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Chronic intermittent toluene inhalation initiated during adolescence in rats does not alter voluntary consumption of ethanol in adulthood

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Abstract

Voluntary inhalation of organic solvents, such as toluene, is particularly prevalent in adolescent populations and is considered to be a contributing factor to substance use and dependence later in life (Lubman, Yücel, & Lawrence, 2008; White, 2001). While inhalants are often the initial “drug” experienced during this period, alcohol is another substance readily abused by adolescent populations. Although both substances are thought to have similar actions within the brain, our understanding of the implications of adolescent inhalant abuse upon subsequent exposure to alcohol remains to be investigated. Thus, this study aimed to assess locomotor responses to acute ethanol and voluntary ethanol consumption following a period of toluene inhalation throughout adolescence/early adulthood. Adolescent male Wistar rats (postnatal day [PN] 27) inhaled air or toluene (3,000 ppm) for 1 h/day, 3 days/week for 4 (PN 27–52) or 8 weeks (PN 27–80) to mimic the patterns observed in human inhalant abusers. Following the exposure period, cross-sensitization to acute ethanol challenge (0.5 g/kg, intra-peritoneally [i.p.]), and voluntary consumption of 20% ethanol in a chronic intermittent 2-bottle choice paradigm, were assessed. Hepatic ethanol and acetaldehyde metabolism and liver histopathology were also investigated. Chronic intermittent toluene (CIT) exposure throughout adolescence for up to 8 weeks did not alter the behavioral response to acute ethanol or voluntary consumption of ethanol in adulthood, although an age-dependent effect on ethanol consumption was observed ($p < 0.05$). Both liver function and pathology did not differ between treatment groups. Thus, in the paradigm employed, CIT exposure throughout adolescence and early adulthood did not predispose rats to subsequent locomotor sensitivity or voluntary consumption of ethanol in adulthood.

Keywords: inhalant abuse, 2-bottle choice, alcohol, liver

Introduction

Inhalation of volatile organic solvents is particularly prevalent among adolescent populations, with inhalants commonly being the initial “drug” experienced (Lubman, Hides, & Yücel, 2006). Accordingly, the incidence of inhalant abuse is highest in adolescents aged between 12–18 years of age, with at least 26% of Australian secondary school students having experimented with inhalants (White, 2001), and over 77% continuing to abuse inhalants for periods greater than 1 year (Neumark, Delva, & Anthony, 1998). Furthermore, in the 2010 National Drug Strategy Household Survey conducted by the Australian Government, a 23% increase in inhalant abuse was reported between 2007–2010, while both alcohol and tobacco use decreased during this period (AIHW, 2011). Thus, within Australian communities, as in many other countries such as the United States, Mexico, Brazil, and Japan, adolescent inhalant misuse is a significant growing public health concern (for review see Dell, Gust, & MacLean, 2011).

The prevalence of inhalant misuse in adolescence is thought to be due to the fact that they are cheap, legal, readily accessible, and provide a rapid intoxicating effect (Lubman et al., 2008). Preferentially abused products such as paints, glues, and aerosols contain a variety of volatile compounds, yet it is the volatile organic solvent toluene that is considered to have the highest abuse potential. Indeed, toluene elicits a conditioned place preference in rodents (Funada, Sato, Makino, & Wada, 2002) and is self-administered in mice (Blokina, Dravolina, Beshpalov, Balster, & Zvartau, 2004) and non-human primates (Weiss, Wood, & Macys, 1979). Furthermore, like other drugs of abuse, toluene’s ability to regulate signaling within the mesocorticolimbic dopaminergic (DA) system is thought to underlie its acute hedonic and reinforcing properties (Beckley, Evins, Fedarovich, Gilstrap, & Woodward, 2013; Riegel, Zapata, Shippenberg, & French, 2007).

Chronic abuse of products containing toluene is associated with both cognitive and neurobiological abnormalities (Dick, Axelsson, Lawrence, & Duncan, 2013; Yücel, Takagi,

Walterfang, & Lubman, 2008). Inhalation of such products during adolescence is of particular concern as it has a significant co-morbidity with neuropsychiatric disorders and is a predictor of substance abuse later in life (Wu, Pilowsky, & Schlenger, 2004). The vulnerability of the adolescent brain to substances of abuse is believed to be driven, in part, by the fact that adolescence encompasses a significant maturational period in which dynamic reorganization occurs within the brain (for review see Lubman, Yücel, & Hall, 2007; Spear, 2000). As such, the long-term adverse consequences of inhalant abuse are associated with the age of abuse onset (Wu et al., 2004), as well as the duration of abuse history in humans (Yücel et al., 2008) and rodents (Duncan et al., 2012).

