Polysubstance addiction vulnerability in mental illness: Concurrent alcohol and nicotine self-administration in the neurodevelopmental hippocampal lesion rat model of schizophrenia

Running Title: Co-addictions in NVHLs
2nd Submit to Addiction Biology 10/25/2018

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Abstract: 250 words (250)
Introduction: 722
Methods: 1254
Results: 1048
Discussion: 1734
Overall length: 4763 (5000)
References: 49
Figures: 8
Tables: 0
Supplementary Material: 0

ABSTRACT

Multiple addictions frequently occur in patients with mental illness. However, basic research on the brain-based linkages between these comorbidities is extremely limited. Toward characterizing the first animal modeling of polysubstance use and addiction vulnerability in schizophrenia, adolescent rats with neonatal ventral hippocampal lesions (NVHLs) and controls had 19 weekdays of 1 hour/day free-access to alcohol/sucrose solutions (fading from 10% sucrose to 10% alcohol/2% sucrose on day 10) during post-natal days (PD 35-60). Starting in adulthood (PD 63), rats acquired lever pressing for concurrent oral alcohol (10% with 2% sucrose) and iv nicotine (0.015 mg/kg/injection) across 15 sessions. Subsequently, 10 operant extinction sessions and 3 reinstatement sessions examined drug-seeking upon withholding of nicotine, then both nicotine and alcohol, then reintroduction. Adolescent alcohol consumption did not differ between NVHLs and controls. However, in adulthood, NVHLs showed increased lever pressing at alcohol and nicotine levers that progressed more strongly at the nicotine lever, even as most pressing by both groups was at the alcohol lever. In extinction, both groups showed expected declines in effort as drugs were withheld, but NVHLs persisted with greater pressing at both alcohol and nicotine levers. In reinstatement, alcohol re-access increased pressing, with NVHLs showing greater nicotine lever activity overall. Developmental temporal-limbic abnormalities that produce mental illness can thus generate adult poly-drug addiction vulnerability as a mechanism independent from putative cross-sensitization effects between addictive drugs. Further pre-clinical modeling of 3rd order (and higher) addiction-mental illness comorbidities may advance our understanding and treatment of these complex, yet common brain illnesses.

Keywords: addiction, alcohol, comorbidity, mental illness, neurodevelopmental, nicotine

INTRODUCTION

Complex comorbidities of psychiatric illness and substance use disorders are mainstream brain health conditions, frequently involving serious illnesses such as schizophrenia where about half of patients have alcohol and/or illicit substance disorders, and more than 75% are nicotine-addicted (Dixon, 1999; Morisano et al., 2009; Mueser et al., 1992; O Brien et al., 2004; Regier et al., 1990). Although often termed 'dual diagnosis' implying comorbidity of just 2 disorders (i.e. one mental illness and one addiction), many dual diagnosis cases involve higher order combinations of multiple addictions with one or more mental illnesses (Barnett et al., 2007; Lambert et al., 2005; Sajid et al., 2016). These 'high order' dual diagnosis cases represent significant treatment challenges not only because these patients are sicker, but because behavioral health care remains largely fragmented into segregated mental health vs. addiction services that are unable to provide integrated care (Balhara et al., 2016; Chambers et al., 2010a; Schmidt et al., 2011).

To advance prevention and treatment of 3rd (and higher) order dual diagnosis conditions, more research is needed to better understand and counteract biological mechanisms that link severe mental illnesses like schizophrenia and poly-addiction vulnerability (Chambers, 2018; Chambers et al., 2001). The present study pursues this goal pre-clinically by examining concurrent poly-substance self-administration in a widely studied and well-validated neurodevelopmental animal model of schizophrenia. Specifically, we examined concurrent adult self-administration of both alcohol and nicotine in the neonatal ventral hippocampal lesion (NVHL) model, which is produced by delivery of axon-sparing neurotoxic lesions to 7-day old rat pups (Lipska et al., 1993). Both alcohol and nicotine are commonly used in the general population, and by schizophrenia patients in addictive patterns at rates at least 2 times greater

than in the general population (DiFranza and Guerrera, 1990; Falk et al., 2006; Kandel et al., 1997; Lasser et al., 2000).   Thus, our study design provides a first animal modelling approach to a commonly encountered mental illness/poly-substance combination that allows for measurement of multi-drug-addiction vulnerability in the context of concurrent poly-drug use.

