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Different chronic ethanol exposure regimens in adolescent and adult male rats: Effects on tolerance to ethanol-induced motor impairment

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Abstract

Findings are mixed regarding the expression of tolerance after repeated ethanol exposure, perhaps in part due to dose/frequency variations in exposure regimens. The present study compared age-related differences in tolerance development following 10 days of 1 g/kg twice daily, 2 g/kg once daily, or intermittent 4 g/kg ethanol exposure regimens. To measure expression of chronic tolerance and acute tolerance, ethanol-induced motor impairment was assessed on day 12, with functionally equivalent ethanol doses administered across age (2 g/kg--adolescents; 1.5 g/kg--adults). Subsequent challenge doses resulted in lower brain ethanol concentrations in both age groups as a function of the chronic ethanol regimens. Expected age-related differences emerged in acute tolerance expression in non-manipulated animals, with adolescents, but not adults showing acute tolerance. Regimens sufficient to induce alterations in ethanol metabolism did not result in chronic functional tolerance at either age, although chronic injections were sufficient to induce acute tolerance in adults.

Keywords

Chronic Ethanol; Acute Tolerance; Chronic Tolerance; Adolescence; Motor Impairment; Sprague-Dawley Rats; Males

Adolescence is a period of development in which not only initiation of alcohol use, but binge-level consumption is commonly reported, with 8.1% of 8th graders, 16% of 10th graders and 24.6% of 12th graders reported to have had 5 or more drinks in a row within the past two weeks according to a 2008 Monitoring the Future Survey [1]. Given the prevalence of alcohol use, research is critical to determine contributors to and potential adaptations of pervasive alcohol use during adolescence. One factor that may contribute to adolescents' propensity to consume binge amounts of ethanol is their relative insensitivity to many acute ethanol effects compared to adults, such as ethanol-induced sedation [2 & 3], motor impairment [4], and social impairment [5], all of which may serve as cues to terminate further consumption of ethanol. This decreased ethanol sensitivity typically observed in adolescent animals could be due at least in part to their greater ability to adapt to and counter

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the effects of ethanol within a single session of ethanol exposure -- a form of ethanol adaptation known as acute tolerance (AT). Expression of AT is characterized by within session recovery from ethanol impairment that occurs more rapidly than the decline in blood or brain ethanol concentration (BEC & BrEC, respectively) [see 6 &7 for review]. Indeed, a number of previous studies have reported that adolescent rats exhibit more AT than adults to sedative [2, 8 & 9] and social impairing [10] effects of ethanol.

Chronic tolerance (CT) [see 6 for review], characterized by a diminished response to a given dose of ethanol after repeated ethanol administrations, may be another factor that could contribute to elevated ethanol use during adolescence, possibly contributing to continued use and increasing the risk for development of future alcohol use disorders [11 & 12]. Unlike AT data, findings are more mixed regarding the acquisition of chronic tolerance (CT) in adolescent rodents, with some [13 & 14], but not all [15 & 16] studies indicating greater or equivalent CT acquisition in adolescents relative to adults. The mixed findings may be attributed to differences in experimental parameters across studies, such as length, dose and frequency of chronic exposure, species and genotype differences, as well as the task used to measure tolerance.

Although adolescents are predisposed to displaying greater AT to many ethanol effects upon initial exposure than adults, it remains to be determined whether similar age differences in AT are apparent following repeated exposures to ethanol, and whether age differences in these adaptations are influenced by the emergence of CT. Given that frequency/dose of ethanol exposure may influence tolerance expression, the current study examined AT and CT to ethanol-induced motor impairment in adolescent and adult rats after repeated exposure to one of three different ethanol regimens: 1 g/kg twice daily, 2 g/kg once daily or 4 g/kg every other day for 10 days.

A total of 200 juvenile/adolescent and adult male Sprague-Dawley rats bred and reared in our colony at Binghamton University were used in this experiment. On the day after birth, postnatal day (P) 1, litters were culled to 8–10 pups, with a sex ratio of 6 males and 4 females retained whenever possible. Pups were housed with their mother in a standard clear plastic tub with pine shavings until the time of weaning. Offspring were weaned at P21 and housed in same-sex littermate pairs; female offspring were used in other projects. All animals were maintained in a temperature-controlled vivarium on a 12:12-h light: dark cycle (lights on 0700) with ad libitum access to food (Purina Rat Chow, Lowell, MA) and water. Animals used in this experiment were maintained and treated in accordance with guidelines for animal care established by the National Institutes of Health (1986), using protocols approved by the Binghamton University Institutional Animal Care and Use Committee.