Similarly, alcohol abuse is prevalent in adolescent populations and is a predictor of alcohol use and dependence later in life (Chassin, Pitts, & Prost, 2002). These observations have been supported by rodent models showing adolescent ethanol exposure elicits increased ethanol consumption and/or preference in adulthood (Criado & Ehlers, 2013; Maldonado, Finkbeiner, Alipour, & Kirstein, 2008; Pascual, Boix, Felipo, & Guerri, 2009). Interestingly, like ethanol and other classic central nervous system depressants, toluene exerts biphasic locomotor effects inducing motor excitation at low concentrations (< 4,000 ppm) and motor impairment, sedation, and anesthesia at high concentrations (> 6,000 ppm) (Bowen & Balster, 1998). This is thought to be due in part to several neuropharmacological similarities between toluene and ethanol (Cruz, Mirshahi, Thomas, Balster, & Woodward, 1998; Lovinger, White, & Weight, 1990). Toluene inhalation also acts as an ethanol-like discriminative stimulus in rodents, suggestive of a degree of similarity in the hedonic reinforcing properties of these substances (Bowen, 2009; Rees, Knisely, Breen, & Balster, 1987). Thus, due to the similar actions mentioned above, adolescent toluene exposure may also alter subsequent consumption of ethanol or administration of other drugs of abuse in adulthood, although this remains to be investigated.

Consequently, the present study aimed to investigate the effect of CIT exposure at a positively reinforcing concentration (3,000 ppm) (Funada et al., 2002) during adolescence and early adulthood upon subsequent ethanol-induced locomotor activity and voluntary ethanol consumption in adulthood. As it has been previously demonstrated that the duration of CIT exposure results in differential neuropathological and behavioral effects in this model (Duncan et al., 2012), this study investigated the effects of ethanol following exposure confined primarily to adolescence (PN 27–52) or both adolescence and early adulthood (PN 27–80). As age-dependent effects on voluntary ethanol consumption have been observed in rats (Schramm-Sapota et al., 2013), this paradigm also enabled the investigation of the effects of age upon voluntary ethanol consumption. Due to the potential hepatotoxic nature of adolescent CIT exposure, endogenous liver enzyme activity and liver pathology were also assessed. It was hypothesized that CIT exposure for up to 8 weeks would alter both the locomotor response to ethanol and voluntary consumption of ethanol in adulthood, and that the effect of toluene would be related to duration of exposure.

Materials and Methods

Animals

Adolescent male Wistar rats (PN 24) were obtained from the Australian Resources Centre (Perth, Australia). In rats, adolescence ranges from weaning at PN 21 to adulthood at PN 60 (for review see Spear, 2000). Rats were pair-housed, maintained on a 12-h light/dark cycle (lights on at 7:00 AM) and given access to food and water *ad libitum*, with 2 water bottles available to reduce novelty in the 2-bottle choice paradigm. Rats were acclimatized for 3 days prior to any experimental manipulation. All experiments were approved in advance by the Florey animal ethics committee and were performed in accordance with the Prevention of Cruelty to Animals Act, 1986 under the guidelines of the National Health and Medical

Research Council Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

Toluene inhalation exposure

Exposure to toluene inhalation was conducted as previously described (Duncan et al., 2012). Briefly, rats were acclimatized to the laboratory at least 1 h prior to exposure to toluene or air during which time their body weights were recorded. Exposure to vaporized toluene was conducted in specialized chambers (4.76 L; $17.6 \times 16.5 \times 16.4$ cm) connected to a toluene vapor system whereby air was pumped through liquid toluene (1.08389, purity 99.8%, Merck, Vic, Australia) in a gas wash-bottle to produce toluene vapor. Flow meters allowed the regulation of the desired concentration of toluene vapor, which was verified using a previously calibrated inline gas chromatography system (Shimadzu Corporation, Kyoto, Japan). A minimum of 3 readings were taken per session with deviations greater than 100 ppm of the desired toluene concentration being corrected. Chambers of similar design but exposed to room air only were utilized for control animals (0 ppm exposure).

Rats were randomly assigned to inhale either air ($n = 24$) or toluene (3,000 ppm, $n = 24$) for 1 h per day, 3 days per week (Monday, Wednesday, Friday), for 4 weeks (air $n = 12$; CIT $n = 12$) or 8 weeks (air $n = 12$; CIT $n = 12$). Thus, the exposure period took animals from early adolescence (PN 27) to late adolescence (PN 52, 4 weeks exposure) or early adulthood (PN 80, 8 weeks exposure). The chronic intermittent paradigm employed was designed to mirror the human pattern of toluene abuse (Lubman et al., 2008). After 1 h of exposure, rats were placed back into their home cages and isolated from other rats for 1 h to avoid the possible confounds of olfactory stimulation by toluene scent on the fur. All chambers were briefly cleaned with 70% ethanol between sessions. Exposures were conducted at room temperature (~ 21 °C) under normal lighting and each rat was exposed at approximately the same time each day ($\sim 9:00$ – $11:00$ AM or 2–4 h into the light cycle).