The applicability of the NVHL model in this approach is suggested by an accumulation of over 100 studies characterizing the mental illness and/or the addiction vulnerability features of the model (Tseng et al., 2009). NVHLs generate a developmentally progressive syndrome that encompasses cognitive and negative symptom domains of human schizophrenia with positive-range symptoms that worsen after adolescence (Tseng et al., 2009). The model also produces frontal cortical-striatal-limbic circuit dysfunction, that mimics core histopathological, neuroimaging, and neurochemical features schizophrenia, including markers of prefrontal dysfunction (i.e. 'hypofrontality') and striatal network hypersensitivity to the effects of mesolimbic dopamine release (Chambers et al., 2013; Chambers et al., 2010b; Chambers et al., 2010c; O'Donnell, 2012; Tseng et al., 2009; Tseng et al., 2007).

These same biological attributes likely underpin impairments of decision-making and impulse control in the NVHL model that emulate human endophenotypes of addiction vulnerability measured before drug exposure (Chambers et al., 2005; Placek et al., 2013; Rao et al., 2016). With drug exposure, NVHLs show acceleration/amplification of the addictive disease process in a non-drug specific way as measured by both experimenter delivered (i.e. behavioral sensitization) to cocaine, alcohol or nicotine (Berg and Chambers, 2008; Chambers and Taylor, 2004; Conroy et al., 2007) and self- administration (i.e. instrumental learning reinforcement) to all of these same 3 drugs (Berg et al., 2011; Berg et al., 2014; Chambers and Self, 2002;

Jeanblanc et al., 2015; Karlsson et al., 2013; Khokhar and Todd, 2018) and methamphetamine (Brady et al., 2008).

This present study is the first to compare NVHL vs. SHAM-operated (healthy) rats in the acquisition of instrumental responding for any two drugs (in this case alcohol and nicotine) self-administered concurrently, followed by tests of drug-seeking during extinction and drug-induced relapse. To increase the likelihood that rats would concurrently self-administer both drugs in adulthood, our design was informed by the capacity of adolescent drug exposure to enhance adult addiction risk (Chambers et al., 2003). Specifically, given our estimation that the rats might be slower to acquire operant responding for access to oral alcohol compared to iv nicotine delivery, and given prior work by Jeanblanc et al, showing that adult alcohol consumption is amplified in NVHL rats after adolescent alcohol drinking (Jeanblanc et al., 2015), we first exposed both NVHL and SHAM rats to free-access alcohol drinking during adolescence, followed by concurrent instrumental operant acquisition to both alcohol and nicotine in adulthood.

METHODS

Subjects and Neonatal Surgeries

Sprague Dawley litters born from rats arriving 14-17 days gestation (Harlan, Indianapolis) were culled to males on post-natal day (PD)-3 in preparation for surgeries on PD-7. Thirty-six pups weighing 16-19g were randomized evenly to NVHL or SHAM surgeries as detailed in (Chambers and Lipska, 2011). Briefly, ibotenic acid (3.0 µg; Sigma) in 0.3 µL artificial CSF (or aCSF only for SHAMs) was delivered under hypothermic anesthesia via

infusion into the ventral hippocampus bilaterally (A/P - 3.0 mm, M/L ± 3.5 mm, DV -5.0 mm from bregma). Pups were reared under standard conditions until weaning (PD-21), then housed in lesion-like pairs until adulthood, when they were individually housed following jugular venous catheterization. Surgical and experimental procedures (**Figure 1**) were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and Indiana University IACUC-approved protocols.

Drug preparation and adolescent alcohol exposure

Nicotine hydrogen tartrate salt (Sigma) was dissolved in 0.9% normal saline to a stock solution of 0.25 mg/ml free base and adjusted to pH of 7.4 (Matta et al., 2007). For iv self-administration, doses were prepared daily on a per rat basis from stock to achieve 0.015 mg/kg/infusion. Stock also provided subcutaneous injections at a volume of 1ml/kg during reinstatement.

