01$. Post hoc analysis showed that the PD28 NO subjects were significantly different from all other PD28 groups and from all of the PD70 treatment conditions. None of the PD70 groups were significantly different from each other, and as would then be expected, the repeated measure ANOVA for these subjects was also not significant. However, the repeated measure ANOVA for the PD28 subjects showed that the NO group was different from the EO group at hour 5, $F(3, 155) = 3.52, p < 0.05$; from the CO group at hour 6, $F(3, 155) = 2.52, p < 0.05$; from the EO, EN, and CO groups at hour 8, $F(3, 155) = 6.55, p < 0.01$ and also at hour 9, $F(3, 155) = 5.89, p < 0.01$; and from only the CO group at hour 10, $F(3, 155) = 3.78, p < 0.01$.

For the 24-hour AUC, the two-way ANOVA was again significant, $F(7, 220) = 10.20, p < 0.01$, as was age, $F(1, 220) = 17.12, p < 0.01$; phase I treatment, $F(3, 220) = 5.70, p < 0.01$; and their interaction, $F(7, 220) = 3.70, p < 0.01$. Post hoc analysis showed that only the PD28 NO subjects were significantly different from all other PD28 and PD70 groups.

Figure 1, which shows the ethanol withdrawal patterns for the PD28 subjects by treatment condition and from which the AUC values were derived (PD70 groups are not shown for simplicity), clearly shows that the NO subjects had greater seizure activity in comparison to all of the other treatment groups. It is also interesting to note that despite receiving nicotine, the PD28 EN subjects were not different from the other treatment groups. The PD28 and PD70 AUC-10 and AUC-24 values by treatment group are graphically shown in Figures 2a and 2b, respectively.

DISCUSSION

There were significant weight gain differences in the treatment groups for both age cohorts during phase I, but all subjects had comparable body weights by the start of phase II. Clearly, these weight gain difference were directly related to ethanol intoxication as evidenced by the fact that the ethanol exposed groups had greater weight loss compared to the non-ethanol exposed groups. It is important to note that all four treatments resulted in weight loss during the phase I treatment regimen. However, it is likely that calorie restriction due to intoxication played a role in the greater loss of weight among ethanol-exposed animals. More importantly, there were no significant weight differences in phase II for any treatment group or age cohort. Thus, it is unlikely that these phase I weight losses caused any differences in the overall health of the subjects and thus did not influence their ethanol withdrawal severity.

There was also a statistically significant difference in BEC among the PD28 treatment groups, with the PD28 ethanol only (EO) group showing an elevated BEC compared to controls (CO). We do not know what caused this difference, but the magnitude of this difference was

apparently not sufficient to influence the withdrawal severity for these two groups as their AUC10 values were not significantly different. In addition, since the significant finding of this research is that a chronic adolescent exposure to nicotine only (NO) exacerbates ethanol withdrawal seizures in adulthood, and given that the NO group had comparable BEC levels to all other groups, this result was not likely related to BEC.

The major finding of this research is that the adolescent developmental period contains a critical period that is affected by a chronic exposure to nicotine, and which produces an altered withdrawal ethanol response in adulthood. Moreover, this exacerbation of ethanol withdrawal seizures was not seen when the chronic exposure to nicotine occurred during adulthood, further supporting this finding.

A number of possibilities could account for this finding. For example, much of the cholinergic system's ontogenesis occurs during the first 3-4 postnatal weeks in rodents, which means that the sensitivity of the periadolescent brain is vulnerable to very low doses of nicotine and anticholinesterase inhibitors in comparison to adults (Eriksson et al., 2000; Smith et al., 2006). This could result from significant changes in the nicotinic acetylcholine receptors (nAChRs) as a consequence of nicotine exposure. For example, studies have shown that an adolescent exposure to nicotine causes permanent and profound changes in nAChR composition (Eisile et al., 1993); and in the number, distribution, sensitization (Wonnacott et al., 2000), and overall function of these receptors (Adriani et al., 2004). Moreover, it has been shown that ethanol has a direct effect on nAChR function (Aistrup et al., 1999; Criswell et al., 1993), which suggests that in addition to ethanol exerting its effects through various modulatory systems such as GABA_A, NMDA, and voltage-gated calcium channels, it also acts on nAChRs. Additionally, it has been shown that a single systemic injection of nicotine can cause profound changes in nAChR function that can persist for four weeks (Oliff and Gallardo, 1999). Moreover, Slotkin (2002) demonstrated that adolescent nicotine exposure causes a substantially different pattern of nAChR upregulation from the pattern seen in adults, which persisted for more than a month after nicotine had been cleared from the system.