Ethanol cross-sensitization

To test for locomotor cross-sensitization to ethanol following adolescent CIT exposure, air- and CIT-exposed rats were subjected to an acute ethanol challenge. Commencing 3 days following the final air/CIT exposure, rats were habituated to locomotor cells (TruScan™ Photobeam; Coulbourn Instruments, Allentown, PA, USA) over 3 daily habituation sessions, where locomotor activity was monitored for 15 min followed by administration of saline (10 mL/kg, i.p.) and monitoring of locomotor activity for a further 30 min. Following the habituation period, rats were administered ethanol (0.5 g/kg, i.p.) under the same conditions as the saline exposure paradigm (i.e., 15-min habituation, injection and locomotor activity monitored for a further 30 min; PN 61, 4-week cohort; PN 86, 8-week cohort). Horizontal (HP) and vertical plane (VP) activity was recorded throughout all sessions.

2-bottle choice paradigm

Following ethanol cross-sensitization, rats were single housed, and the effect of adolescent CIT exposure upon voluntary ethanol consumption was assessed in a chronic intermittent 2-bottle choice paradigm as previously described (Simms et al., 2008). Commencing ~10 days following the exposure period (PN 63, 4-week cohort; PN 89, 8-week cohort) rats were given home cage access to 1 bottle of ethanol (20% v/v in H₂O) and 1 bottle of water for 24 h, 3 days per week for up to 10 weeks and consumption recorded.

Histopathology

Upon cessation of experimentation, rats were euthanized with an overdose of Lethobarb (1 mL/kg, i.p.) and the liver was removed and weighed, and the right lateral lobe was rapidly frozen over liquid nitrogen. In all cases ($n = 12$ per group per cohort), the upper portion of the right lateral lobe of the liver was serially sectioned at 10 μ m (2 sections per slide separated by 100 μ m) and stained with hematoxylin and eosin. A minimum of 4

sections per case were scored (0–4) for the presence of overt tissue pathology including: 1) steatosis: 0 = no lipid droplets per cell, 1 = 1 lipid droplet per cell in localized regions, 2 = 1 lipid droplet per cell throughout tissue, 3 = ≥ 2 lipid droplets per cell in localized regions, 4 = ≥ 2 lipid droplets per cell throughout tissue; 2) evidence of tissue infarct/necrosis (degenerating cells and/or fibrous scar tissue): 0 = no overt changes; 1 = minor localized overt changes; 2 = minor overt changes located throughout the tissue; 3 = moderate overt changes; 4 = extensive overt changes.

Liver assays

Tissue collection and preparation

Processing of liver samples to obtain cytosolic and mitochondrial fractions was performed as previously described (Lodge & Lawrence, 2003). Briefly, the lower segment of the right lateral lobe was divided into 4 pieces and incubated for 1 h at 37 °C in carbogenated physiological saline solution (composition in mM: NaCl, 118.0; KCl, 4.7; NaH₂PO₄, 1.0; MgCl₂, 1.2; CaCl₂, 1.3; NaHCO₃, 25.0; and ethylenediaminetetraacetic acid [EDTA], 0.04), and subsequently manually homogenized in 12 mL ice-cold sucrose buffer (0.25 M sucrose, 5 mM TRIS, 0.5 mM EDTA, and 0.5 mM dithiothreitol; pH 7.5). The homogenates were centrifuged (4 °C) at 700 × g for 10 min and the resultant supernatant centrifuged (4 °C) at 10,000 × g for 30 min. The pellet was resuspended in 10 mL of pyrophosphate buffer (50 mM sodium pyrophosphate; pH 8.8), this being the mitochondrial fraction. The supernatant was further centrifuged (4 °C) at 48,000 × g for 1 h with the resultant supernatant being the cytosolic fraction. Protein concentration within each fraction was then determined in duplicate with a DCTM Protein assay kit (Bio-Rad Laboratories, CA, USA) as per manufacturer's instructions. The cytosolic fraction was used to assess alcohol dehydrogenase (ADH) activity, and the mitochondrial fraction was used to assess aldehyde dehydrogenase (ALDH) activity. Liver fractions were stored at –80 °C until required.