Sucrose/alcohol solutions were prepared for the adolescent pre-exposure and adult self-administration experiments as delivered via sipper tube or trough/mechanical dipper, respectively. The pre-exposure sucrose/alcohol fade was based on protocols in (Czachowski et al., 1999) and (Berg et al., 2011) which reliably produce alcohol consumption leading to a final solution that has carbohydrate and alcohol concentrations comparable to human alcoholic beverages. Over 19 days, adolescent rats had access to the following: 3 days of 10% sucrose; 2 days of 10% Sucrose/2% ETOH (10s/2e); 2 days of 10s/5e, 1 day of 10s/10e; 1 day of 5s/10e; then 2s/10e for 10 more sessions. This regimen was delivered over a 4-week (Monday-Friday) schedule (PD-35-60), except for on the 4th Monday (PD 56) when rats underwent iv catheterization surgeries. Rats had access to these solutions (via sipper tubes) for 1 hour/day in Plexiglas cages away from their home cages.

Jugular Catheterization Surgery

On PD-56, subjects underwent jugular venous catheterization as described previously (Chambers and Self, 2002), with Silastic tubing (Dow Corning, Midland, MI) threaded into the right jugular vein coursing subcutaneously over the shoulder to exit the back via 22-gauge cannula (Plastics One, Roanoke, VA). To maintain patency, catheters were flushed before and after operant sessions and once daily on weekends with 0.3 ml of 20 u/ml heparinized saline containing 0.13 mg/ml gentamicin. Patency was verified after Friday operant sessions by pushing 0.1mg/0.1ml iv of methohexital sodium (McKesson, USA) which produces a brief loss of consciousness. Rats with failed or infected catheters were excluded from the experiment.

Operant Co-Self-Administration of Oral Alcohol and IV Nicotine

Concurrent self-administration was conducted in Med Associates chambers (St. Albans, VT) interfaced with Med PC software that controlled lighting and drug deliveries while recording instrumental activity. Our eight units, modified specifically for concurrent instrumental delivery of alcohol and nicotine (as in simulation of the traditional 'Pub') were termed 'Pub-Med' and equipped with 3 non-retractable levers across the right wall of the chamber with only a house light on the opposing (left) wall. The 1st lever (left most, from the rat's perspective) activated a magazine with a 0.1ml dipper cup that retrieved 2s/10e solution from a trough; the 2nd lever (middle, 'blank') was completely inactive; the 3rd lever (rightmost) initiated an iv infusion that

delivered 0.015mg/kg of nicotine. Cue lights were positioned above the levers and the magazine

delivering the alcohol dipper (located between alcohol and blank levers).

Self-administration sessions in 'Pub Med' began on PD-63 for 5 days/week (M-F) for 7

and a half weeks. To promote exploratory behavior for the operant procedure, rats were food

deprived 24 hours preceding the first operant session and remained food restricted to greater than

85% of PD-63 body weight with delivery of 2-3 pellets daily of rat chow after sessions through

the acquisition phase. Over this 3-week phase (15 x 1 hour sessions), both drug-paired levers

delivered reinforcers on an FR1 schedule. The house light was on for the duration of the

sessions. Presses on the alcohol lever activated the cue lights above the lever and the magazine

for the 10-second dipper presentation bearing 2s/10e solution. Presses on the alcohol lever

during this 10-second interval had no consequences and were recorded as time out presses.

Active presses on the nicotine lever delivered a 0.015 mg/kg/infusion over 3 seconds followed

by a 7 second time out phase. The cue light above the nicotine lever was on for 10 seconds

including during the infusion and time out phase; more nicotine lever presses during this phase

had no consequences but were recorded as time out presses. Presses on the middle 'blank' lever

were also recorded. All levers were able to operate independently and both drugs could be

consumed simultaneously or in any back and forth pattern. Cue lights and software

programming remained consistent through all phases of self-administration (**Figure 1**) including

acquisition (sessions 1-15), nicotine-only extinction when 2s/10e was still available (sessions 16-

20), during extinction from both nicotine and alcohol (sessions 21-25); and over 3 sessions of

reinstatement (sessions 26-28). Although it would have been informative to extinguish either

nicotine or alcohol first, we chose nicotine first, since the drug (being delivered iv) was likely

more reinforcing than the alcohol. Tail bloods were collected 30+/-10 minutes of the sessions on

Fridays of week 1 and 2 (sessions 5 and 10) of acquisition to confirm alcohol consumption. Rats were given a week off from 'Pub Med' testing after both the nicotine and nicotine/alcohol extinction phases. For reinstatement, on session 26, rats were given a 1 ml/kg subcutaneous injection of saline 30 minutes prior to the start of the session. On session 27, rats were given a 0.25 mg/kg sc nicotine injection 30 minutes prior. For the last session, rats were again given nicotine 30 minutes prior and troughs were filled with 2s/10e.