Eight - 11 rats were assigned to each group defined by the 2 (age) × 5 (exposure condition: non-manipulated [NM], saline [SAL], 1 g/kg ethanol [E1], 2 g/kg ethanol [E2], or 4 g/kg intermittent ethanol [E4]) × 2 (test day injection-test interval: 10 vs. 60 min) factorial design of the study, with no more than one animal from a given litter placed into any one condition [17 & 18]. The E1 group was administered a 1 g/kg dose of ethanol twice daily (8–9 AM and 3–4 PM), whereas animals in the E2 group received 2 g/kg ethanol every 24 hours. A saline injection equivalent to a 4 g/kg ethanol injection volume was given to animals in the E4 group on Day 1 and continuing every other day (i.e., the odd-numbered days) for the 10 day exposure period. Starting on Day 2, E4 animals received a 4 g/kg dose of ethanol every 48 hours (i.e., on the even-numbered days during the exposure period). Thus, the overall amount of ethanol administered to each animal was equated across groups while varying the dose of ethanol and schedule of administration. Chronic SAL animals were administered saline at an equivalent volume to a 4 g/kg ethanol dose every 24 hours for 10 days. Saline (0.9% w/v) and ethanol (18.9% v/v in saline) were administered intraperitoneally (i.p.) at

room temperature between 11:00 AM and 12:00 PM unless otherwise specified (E1 group). Dose of ethanol was adjusted by volume rather than concentration to avoid concentration induced alterations in ethanol absorption [see 19]. NM animals were not handled during the 10 day exposure period.

The motor impairment test used was a slightly modified version of the procedure originally described by Ramirez and Spear [20], with three massed baseline tests given to each animal instead of one, and the best score (shortest latency) used as the baseline measure. Animals were tested for the negative geotaxis reflex (latency to rotate 180°) on a stationary inclined plane 48 hours after the final exposure day. Prior to testing, each animal was weighed and placed individually into a clean holding cage until baseline trials commenced. Immediately following the third baseline trial, animals were injected i.p. with ethanol and then returned to their holding cage for the duration of the pre-assigned injection-test interval (10 or 60 min). Given that ontogenetic differences in initial sensitivity could affect expression of tolerance, impairment level was equated across age by using different ethanol challenge doses for adolescents (2 g/kg) and adults (1.5 g/kg) [based on preliminary data, in progress]. Animals were then given a test trial either 10 or 60 minutes following ethanol injection and sacrificed immediately thereafter via decapitation. Animals unable to complete the task post ethanol, including animals that fell off the apparatus, were assigned a latency score of 30 seconds given that animals have been previously found to be able to complete this task within that time frame [20], and thereby allowing data from all animals to be included in the regression analyses across the 10 and 60 minute post-injection-test intervals for assessment of AT. Trunk blood and brains were collected, rapidly frozen and maintained at a temperature of −80 °C until analysis of blood and brain ethanol concentrations (BEC and BrEC, respectively) via gas chromatography [see 15 for details]. BECs and BrECs were highly correlated at both ages (adolescent: $r = .84$, $p<.001$; adult: $r=.84$, $p<.001$) and at each injection-test interval (10 min: $r = .77$, $p<.001$; 60 min: $r = .79$, $p<.001$). Data analyses were focused on BrEC data given the behavioral task emphasis of this study.

AT was indexed as a greater within session decline in motor impairment relative to brain ethanol concentration (BrEC) across time in each condition. For these determinations, impairment scores (each animal's own post ethanol latency – baseline latency) were divided by each animal's BrEC and then subjected to linear regression at each age and pre-exposure condition. Regressions yielding negative slopes significantly different from zero indexed AT [21]. In these analyses, significant negative slopes emerged in the regression of impairment ratios across the 10 and 60 minute injection-test intervals for all age × condition groups except for NM adults (Table 1, left panel). Thus, evidence for AT emerged regardless of exposure condition in adolescents, whereas only adults that were exposed to some sort of chronic perturbation (i.e., chronic injections) exhibited AT.

Expression of AT was also confirmed using Radlow's method [see 7, 10 & 22 for details]. Using this method, BrEC and impairment scores were first transformed into percent maximum values for each age and condition. BrEC (% max) – impairment (% max) difference scores were then calculated for each animal and subjected to linear regression at each age and pre-exposure condition, with positive slopes significantly different from zero indexing AT. These regression analyses of Radlow's AT difference scores yielded identical findings, although in this case, significant positive, rather than negative slopes indexed AT (see Table 1, right panel).