Alcohol dehydrogenase assay

The endogenous activity of hepatic ADH was determined for each animal by the nicotinamide adenine dinucleotide-induced increase in absorbance at 340 nm on a microplate spectrophotometer (Bio-Rad Benchmark Plus; Bio-Rad Laboratories, CA, USA) as previously described (Bird, Kirchhoff, Djouma, & Lawrence, 2008; Lodge & Lawrence, 2003). In brief, the assay was performed in duplicate at 37 °C in a final volume of 1.25 mL glycine buffer (0.1 M glycine; pH 10) containing 2.4 mM of β -NAD⁺ and a volume of cytosolic fraction equivalent to 1 mg protein. After 5 min of equilibration (37 °C), the reaction was initiated by the addition of ethanol (100 μ M–100 mM) and incubated for 5 min (37 °C) before the increase in absorbance was measured relative to a blank reaction (no ethanol added). After construction of a concentration–response curve, the effect of the ADH inhibitor pyrazole (10 mM) was examined on the ethanol concentration that yielded maximal ADH activity (10 mM ethanol).

Aldehyde dehydrogenase assay

The endogenous activity of hepatic ALDH was determined for each animal by the nicotinamide adenine dinucleotide-induced increase in absorbance at 340 nm on a microplate spectrophotometer (Bio-Rad Benchmark Plus; Bio-Rad Laboratories, CA, USA) (Bird et al., 2008; Lodge & Lawrence, 2003). The assay was performed in duplicate at room temperature in a final volume of 1.25 mL pyrophosphate buffer (50 mM sodium pyrophosphate; pH 8.8; containing 1.5 mM β -NAD⁺; pyrazole [0.1 mM]; rotenone [2 μ M in dimethylsulfoxide: 0.2% of final volume]; sodium deoxycholate [0.01% w/v]), and a volume of mitochondrial fraction corresponding to 1 mg of protein. After 5 min equilibration, the reaction was initiated by the addition of acetaldehyde (1 mM–1 M) and incubated in a sealed 48-well plate (to minimize acetaldehyde evaporation) for 15 min before the increase in absorbance was measured relative to a blank reaction (no acetaldehyde added). After the construction of a

concentration-response curve, the effect of disulfiram (0.1 mM in ethanol: 0.2% of final volume) was examined on the concentration of acetaldehyde that yielded maximal ALDH activity (100 mM acetaldehyde).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 20 (IBM, USA) and SigmaStat 3.5 (Jandel, San Jose, CA, USA), and all graphs were produced with GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Body weights throughout the exposure periods were analyzed utilizing a 2-way repeated-measures analysis of variance (RM ANOVA), with treatment and day as factors. Body and liver weights (corrected for body weight) at *post mortem* were analyzed using unpaired *t* tests. Cross-sensitization and 2-bottle choice data (ethanol and total fluid consumption in acquisition phase; ethanol exposures 1–15) were analyzed utilizing 2-way RM ANOVAs. Ethanol consumption data over the final 3 ethanol exposure days were averaged for each animal and analyzed with unpaired *t* tests. Ethanol consumption was corrected for body weight to calculate consumption in g/kg/day. Results from both the ADH and ALDH assays were averaged for each individual animal and subjected to a 2-way RM ANOVA with treatment and substrate concentration as factors. Liver histopathology scores were assessed using a non-parametric Mann-Whitney *U*-test. Data are presented as mean (or mean of means; last 3 days of ethanol consumption) $\pm$ SEM. For all statistical analysis, significance was accepted at $p < 0.05$.

Results

Effect of adolescent CIT exposure on body and liver weight

Both air- and CIT-exposed rats displayed an increase in body weight throughout the exposure period (Fig. 1A & B). Analysis of body weights revealed main effects of time (4-week cohort; $F[2,44] = 3487.45$, $p < 0.001$, Fig. 1A; 8-week cohort; $F[4,88] = 972.17$, $p < 0.001$, Fig. 1B) with no main effects of treatment or factor interactions. Analysis of body

and liver weights upon the cessation of experimentation revealed no significant differences between groups in either the 4-week (Fig. 1C & E) or 8-week cohort (Fig. 1D & F).

Ethanol cross-sensitization following adolescent CIT exposure

Throughout habituation, all animals displayed acclimatization (decreased locomotor activity) to the test apparatus and saline injections with no significant differences in HP activity between groups (main effects of time; 4-week cohort $F[8,176] = 91.22, p < 0.001$; 8-week cohort $F[8,176] = 91.22, p < 0.001$; no main effects of treatment or factors interaction; data not shown). Analysis of the time course of HP activity upon ethanol challenge (0.5 g/kg, i.p.) revealed main effects of time (4-week cohort; $F[8,176] = 91.22, p < 0.001$; 8-week cohort; $F[8,176] = 103.27, p < 0.001$) as animals increased HP activity acutely (within 5 min) upon ethanol injection followed by a decrease to the end of the session (data not shown). There were no main effects of treatment or factor interactions between air- and CIT-exposed rats. In addition, there were no differences between air- and CIT-exposed rats upon analysis of VP activity in either cohort (data not shown).