Histological Lesion Verification

Following Pub Med sessions and sacrifice, brains were removed whole and cryostat cut into 40 µM coronal sections through the rostral-caudal extent of the hippocampus. Mounted sections were fixed and 0.5% thionin stained. Microscopic examination of both lesioned and SHAM brains were performed blind to behavioral data. Rats showing bilateral evidence of atrophy, paucity of nuclei, and cellular disarray in the ventral hippocampus with some lateral ventricular enlargement, were included. Brains with unilateral damage, dorsal hippocampal damage or direct damage encompassing structures adjacent to the ventral hippocampus were excluded from the study (Chambers and Lipska, 2011). Five lesioned rats were excluded yielding final totals of 13 NVHL and 18 SHAMS rats (**Figure 2**).

Data Analysis

Analyses of adolescent drinking and adult self-administration data generally utilized mixed model repeated measures ANOVAs with lesion status as the main independent factor and alcohol

consumption or bar pressing as dependent variables. Adolescent drinking (10 sessions at the 2s/10e concentration), adult acquisition (15 sessions), nicotine extinction (5 sessions), alcohol & nicotine extinction (5 sessions) and reinstatement (3 sessions) were each examined independently with separate analyses on each lever. Active presses, and time out or total presses were analyzed separately. For acquisition sessions, simple post-hoc t-tests where applied to compare NVHL vs. SHAM pressing on each day to assist with interpretation of the initial ANOVAs that revealed simple lesion or lesion x day interactions. All significant statistical results (assumed at $p<0.05$), and informative negative results are reported with group means $\pm$ SEMS throughout.

RESULTS

Adolescent Drinking and Adult Acquisition of Co-Self-Administration

Across the final 10 weekday sessions of adolescent drinking (PD 46-60) at the 2s/10e concentration, rats significantly increased their consumption (day: $F_{(9,261)}=6.3$, $p<0.001$) so that alcohol intake increased from 0.13 ± 0.03 gm ETOH/kg rat on day 1 to 0.47 ± 0.08 g/kg on day 10. There was no lesion based difference in consumption (lesion: $F_{(1, 29)}=0.8$, NS), or lesion x day interaction ($F_{(9,261)}=0.8$, NS).

Over the 15 concurrent alcohol (2s/10e)/nicotine acquisition sessions, active presses on the alcohol lever (**Figure 3A**) increased for all animals (day: $F_{(14, 406)}=10.1$, $p<0.00$), with NVHLs showing greater active pressing (lesion: $F_{(1,29)}=7.7$, $p<0.01$) but no lesion x day interaction $F_{(14, 406)}=0.7$, NS), even as NVHL and SHAMs showed the same level of active presses on session 1. Time out responding on the alcohol lever (**Figure 3B**) also increased then

stabilized over the 15 sessions (day: $F_{(14,406)}=6.6$, $p<0.001$), but there were no lesion ($F_{(1,29)}=1.9$, NS) or lesion x day interaction ($F_{(14, 406)}=0.6$, NS). To confirm that animals were actually drinking alcohol presented to them via active alcohol lever presses, tail blood alcohol levels were analyzed using linear regressions between the number of dipper presentations (x-variable) and tail blood alcohol levels (y-variable). After the 5th session, 12 NVHL and 13 SHAM rats yielded adequate blood samples showing a significant linear relationship (($F_{(1,24)}=16.8$, $p<0.001$); R=0.65; y= 0.69x +5.2) (**Figure 4A**). After the 10th session, a better yield of tail bloods from all 13 NVHL and 18 SHAMs also showed a significant relationship between blood alcohol levels and active alcohol lever presses (($F_{(1,30)}=6.9$, $p<0.05$); R=0.44; y=0.35x+6.3) (**Figure 4B**).