A 2 (age) × 2 (time point: 10 and 60) × 5 (condition: NM, SAL, E1, E2, and E4) factorial ANOVA of baseline turn latencies revealed only a significant main effect of age [$F(1,175)= 15.21$, $p<.001$], with adolescents overall displaying shorter turn latencies (3.84 ± .16) than adults (4.98 ± .24). Thus, the chronic exposure regimens did not influence baseline motor

performance in this task at either age. The chronic exposure regimens were effective, however, in influencing body weight gain. A 2 (age) × 5 (condition: E1, E2, E4, SAL and NM) factorial ANOVA of body weight on test day, Day 12, revealed significant main effects of age [$F(1,282)= 5563.32$; $p<.001$] and condition [$F(4,282)= 24.97$; $p<.001$]. At test, adolescents of course weighed significantly less than adults. Regardless of age, animals in the E4 condition weighed significantly less than animals in all other exposure conditions and animals in the E2 condition weighed significantly less than their NM counterparts (Table 2).

Given the different ethanol challenge dose used at each age, BrEC data were analyzed separately at each age. The 2 (time point: 10 and 60 min) × 5 (condition: NM, SAL, E1, E2, and E4) factorial ANOVA of the adolescent BrEC data revealed a significant main effect of time point [$F(1,89)= 490.34$; $p<.001$] and a significant condition × time point interaction [$F(4,89)= 2.5$; $p<.05$]. As expected, BrECs decreased significantly from 10 to 60 minutes. At the 60 minute time point, adolescents in the E2 and E4 conditions had significantly lower BrECs compared to their SAL and NM age-mates, with similar trends for comparisons with E1 animals ($p=.08$, $p=.06$, respectively) (see Table 3, left panel). In the analysis of adult BrECs, significant main effects of condition [$F(4,86)= 8.63$; $p<.001$] and time point [$F(1,86)= 490.06$; $p<.001$], as well as their interaction [$F(4,86)= 6.38$; $p<.001$] emerged. Again, BrECs were significantly lower at 60 than at 10 minutes. At the 10 minute time point, SAL, E1 and E2 adults had lower BrECs than NM adults, whereas adults in the E4 condition had significantly higher BrECs than SAL-exposed adults. By 60 minutes, all chronic exposure conditions exhibited significantly lower BrECs than NM animals, with animals in the E4 condition also having significantly lower BrECs than all of the other exposure conditions, and adults in the E1 group having significantly lower BrECs than SAL-exposed adults (see Table 3, right panel).

A 2 (age) × 2 (time point: 10 and 60) × 5 (condition: NM, SAL, E1, E2, and E4) factorial analysis of impairment scores (post ethanol latency - baseline latency) revealed only a significant main effect of time [$F(1,175)= 252.79$, $p<.001$], with lower impairment scores at 60 minutes compared to 10 minutes (see Table 3, left panel). The lack of a main effect of age in the ANOVA confirmed that the ethanol dose chosen for use at each age was effective in equating impairment between adolescents and adults (impairment latencies collapsed across time point and exposure condition of 16.8 sec ± 1.1 and 16.4 sec ± 1.1, respectively). The lack of effect of chronic exposure condition, suggested that ethanol-induced motor impairment as reflected by impairment scores in this ANOVA did not differ as a function of repeated ethanol exposure.

Chronic ethanol effects on impairment ratios (impairment score/BrEC) were analyzed using the same 2×2×5 factorial ANOVA design. This analysis revealed significant main effects of time [$F(1,175)= 99.55$, $p<.01$ and age [$F(1,175)= 6.4$, $p<.05$], with lower impairment ratios seen at 60 (0.25 ± 0.03) than 10 minutes (0.54 ± 0.01) post-injection, and adolescents (0.34 ± 0.02) showing lower ratios than adults (0.44 ± 0.03). This analysis again revealed no effect of chronic exposure condition, suggesting chronic functional tolerance did not emerge.