Ethanol consumption following adolescent CIT exposure

Analysis of the acquisition of voluntary ethanol consumption in a chronic intermittent 2-bottle choice paradigm following 4 or 8 weeks of air or CIT exposure revealed main effects of day (4-week cohort, $F[14,308] = 3.838, p < 0.001$, Fig. 2A; 8-week cohort, $F[14,294] = 7.938, p < 0.001$, Fig. 2B), with no main effects of treatment or factor interactions. The effect of day is evident as an increase in ethanol consumption throughout the initial 15 ethanol exposures (Fig. 2A & B). Similarly, analysis of total fluid consumption revealed main effects of day (4-week cohort, $F[14,308] = 6.642, p < 0.001$, Fig. 2C; 8-week cohort, $F[14,294] = 8.202, p < 0.001$, Fig. 2D), as rats decreased total fluid consumption over the acquisition phase of the experiment, yet there were no main effects of treatment or factor interactions. Analysis of stable voluntary ethanol consumption across the final 3 ethanol

exposures revealed similar ethanol consumption between groups in both the 4-week (Fig. 2E) and 8-week (Fig. 2F) cohorts.

Analysis of voluntary ethanol consumption between the 4-week (commenced PN 63) and 8-week cohorts (commenced PN 89) within each treatment group was undertaken to investigate the effect of age in this study (Fig. 3). Within air-exposed rats, analysis of the acquisition phase of ethanol consumption revealed main effects of age (4- vs. 8-week cohort; $F[1,21] = 7.472, p = 0.012$) and day ($F[14,294] = 4.982, p < 0.001$), and a significant age $\times$ day interaction ($F[14,294] = 2.304, p < 0.01$; Fig. 3A). *Post hoc* analysis revealed significantly increased ethanol intake in the 4-week cohort on ethanol exposure days 3–10, 12, 13, and 15 compared to the 8-week cohort. Similarly, analysis within CIT-exposed rats revealed a main effect of age ($F[1,22] = 6.718, p = 0.017$) and day ($F[14,308] = 2.702, p < 0.01$), but no significant factors interaction (Fig. 3B). *Post hoc* analysis revealed significantly increased ethanol intake in the 4-week cohort on ethanol exposure days 4, 6–10, and 13 (Fig. 3B), compared to the 8-week cohort.

Endogenous liver enzyme activity

ADH activity assay

To examine whether ethanol consumption may have been affected by dysfunction of hepatic metabolism in CIT-exposed rats, endogenous ADH and ALDH activity was assessed in livers collected at the cessation of experimentation. Spectrophotometric analysis revealed that cytosolic ADH activity was concentration-dependent in all rats as indicated by main effects of concentration in both the 4-week ($F[3,30] = 14.481; p < 0.001$; Fig. 4A) and 8-week ($F[3,30] = 33.058; p < 0.001$; Fig. 4B) exposure cohorts with no main effects of treatment or factor interactions. Pyrazole (10 mM) completely inhibited maximal ADH activity (10 mM ethanol) of rats in both the 4-week (air – $147.2 \pm 6.9\%$; CIT – $141.2 \pm 6.6\%$; $p < 0.001$) and 8-week (air – $135.2 \pm 17.5\%$; CIT – $121.4 \pm 3.5\%$; $p < 0.01$) cohorts.

ALDH activity assay

Spectrophotometric analysis of mitochondrial ALDH activity revealed enzyme activity was concentration-dependent in all rats as indicated by main effects of concentration in both the 4-week ($F[3,30] = 11.904$; $p < 0.001$; Fig. 4C) and 8-week ($F[3,30] = 14.481$; $p < 0.001$; Fig. 4D) cohorts, with no main effects of treatment or factor interactions. Disulfiram (0.1 mM) significantly attenuated maximal ALDH activity (100 mM acetaldehyde) in both the 4-week (air – $50.6 \pm 17.9\%$; CIT – $28.62 \pm 3.760\%$; $p < 0.05$) and 8-week (air – $51.9 \pm 9.7\%$; CIT – $44.5 \pm 8.9\%$; $p < 0.05$) cohorts.