Active nicotine lever presses also increased steadily over the 15 acquisition sessions (day: $F_{(14,406)}=16.5$, $p<0.001$), after NVHL and SHAM rats started out at similar low levels on day 1 (**Figure 3C**). NVHLs also showed greater overall active nicotine lever presses (lesion: $F_{(1,29)}=10.9$, $p<0.01$) with a significant day x lesion interaction ($F_{(14,406)}=2.2$, $p<0.01$) that, as suggested by simple post-hoc comparisons on each day, was generated by a progressive widening of group differences expressed over the final 8 sessions. Time out responding on the nicotine lever showed similar patterns but with less statistical strength in terms of group differences (**Figure 3D**) with overall increases in pressing (day: $F_{(14, 406)}=5.3$, $p<0.001$), where NVHLs produced more presses overall (lesion: $F_{(1,29)}=7.7$, $p<0.05$) and in a significant lesion x day interaction ($F_{(14,406)}=1.8$, $p<0.05$).

Analysis of responding on the blank lever (**Figure 3E**) also showed more subtle but still significant increases in lever responding for all rats across the 15 sessions (day: $F_{(14,406)}=2.7$, $p<0.001$). There was also greater NVHL pressing (lesion: $F_{(1, 29)}=5.7$, $p<0.05$) without the day

x lesion interaction (F(14, 406)=1.1, NS) on the blank lever.   Examination of the fractions of

total activity directed at each lever (**Figure 5**) shows that relative levels of responding evolved

on all three levers (alcohol (days: F(14,406)=8.7, p<0.001) , nicotine (day: (F(14,406)=3.0,

p<0.001) blank (F(14,406)=5.6, p<0.001)) with the proportion of alcohol responding growing

faster than nicotine responding over the first five days, then stabilizing at about 2:1 ratios

(alcohol to nicotine) over days 5-15 with blank lever pressing being relatively extinguished.

NVHLs did not differ from SHAMs in overall proportions of type of lever responses but did

show a significant growth in the relative proportion of presses at the nicotine lever (day x lesion:

F(14,406)=1.9, p<0.05).

Single and Poly-Drug Extinction

In sessions 16-20 with nicotine but not alcohol delivery withheld, there was no overall

change in alcohol lever responding examined as active presses (**Figure 6A;** day: F(4, 116)=1.0,

NS), or total alcohol lever presses (active + time out hits are examined for better comparison to

total nicotine lever presses, which has no active vs. time out component) (**Figure 6B;** day:

F(4,116)=0.7, NS)). However, NVHLs continued to show greater alcohol lever responding for

both active (lesion: F (1, 29)=6.6, p<0.05) and total presses (F(1, 29)= 6.3, p<0.05). In contrast,

there was a significant decline in total lever pressing on the nicotine lever (**Figure 6C**) consistent

with extinction (day: F(4, 116)=8.1, p<0.001) while NVHL rats continued to press for nicotine to

a greater extent (lesion: F(1,29)=11.1, p<0.01) than SHAMs across these sessions, without a day

x lesion interaction (F(4,116)=0.8, NS)).

In sessions 21-25, with delivery of both alcohol and nicotine denied, total alcohol lever

pressing (**Figure 7A**) declined significantly consistent with extinction (day: F(4, 116)= 19.2,

p<0.001), while NVHL rats persisted in pressing for ETOH at greater rates than SHAMs (lesion: $F_{(1,29)}=4.6$, p<0.05) without a day x lesion interaction: $F_{(4,116)}=0.4$, NS). On the nicotine lever (**Figure 7B**), in continuation of patterns observed in the prior nicotine-only extinction-sessions (**Figure 6**) rats continued to show ongoing declines in total pressing (day: $F_{(4,116)}=13.5$, p<0.001), with NVHL rats still pressing more for nicotine overall (lesion: $F_{(1,29)}$: 5.4, p<0.05; day x lesion : $F_{(4,116)}=0.28$, NS). Analysis of blank lever pressing during the final 5 extinction sessions showed ongoing declines in low level pressing (day: $F_{(4,116)}=3.3$, p<0.05; means of all rats from 7.4 ± 1.0 presses/hr (session 21) to 4.5 ± 1.2 presses/hr (session 25), with no differences based on lesion (lesion: $F_{(4,29)}=1.7$, NS; day x lesion: $F_{(4,116)}=0.45$, NS). This blank lever pressing differed from blank lever pressing when ETOH was still being delivered during nicotine-only extinction (sessions 16-20). Over these sessions, although overall blank lever responding was also quite low and still extinguishing (day: $F_{(4,116)}=4.9$, P<0.01), NVHL rats were pressing more (lesion: $F_{(1,29)}=14.4$ p<0.01) and showed steeper overall declines in responding (day x lesion: $F_{(4,116)}=2.7$, p<.05), such that NVHLs declined from 9.9 ± 2.2 presses/hr (session 16) to 4.0 ± 0.8 presses/hr (session 20), compared to SHAMs with $3.8+0.8$ (session 16) and 2.5 ± 0.7 presses/hr (session 20).