The combined exposure to ethanol and/or nicotine during adolescence has been shown to alter nAChR upregulation and to be accompanied by persistent alterations in the cholinergic, noradrenergic, dopaminergic, and serotonergic systems (Ribeiro-Carvalho et al., 2008; Slotkin, 2002; Slotkin et al., 2008). This is important as numerous other studies have shown that nAChRs regulate the release of several CNS neurotransmitters, including glutamate, GABA, dopamine, serotonin, and acetylcholine (Lloyd and Williams, 2000; Mansvelder et al., 2002; Wonnacott et al., 2000). This suggests that nAChRs may serve to amplify and modulate CNS activities by regulating the release of other neuromodulators (Aramakis and Metherate, 1998), many of which are implicated in ethanol-related activity, including withdrawal. Given this, a chronic exposure to nicotine during adolescence could cause profound changes in normal neuronal development as a result of changes in nAChR composition or by nicotine-mediated changes in these other neuromodulatory systems.

Moreover, since nicotine and ethanol interact with many common receptors, the chronic exposure to nicotine during adolescence could have acted directly on ethanol's targets and produced persistent and fundamental changes in those targets. For example, both NMDA and GABA receptors have been implicated in ethanol withdrawal, and nicotine is known to affect the GABA and NMDA pathways, in that it persistently depresses GABA release through nAChR desensitization (Mansvelder et al., 2002); and through its facilitated release of glutamate and calcium, nAChR activation enhances NMDA activity (Fallon et al., 2006). Given the dynamic state of development during adolescence and nicotine's influence on that development, a chronic adolescent exposure to nicotine could modify the GABA and NMDA neurochemical pathways, thereby producing an altered adult environment with respect to

subsequent drug response in adulthood. That is to say that, the exposure to ethanol during adulthood may have interacted directly with nAChRs, and/or neurocircuitry they modulate, that had been permanently altered by the chronic nicotine exposure during adolescent development resulting in the exacerbation of ethanol withdrawal-related seizures. Whereas the same exposure to nicotine during adulthood did not result in this same alteration to the nAChRs, and/or the neurocircuitry they modulate, and consequently did not affect the severity of ethanol withdrawal seizures.

An additional and interesting observation of this research is that ethanol withdrawal seizure activity was not exacerbated when both ethanol and nicotine were administered during the adolescent developmental period. It has been previously shown that a chronic co-exposure of nicotine with ethanol reduces ethanol withdrawal-induced toxicity (Prendergast et al., 2002), and our results imply that the co-administration of these two drugs during adolescence can dampen subsequent ethanol withdrawal seizure activity in adulthood. Given the direct interaction of ethanol and nicotine at the receptor level, it is likely that the presence of ethanol during adolescent nicotine exposure interacted with nicotine's perturbation of neurocircuitry that modulate adult ethanol withdrawal-induced seizure activity. Another possible explanation for this interaction is that the clearance of one or both of these agents is influenced by the other. One study reported that ethanol may increase the metabolism of nicotine and that nicotine, in turn, may increase the elimination of ethanol (Schoedel and Tyndale, 2003). However, it is difficult to generalize this finding to the present result because the work was carried out on adult male rats. In addition, Collins et al. (1988) investigated cross-tolerance between ethanol and nicotine in female DBA mice and found that chronic exposure to either drug did not affect elimination of the other drug. Finally, our results showed an elevated BEC in adults exposed to only ethanol in adolescence, but even then only in comparison to controls. Clearly then, the influence of an adolescent nicotine and/or ethanol exposure on subsequent ethanol elimination in adulthood is an important consideration and warrants further study. However, given the design of the present experiment where nicotine is administered 6 weeks prior to ethanol withdrawal, it is unlikely that the nicotine dosing received during adolescence would influence the elimination of ethanol during withdrawal testing.