Liver Histopathology

Histopathological analysis of the right lateral lobe of livers from air-exposed rats in the 4-week (Fig. 5A) and 8-week (Fig. 5B) cohorts revealed single hepatocytes arranged in rows radiating out from the central vein and separated by sinusoidal space. There was no evidence of overt changes to liver pathology in either cohort between air- (Fig. 5A & B) and CIT-exposed rats (Fig. 5C & D), including lipid accumulation in the cytoplasm of hepatocytes (4-week cohort air: 0.23 ± 0.16 ; 4-week cohort CIT: 0.21 ± 0.14 ; 8-week cohort air: 0.17 ± 0.25 ; 8-week cohort CIT: 0.25 ± 0.13). There was no evidence of active tissue necrosis, and fibrous tissue was only observed on rare occasions. If present, the area was minor (4-week cohort air: 0.50 ± 0.18 ; 4-week cohort CIT: 0.33 ± 0.21 ; 8-week cohort air: 1.04 ± 0.32 ; 8-week cohort CIT: 0.71 ± 0.24).

Discussion

The current study demonstrates that, under the conditions employed, CIT exposure in rats at a positively reinforcing concentration (3,000 ppm) throughout adolescence or adolescence and early adulthood does not result in cross-sensitization to ethanol or alter voluntary consumption of ethanol in adulthood. These experiments were not confounded by altered body weights following CIT exposure, with no effects upon long-term liver function

or pathology. However, age-dependent effects on voluntary ethanol consumption were present in both air- and CIT-exposed rats.

In humans, earlier onset of alcohol use in adolescence is a predictor of alcohol abuse later in life (Chassin et al., 2002). Similarly in rats, ethanol exposure during adolescence results in increased consumption and/or preference for ethanol in adulthood (Criado & Ehlers, 2013; Maldonado et al., 2008; Pascual et al., 2009). However, this appears to be dependent on the ethanol-exposure paradigm employed with models of “binge” adolescent ethanol exposure opposed to voluntary consumption eliciting increases of ethanol intake in adulthood (Broadwater, Varlinskaya, & Spear, 2011, 2013; Vetter, Doremus-Fitzwater, & Spear, 2007). Adolescent toluene abuse is also thought to be a risk factor for substance abuse and dependence later in life (Wu et al., 2004), due in part to its reinforcing and neuroadaptive properties (Beckley et al., 2013; Funada et al., 2002; Williams, Stafford, & Steketee, 2005). Furthermore, toluene induces acute ethanol- (Bowen, 2009; Rees et al., 1987) and amphetamine-like discriminative stimulus effects in rodents (Bowen, 2006), suggesting that toluene’s hedonic and reinforcing properties share a degree of commonality with other known substances of abuse including ethanol. In agreement with this, several neuropharmacological similarities have been demonstrated between ethanol and toluene, including antagonism of *N*-Methyl-D-aspartic acid (NMDA) receptors (Cruz et al., 1998; Lovinger et al., 1990), and positive allosteric modulation of γ -aminobutyric acid A receptors (Beckstead, Weiner, Eger, Gong, & Mihic, 2000; Wallner, Hancher, & Olsen, 2003), although differential pharmacological properties have also been elucidated (Bale, Smothers, & Woodward, 2002; Cardoso et al., 1999; Del Re, Dopico, & Woodward, 2006; Kobayashi et al., 1999).

Despite the notion that ethanol and toluene have similar modes of action within the brain, cross-sensitization to an acute ethanol challenge in adulthood following up to 8 weeks CIT exposure initiated in early adolescence was not observed in the present study. Notably,

toluene's acute locomotor effects involve DA signaling within the mesocorticolimbic DA system (Riegel, Ali, & French, 2003). Specifically, toluene regulates the activity of DA neurons within the ventral tegmental area (Riegel et al., 2007), induces synaptic plasticity at mesoaccumbal terminals within the nucleus accumbens (NAc) (Beckley et al., 2013), and alters DA release within the prefrontal cortex following inhalation (Gerasimov et al., 2002). Thus, like other drugs of abuse, toluene inhalation has the potential to induce long-term alterations within reward-related brain regions, which may confer the susceptibility of adolescent inhalant abusers to substance abuse in adulthood, as seen in human populations (Wu et al., 2004). Adolescent CIT exposure, albeit at a higher concentration (10,000 ppm), results in altered locomotor responses to amphetamine (1.0 mg/kg, i.p.), as well as the non-competitive NMDA receptor antagonist MK-801 (0.5 mg/kg, i.p.) in late adolescence/early adulthood, suggestive of long-term glutamatergic dysfunction in this context, which may alter subsequent behavioral responses to other drugs of abuse (Duncan, Gibbs, & Lawrence, 2014). Indeed, in adult rats chronic toluene inhalation (8,000 ppm, 30 min for 10 sessions) elicits cross-sensitization to cocaine (15 mg/kg, i.p.), which is associated with increased extracellular DA release in the NAc following cocaine challenge in toluene- compared to air-exposed rats (Beyer, Stafford, LeSage, Glowa, & Steketee, 2001). Chronic ethanol administration (2.2 g/kg for 21 days) also results in cross-sensitization to intra-NAc administration of MK-801, suggesting the involvement of NMDA receptor signaling in behavioral sensitization to ethanol (Abraham & Souza-Formigoni, 2012). However, despite evidence of altered locomotor responses to MK-801 following adolescent CIT exposure at a higher toluene concentration (Duncan et al., 2014), results from the present study suggest that adolescent CIT exposure under the conditions employed herein, although reinforcing in rodents (Funada et al., 2002), were insufficient to induce persistent neuroadaptations implicated in behavioral sensitization to ethanol as measured by locomotor activity.