Single and Poly-Drug Re-instatement of Drug Seeking

Across all 3 reinstatement sessions (**Figure 8**) there was no change in blank lever pressing (day: $F_{(2,58)}=2.5$, NS) or lesion-based differences in blank lever pressing (lesion: $F_{(1,29)}=1.8$, NS; day x lesion: $F_{(2,58)}=1.8$, NS). However, there was an overall increase in total lever pressing on the alcohol lever (day: $F_{(2,58)}=26.5$, p<0.001) as expected, with substantial increases when alcohol became available on day 3. These changes were not

accompanied by lesion-based differences in alcohol lever presses (lesion: $F_{(1,29)}=0.07$, NS; day x lesion: $F_{(2,58)}=2.9$, NS). On the nicotine lever, NVHLs continued to press more (lesion: $F_{(1,29)}=17$, $P<0.001$), without substantial increases across sessions overall (day: $F_{(2,58)}=0.7$, NS) or according to lesion status (day x lesion: $F_{(2,58)}=2.9$, NS).

DISCUSSION

This study is to our knowledge the first to demonstrate concurrent instrumental oral alcohol and iv nicotine self-administration in a neurodevelopmental model of mental illness, and to show that addictive behaviors with respect to both drugs are simultaneously increased by the mental illness model. Le et al have previously show that healthy Wistar rats will concurrently self-administer oral alcohol and iv nicotine (Le et al., 2010), and that in Long Evans rats, initial self-administration of nicotine will enhance subsequent self-administration of alcohol (Le et al., 2014). The latter finding is consistent with a 'cross-sensitization' or 'gateway effect', whereby use of, or addiction to one drug biologically predisposes to addiction to one or several others via shared or synergistic pharmaco-biological effects (e.g. in the mesolimbic reward pathways) (Nestler, 2005). While providing a compelling explanation for the occurrence of polysubstance use disorders, e.g. where rates of nicotine addiction are 2-3 fold higher in patients with alcoholism, than in the general population (Falk et al., 2006), the present findings are among the first to suggest an additional causal dynamic: The co-occurrence of 2 drugs used in addictive patterns may be caused by their shared vulnerability or 'gravitation' toward a third pathological entity—the mental illness. This possibility has been preliminarily supported by prior work in the NVHL model showing that it confers elevated addiction vulnerability to both nicotine and alcohol, completely independently of one another (Berg et al., 2011; Berg et al., 2014; Jeanblanc

et al., 2015), whereas the present study confirms it in the context of concurrent self-administration of both drugs.