This study was uniquely designed to assess the effects of chronic exposure to different dose/frequency of ethanol on tolerance development in adolescents and adults. It is important to note that, unlike the acute challenge dose, the perturbations associated with these chronic exposure regimens may not be equivalent across age, given reports of increased ethanol metabolism in adolescents relative to adults [23 & 24]. Even if doses were chosen to produce equivalent BECs at the start of the exposure period, it would be difficult to ensure equivalency throughout the exposure period given possible age differences in the emergence of pharmacokinetic alterations over time. Thus, the strategy used here was to hold the dosing regimens constant across age while exploring the question of whether adolescents require a

higher ethanol dosing regimen to demonstrate tolerance to a moderate ethanol challenge dose.

Taken together, these data correspond to previous reports of age-related differences in acute tolerance expression [3, 8–10 & 25], with non-manipulated adolescents exhibiting acute tolerance to ethanol-induced motor impairment whereas acute tolerance was not evident in their non-manipulated adult counterparts. Following repeated perturbations, however, acute tolerance was robustly expressed in all chronic exposure groups regardless of age, suggesting that within-session adaptations emerge in adults in response not only to repeated ethanol exposure but also to the mild stress of the chronic injection procedure per se. These data are reminiscent of prior work suggesting that a single i.p. injection of saline given the day before testing was sufficient to allow expression of acute tolerance to ethanol's sedative effects in adults [26]. Collectively, these data support the suggestion that age-related differences in acute tolerance expression may be ameliorated by prior procedural manipulations rather than prior ethanol exposure per se.

The chronic exposure regimen also induced possible pharmacokinetic adaptations, as well as changes in weight gain; however, these alterations were evident at both ages, and were regimen-dependent. Adults from all exposure conditions (including chronic saline) had significantly lower BrECs relative to NM adults at 60 minutes post-challenge, with BrECs of E4 adults also lower than adults from all other conditions. Significantly lower BrECs were also seen at 60 minutes following ethanol challenge on test day among adolescent animals chronically exposed to the higher doses of ethanol (E2 and E4). Although rates of ethanol metabolism were not directly measured, the attenuated BrECs seen in these exposure groups at 60 minutes post-injection likely reflect increases in ethanol metabolism as a result of ethanol pre-exposure at both ages, as well as following saline pre-exposure in adults. Evidence for enhanced ethanol metabolism after chronic ethanol exposure has been reported in some [e.g., 14, 15, 27 & 28], but not all studies [e.g., 21, 29 & 30]. While evidence in the literature regarding increased ethanol metabolism after chronic saline injections is scarce due to limited inclusion of NM control groups which are necessary to determine potential effects of the chronic injection procedure per se, evidence for increased ethanol metabolism after repeated saline exposure in adults has been previously observed in our laboratory [15]. Importantly, this effect was not seen in adolescents, suggesting that adolescents may not be as sensitive as adults to chronic stress of the injection/handling procedure. These results are reminiscent of a study conducted by Ristuccia and Spear [31] that found an attenuation of ethanol hypothermia after chronic injection/handing in adults, whereas adolescents' hypothermic response to ethanol remained unchanged. In contrast, Varlinskaya & Spear [32] reported an enhanced sensitivity of adolescent relative to adult animals to a mild stress associated with repeated saline injections. In that study, adolescents but not adults demonstrated social anxiety and significant reductions of body weight following repeated saline injections when compared with non-manipulated age-matched controls. Further studies assessing potential corollary alterations in hypothalamic-pituitary-adrenal (HPA) axis functioning in adolescents and adults after chronic saline injections would be valuable in interpreting these results.

Similar to evidence for pharmacokinetic alterations, body weight gain likewise differed as a function of exposure regimen at both ages, with all regimens suppressing weight gain of adults, the two higher doses (E2 and E4) producing weight deficits in adolescent animals, and E4 producing the greatest weight deficit at both ages. Given that total amount of ethanol administered was equated across groups in our study, these results are reminiscent of previous neonatal studies suggesting that peak BECs rather than total daily amount of ethanol administered is a better predictor of adverse ethanol effects [33 & 34], although caution should be exerted when comparing across these developmental stages.