Chronic intermittent access to ethanol in a 2-bottle choice paradigm has previously been demonstrated to initiate stable levels of ethanol intake in both alcohol-preferring P rats and non-preferring Wistar rats, and thus a similar paradigm was employed in the current study (Simms et al., 2008). In agreement with Simms et al., 2008, all rats in the current study acquired a stable level of ethanol intake, yet contrary to the initial hypotheses of this study, voluntary ethanol consumption was not altered following 4 or 8 weeks of CIT exposure. Thus, despite the reported behavioral and neuropharmacological similarities between ethanol and toluene, this study suggests that these substances may have different effects within discrete neural circuits mediating ethanol consumption and locomotor sensitization in rats. Indeed, this hypothesis is supported by differential receptor subunit expression in regions of the mesocorticolimbic DA system following toluene or ethanol exposure (Duncan & Lawrence, 2013; Williams, Stafford, & Steketee, 2005). Alternatively, as persistent neurobiological alterations within regions such as the prefrontal cortex are associated with increased ethanol consumption following adolescent ethanol exposure (Pascual et al., 2009), it is possible that chronic alterations within these circuits related to ethanol consumption may not have occurred following 4 or 8 weeks CIT exposure under the conditions employed. It must also be noted that the 2-bottle choice paradigm is limited to the assessment of ethanol intake and preference, and does not assess more complex aspects of drug-seeking behavior.

Toluene inhalation can induce hepatotoxicity under various exposure paradigms (Gotohda, Nishimura, & Morita, 2009; Tas et al., 2011), as like ethanol, toluene is metabolized in the liver via the hepatic microsomal system, involving the cytochrome P450 enzymes as well as ADH and ALDH, ultimately being excreted as hippuric acid in the urine (Nakajima et al., 1991). Thus, toluene-induced hepatic metabolic dysfunction may alter subsequent ethanol consumption and/or the interpretation of ethanol consumption data. However, ADH and ALDH activity did not differ between air- and CIT-exposed rats in either

the 4- or 8-week cohort, suggesting a lack of long-term metabolic dysfunction consistent with no overt changes to liver pathology at the cessation of experimentation. Moreover, liver regeneration occurs within 5–7 days following hepatectomy (Michalopoulos & DeFrances, 1997), and thus it is unlikely that hepatotoxicity and/or metabolic impairment was present at the commencement of voluntary ethanol consumption ~10 days following the final CIT exposure. Interestingly, increased endogenous ADH activity has also been suggested to be a factor contributing to increased ethanol consumption in alcohol-preferring P rats (Lodge & Lawrence, 2003). Thus, any induction of hepatic metabolism by toluene would likely alter ethanol intake in CIT compared to air-exposed rats in the initial sessions of voluntary ethanol consumption, which was not observed in the current study.

Previous work has demonstrated that pathological and behavioral abnormalities are present following 8 but not 4 weeks of CIT exposure in this model (Duncan et al., 2012). Furthermore, the duration of abuse history is associated with the severity of cognitive and pathological abnormalities in human abusers (Rosenberg, Grigsby, Dreisbach, Busenbark, & Grigsby, 2002), supporting the idea of increased adverse affects with continued exposure. Despite this, cross-sensitization to ethanol or changes to ethanol consumption following 4 or 8 weeks of CIT exposure was not observed. Therefore, it appears that the duration of toluene exposure under the current conditions is not a contributing factor to the subsequent locomotor response to ethanol challenge, or voluntary consumption of ethanol in adulthood. However, in line with previous studies in rats reporting decreased voluntary ethanol consumption with age (Schramm-Sapota et al., 2013; Vetter et al., 2007), increased ethanol consumption in the acquisition phase in rats commencing on PN 63 (young adults; 4-week cohort) opposed to PN 89 (adults; 8-week cohort) for both air- and CIT-exposed rats was observed in the present study. It has been suggested that the decrease in ethanol consumption with age may relate to either developmental factors or differential conditioned-taste aversion in these age groups