It is possible that both the cross-sensitization effect and mental illness-induced vulnerability to addiction may simultaneously, biologically attract all these pathologies together causing high-order dual diagnosis cases that are now routinely encountered in behavioral health care settings (Sajid et al., 2016). Notably, the present study does not rule out the possibility that a cross-sensitization effect between alcohol and nicotine was also in play, nor was it designed to test which pathological attraction, the 'drug-drug' or the 'drug-mental illness' one is greater. Future animal studies are needed to dissect and characterize the relative strengths of these causal dynamics, including the intriguing possibility that mental illness could biologically enhance drug-to-drug sensitization. The majority of work looking at how the NVHL model biologically worsens the addiction process has been limited to cocaine. These studies have shown that while the NVHL model does not increase drug-induced DA release into the ventral striatum as compared to healthy animals (Chambers et al., 2010c), the cumulative neuroplastic and behavioral effects of drug-induced DA release are accentuated by abnormal neuronal activity (Chambers et al., 2010b) and gene expression patterns (Chambers et al., 2013) present in both the prefrontal cortex and dorsal striatum of NVHL rats. So, to the extent that alcohol and nicotine both exert cocaine-like reinforcing effects in schizophrenia (Berg et al., 2014; D'Souza et al., 2006) via their shared effects on increasing meso-striatal DA transmission, it is plausible to speculate that both their independent and synergistic (e.g. drug-drug sensitizing) neuroplastic effects may be expressed and amplified by the NVHL model—post-synaptic to DA transmission within prefrontal cortex and striatal networks.   Consistent with this, GABAergic-interneuron control of prefrontal cortical networks (e.g. required for adult-age capacities for working

memory and impulse control), requires proper maturation during adolescence, and is modulated by meso-cortical DA afferents (Caballero and Tseng, 2016; Lew and Tseng, 2014). NVHLs disrupt the development of these complex regulatory interactions leading to impaired DA modulation of prefrontal GABAergic interneurons in adulthood, and other physiological signs of cortical incoherency in which both GABA and glutamatergic neurons are implicated (Tseng et al., 2008; Tseng et al., 2007; Vohs et al., 2012). Given evidence presented here and in prior studies that addiction vulnerability of NVHLs is more fully expressed after adolescence, and correlates with deficits of working memory and impulse control (Berg et al., 2014; Rao et al., 2016) a complex array of disruptions of information processing and neuroplasticity involving both excitatory and inhibitory neurotransmission within cortical-striatal networks likely contribute to both mental illness symptoms and non-drug specific susceptibility to addiction.

A key design feature of the present study is that rats had a free-access drinking regimen of a sucrose/alcohol solution during adolescence as informed by Jeanblanc et al, to increase the likely-hood of successful acquisition of concurrent instrumental self-administration of both drugs in adulthood (Chambers et al., 2003; Jeanblanc et al., 2015). This approach was also undertaken to help avoid the possibility that heavy use of one drug in the operant boxes could actually drive down use of another drug as has been observed under certain circumstances between alcohol and nicotine (Le et al., 2014) in healthy rats. Our findings suggest our approach worked, while replicating prior findings showing that although the NVHL model does not cause increased alcohol-consumption during adolescence, the alcohol addiction vulnerability phenotype of NVHLS is expressed in adulthood (Jeanblanc et al., 2015). Similarly, we have seen the same kind of effect with nicotine: behavioral sensitization to nicotine is not increased in adolescent NVHLs, although it is in adult NVHLs, who also show increased nicotine self-administration

compared to SHAMs, regardless of adolescent nicotine exposure (Berg and Chambers, 2008; Berg et al., 2014). Notably, because all rats were exposed to alcohol solutions in adolescence, this study was not able to determine if this exposure was necessary for, or caused differential expression of the adult NVHL vs SHAM phenotypes.

The use of a three-lever system in the present experiments with one blank lever, and time-out phases on both the alcohol and nicotine levers, allowed us to examine NVHL-model differences in the specificity of drug pursuit and effort. During acquisition, NVHL-based differences from SHAMs were greater for active lever as compared to time-out responding with respect to both drugs, and, as compared to blank lever hitting. These patterns indicate that NVHLs specifically increased pressing that was most certain to deliver drug, but simultaneously some degree of NVHL-induced non-specific over-flow of pressing (on the nicotine time-out and blank lever) did occur. Even so, this non-specific pressing in NVHLs was strongest for nicotine-lever time-out responding, which emerged in the last half of the acquisition series when nicotine intake reached the greatest levels, whereas NVHL difference in blank hitting became more infrequent with more sessions.