To our knowledge, this is the only murine investigation of the combined effect of nicotine and ethanol exposure during the adolescent developmental period on the severity of a subsequent withdrawal from ethanol. Recently, Abreu-Villaça and colleagues (Abreu-Villaça et al., 2007; Abreu-Villaça et al., 2008) reported that adolescent mice exposed to ethanol and/or nicotine demonstrate differential long-term effects on anxiety and learning. That is, ethanol and nicotine seemed to have both synergistic and less than additive long-term effects when administered during adolescence. Particularly relevant to the present study, nicotine and ethanol exposure during adolescence resulted in a subsequent anxiogenic state. This increase in anxiety has also been reported in rats after adolescent exposure to nicotine and/or alcohol (Slawecki et al., 2004). This possible anxiogenic state would be consistent with the increase in seizure severity found in the present study, leaving the adult in a more vulnerable state for relapse and abuse. Although Abreu-Villaça and colleagues (2007, 2008) did not replicate their study with the initial exposure to the drugs administered during adulthood, as in the present study, our findings suggest that the co-use of these drugs during the adolescent developmental period results in a unique interaction within the developing brain which results in long-term changes. Clearly, the interaction between ethanol and nicotine is interesting and warrants further study.

In summary, our findings suggest that the adolescent developmental period represents a unique period of vulnerability to nicotine which leads to an increased susceptibility to ethanol withdrawal in adulthood. In light of our findings and the evidence supporting a dynamic and sensitive adolescent brain, it is reasonable to surmise that the chronic exposure to nicotine

during this time of development resulted in a nicotine-induced change in brain development and resulted in an increased vulnerability to ethanol withdrawal in adulthood. It is important to note that the present results, although robust, are limited to one inbred strain of mouse and male mice at that. To further elucidate the apparent vulnerability during the adolescent developmental period to nicotine exposure, other murine lines (both inbred and selected lines) should be utilized. In addition, sex differences should also be explored. Nonetheless, these results have important implications with respect to the combined use and abuse of these substances during adolescence which can result in an increased vulnerability to the long-term effects of ethanol in adulthood.

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Figure 1.

Caption: This illustration shows the ethanol withdrawal pattern for PD28 subjects by treatment group over 24 hours. The asterisks denote hours where the NO group differed from other treatment groups. See results section for details.

Figure 2.

Caption: These illustrations show the Areas under the Curve (AUC) for 10- and 24-hours for PD28 and PD70 subjects (figures 2a and 2b, respectively) by treatment group. The asterisk denotes a statistical significant difference for only the PD28 NO treatment group ($p < 0.01$).

Table 1**Handling Induced Convulsion (HIC) Scoring Criteria**

Score	Criteria
0	No activity on tail lift or after a gentle 360° spin
1	No activity on tail lift, but facial grimace after gentle 360 ° spin
2	No activity on tail lift, but tonic convulsion after gentle 360 ° spin
3	No activity on tail lift, but tonic/clonic convulsion after gentle 360 ° spin
4	Facial grimace on tail lift
5	Tonic convulsion on tail lift
6	Tonic/clonic convulsion on tail lift, often delayed by 1-2 seconds
7	Severe tonic/clonic convulsion on tail lift, no delay
8	Severe tonic/clonic convulsion prior to tail lift

Table 2

Phase II BECs and Percent of Weight Change during Phase I and II by treatment group for PD28 and PD70 cohorts

	BEC (mg%) Level at end of Phase II	Phase I (% change in body weight from start to end of Phase I)	Phase II (% change in body weight from start to end of Phase II)
PD28			
CO	153 ± 6	-1.55 ± 0.89	-4.51 ± 0.81
EN	165 ± 6	-7.81 ± 0.83	-5.59 ± 0.52
EO	181 ± 8	-6.99 ± 0.97	-4.09 ± 0.61
NO	171 ± 6	-5.73 ± 1.02	-5.94 ± 0.64
PD70			
CO	149 ± 2	-1.14 ± 0.54	-1.35 ± 1.27
EN	154 ± 4	-4.04 ± 0.79	-2.41 ± 0.68
EO	152 ± 4	-5.36 ± 0.62	-1.89 ± 1.02
NO	155 ± 3	-2.04 ± 0.57	-1.73 ± 0.92