Even at ethanol doses sufficient to disrupt body weight gain and alter ethanol metabolism, chronic functional tolerance was not observed at either age. Several studies have suggested that tolerance development is measure dependent [21, 30 & 35]. For instance, after repeated daily exposures to 8–11 g/kg ethanol intragastrically, animals expressed chronic tolerance to ethanol-induced hypothermia sooner than to ethanol-induced motor impairment, (at 9 vs 17 days, respectively), with no evidence of tolerance to ethanol-induced suppression of startle responses emerging within the 17-day ethanol exposure period of the study [21]. Given the time course of chronic tolerance to motor impairment reported by Pohorecky et al. [21], perhaps the 10 day exposure period used in the current study was insufficient to produce chronic functional tolerance. Alternatively, this lack of chronic tolerance expression could be attributable, at least in part, to the test parameters used. Many animals displayed recovery of motor impairment by 60 minutes, thus tolerance assessment at a time earlier than the 60-minute injection-test interval may have provided a more sensitive measure of possible group differences in recovery. Studies using longer exposure regimens, and/or different testing procedures (e.g., multiple injection-test intervals or ethanol challenge doses) could prove valuable in exploring the relationship between within-session and long-term adaptations to the motor impairing effects of repeated ethanol exposure during ontogeny.

Results from the present study provide further evidence that acute and chronic tolerance are acquired through separate mechanisms, given that acute but not chronic functional tolerance was robustly expressed by all chronic exposure groups at both ages. These results correspond to several other studies that suggest a divergence between these two forms of tolerance with expression of one, but not both forms of tolerance after repeated ethanol exposures [36–40]. Whereas some studies have suggested acute tolerance expression correlates with the ability to develop chronic tolerance [30 & 41], the present ontogenetic findings, however, do not support this notion. Whether the robust within-session emergence of adaptations to ethanol seen in this task obviates the need for longer term adaptations under these test circumstances is unclear [see 6 for discussion], but remains an intriguing possibility for future study.

Research Highlights
< Examined age-related differences in tolerance to ethanol-induced motor impairment < As expected, non-manipulated adolescents, but not adults showed acute tolerance <Chronic functional tolerance did not emerge at either age < Although chronic injections were sufficient to induce acute tolerance in adults

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Table 1

Acute Tolerance assessment via slopes of regression

	Impairment Ratio				Radlow's AT method			
	Adolescent		Adult		Adolescent		Adult	
	slope	p value	slope	p value	slope	p value	slope	p value
NM	−.42	.04	−.28	.19	1.35	.01	−0.30	.48
SALINE	−.46	.01	−.82	<.01	1.43	<.01	1.87	.01
E1	−.66	<.01	−.58	.02	1.94	<.01	1.48	.01
E2	−.57	<.01	−.72	<.01	1.67	<.01	1.71	<.01
E4	−.74	<.01	−.77	<.01	2.1	<.01	1.85	<.01

Table 2

Body Weight

	Body Weight (g) at Test	
	Adolescent	**Adult**
NM	167.5 ± 1.6	395.5 ± 3.9
SALINE	162.7 ± 2.1	389.4 ± 7.0
E1	161.8 ± 3.4	378.6 ± 7.0
E2	151.1 ± 2.7[a]	359.3 ± 5.0[a]
E4	138.2 ± 3.4[b]	353.0 ± 4.9[b]

denotes significance ($p < .05$) between groups of the same age:

[a] relative to NM;

[b] relative to all other exposure groups

Table 3

Motor Impairment and BrEC

	Impairment Score (sec)				BrEC (mg/dl)			
	Adolescent		Adult		Adolescent		Adult	
	10	60	10	60	10	60	10	60
NM	26.0 ± .33	11.5 ± 4.0	24.6 ± 0.9	14.8 ± 3.4	201.6 ± 4.0	154.6 ± 3.8	170.3 ± 2.2	134.0 ± 3.3
SALINE	25.0 ± 1.7	10.1 ± 3.2	24.6 ± 0.9	7.8 ± 4.3	195.6 ± 6.4	154.2 ± 2.6	158.7 ± 6.1[a]	123.8 ± 2.8[a]
E1	26.0 ± .75	6.0 ± 2.1	21.8 ± 2.8	7.6 ± 2.5	201.2 ± 3.3	144.9 ± 1.8	159.5 ± 2.3[a]	114.1 ± 3.1[a,b]
E2	26.6 ± .37	7.8 ± 3.3	25.9 ± 0.3	8.0 ± 2.3	202.8 ± 2.3	142.1 ± 4.1[a,b]	161.1 ± 3.7[a]	117.4 ± 2.7[a]
E4	25.3 ± .65	4.7 ± 2.4	24.6 ± 0.9	5.6 ± 2.7	200.8 ± 3.8	141.0 ± 3.7[a,b]	168.2 ± 2.1[b]	103.9 ± 1.9[a,b,c]

Superscripts denote significance ($p < .05$) within the same age and time point:

[a] relative to NM

[b] relative to SAL

[c] relative to E1 and E2