(Schramm-Sapota et al., 2013; Vetter et al., 2007), although neither of these factors were investigated in the current study. Rats commencing at later stages of adulthood (8-week cohort) steadily increased ethanol consumption over time reaching similar levels as rats commencing in early adulthood (4-week cohort) by the end of experimentation. Interestingly, unlike studies reporting differential ethanol consumption in adolescent compared to adult rats, an effect which is no longer present once both groups reach adulthood (Schramm-Sapota et al., 2013), it was observed that even rats during early adulthood acquire ethanol consumption more readily than rats in later adulthood. Thus, although ethanol consumption did not significantly differ between air- and CIT-exposed rats, the age-dependent effects observed emphasize that age is an important consideration when investigating voluntary ethanol consumption in outbred rat strains not only in adolescence vs. adulthood but also in early vs. later adulthood.

The present study demonstrates that CIT exposure during adolescence or adolescence/early adulthood does not result in altered locomotor responses or voluntary consumption of ethanol in adulthood, which was associated with a lack of long-term liver dysfunction and pathology under the exposure conditions employed. Despite age-dependent effects upon voluntary ethanol consumption in both air- and CIT-exposed rats, no differences were observed between treatment groups in either the 4- or 8-week cohorts, suggesting that duration of exposure was not a critical factor in this context. Thus, unlike the findings of models using “binge” adolescent exposure to ethanol (Criado & Ehlers, 2013; Pascual et al., 2009), CIT exposure at a reinforcing concentration throughout adolescence or adolescence/early adulthood does not predispose rats to altered locomotor sensitivity or voluntary consumption of ethanol in adulthood in the model employed.

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Figure legends

Figure 1. Body weights throughout the exposure period increased in all rats with no differences between air- ($n = 12/\text{cohort}$) and CIT-exposed rats ($n = 12/\text{cohort}$) in either the 4-week (**A**) or 8-week (**B**) cohorts (main effects of day, 2-way RM ANOVAs). At the cessation of experimentation, both body and liver weights (g/kg body weight) were similar between groups in the 4-week (**C, E**) and 8-week (**D, F**) cohorts. Data are mean $\pm$ SEM.

Figure 2. Voluntary ethanol consumption in the acquisition phase (ethanol exposures 1–15) increased but did not differ between air- ($n = 11\text{--}12/\text{cohort}$) and CIT-exposed rats ($n = 12/\text{cohort}$) following 4 weeks (**A**) or 8 weeks (**B**) exposure (main effect of day, 2-way RM ANOVAs). Total fluid consumption decreased throughout this phase in all rats with no differences between treatment groups in either the 4-week (**C**) or 8-week (**D**) cohorts (main effect of day, 2-way RMA ANOVAs). Stable ethanol intake across the final 3 ethanol exposures was also similar between air- and CIT-exposed rats in the 4-week (**E**) and 8-week (**F**) cohorts. Data are mean $\pm$ SEM (**A–D**) or mean of means $\pm$ SEM (**E, F**).

Figure 3. Age-dependent effects upon voluntary ethanol consumption in both air- (**A**) and CIT-exposed (**B**) rats. Stable high levels of ethanol intake were acquired more readily in the 4-week ($n = 12/\text{group}$) compared to 8-week cohort (air $n = 11$; CIT $n = 12$) with significantly increased intake on several days throughout the acquisition phase of the experiment in both treatment groups ($*p < 0.05$, 4- vs. 8-week cohort; 2-way RM ANOVAs with Holm-Sidak *post hoc* analysis). Data are mean $\pm$ SEM.

Figure 4. Following air ($n = 11\text{--}12/\text{cohort}$) or CIT exposure ($n = 12/\text{cohort}$) and up to 10 weeks intermittent access to ethanol, endogenous activity of alcohol dehydrogenase (**A, B**) and aldehyde dehydrogenase (**C, D**) was concentration-dependent with no differences between treatment groups in either the 4-week (**A, C**) or 8-week (**B, C**) cohorts (main effects of concentration, 2-way RM ANOVAs). Data are mean $\pm$ SEM. *NADH* = nicotinamide adenine dinucleotide.

Figure 5. Hematoxylin and eosin-stained liver sections from air- (**A, B**) or CIT-exposed (**C, D**) rats. Overt liver pathology did not differ between treatment groups after 4-weeks (**A, C**) or 8-weeks (**B, D**) exposure and up to 10 weeks intermittent access to ethanol. *V* = central vein, *H* = hepatocyte. Scale bar: 50 μm .

A **B**

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