The behavioral economy of overall lever responding was remarkably similar between the groups with rats settling into patterns by day 4 of acquisition where 50% to 75% of all presses were on the alcohol lever, 20% to 40% were on the nicotine lever and <10% were on the blank lever. It is not entirely clear why the alcohol lever was pressed more frequently than the nicotine lever overall for both active and time out presses, although active nicotine presses were guaranteed to deliver a 3-second iv infusion, whereas active alcohol lever presses just presented the cup of alcohol, still requiring the animal to find and drink from it. This difference may have produced more relative effort to access the alcohol. Also, since all rats were already experienced

with alcohol (in adolescence) but not nicotine, they may have already been more motivationally sensitized to alcohol compared to nicotine. Notably, in anecdotal observation through viewers into the chambers on the first acquisition day, we saw rats immediately maneuvering to the trough where the alcohol was available, presumably drawn by alcohol odor cues. Despite all this, NVHL rats did show a significant growth in the percentage of activity on the nicotine lever compared to SHAMs (but not on the other levers), which led to more robust NVHL-nicotine findings in the extinction and reinstatement sessions.

Over the first extinction series, where nicotine (but not alcohol) delivery was denied, group differences between NVHLs and SHAMs were statistically more significant on the nicotine ($p<0.01$) compared to alcohol lever ($p<0.05$), while significant between session declines in responding were only seen on the nicotine lever. Then, during the next extinction series when both drugs were denied, significant declines in both levers were obtained, with the NVHL rats maintaining increased efforts on both levers. During re-instatement, NVHL activity on the nicotine lever was exclusively higher (compared to the other levers) pervasively across sessions, whereas return of alcohol access significantly boosted alcohol lever activity for all rats regardless of lesions status. Somewhat unexpectedly, nicotine injections introduced in the second reinstatement session did not significantly increase nicotine lever responding compared to levels on the first (saline injection day). It is possible that the nicotine dose used (0.25mg/kg/sc) was not high enough to elicit a strong reinstatement response specific to NVHLS as we've seen by using a 0.5mg/kg dose reported previously (Rao et al., 2016). In any event, the acquisition, extinction and reinstatement data collectively suggest that in the context of the adolescent alcohol pre-exposure, the adult addiction vulnerability phenotype expressed in the NVHL model is generalizable to both alcohol and nicotine but occurs with greater robustness with nicotine.

This interpretation should be qualified by the fact that the alcohol in this paradigm had to be orally consumed, whereas the nicotine was iv injected. This difference created reliability, work-load and pharmacokinetic differences for the animals in terms of how the 2 drugs arrived in their brains, which all could be assumed to decrease the relative addictive potential of alcohol compared to nicotine in this paradigm. Moreover, it is generally accepted that nicotine is relatively more addictive than alcohol, and so our findings are interpretable as indicating that NVHLs increase addiction risk in a way that is more readily expressed with more addictive drugs.

In summary, the present findings, while representing at least the 2nd published replication showing increased addiction vulnerability to either nicotine (Berg et al., 2014; Rao et al., 2016) or alcohol (Berg et al., 2011; Jeanblanc et al., 2015; Khokhar and Todd, 2018) in the NVHL model, are the first to show the comorbidity of these addiction vulnerability phenotypes when both drugs are used concurrently. A huge variety of alternative study designs may be pursued based on the mental illness/poly-addiction model approach described here to investigate the interactive pathologies of these conditions on neurobiology, cognition and motivation. While providing a platform to pre-clinically investigate novel preventative and treatment approaches for 3rd and higher order dual diagnosis patients, these findings point to a fundamental connection between mental illness and multiple addictions that should translate clinically to greater integration of professional training, treatment and research on these complex comorbidities (Barnett et al., 2007; Chambers, 2018).

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

RAC has advisory and/or creative contracts with Enfoglobe (Medical Education and Data Analytics Software), Indigobio (Bioanalytics Technology and Software) and Proniras (Pharmaceutical Research Firm). None of the other authors (AS, RB, EAE) have any conflicts of interests or biomedical interests to report.

AUTHORS CONTRIBUTION

AS, EAE, and RAC were responsible for the fundamental design elements of the experiment, with advice from RLB. Hands on work and data acquisition were conducted by AS and EAE. Data analysis and interpretation involved all of the authors, and primary manuscript drafting was a combined effort of AS and RAC, with EAE and RLB providing critical input on pre-submission revision. All authors approved the final submitted documents.

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FIGURE LEGENDS

Figure 1. Surgical and experimental timeline.

Figure 2. Brain mapping of lesion extent. Maps on left show the range of lesion impact across all N=13 NVHL rats included in the study at sections AP relative to Bregma (Maps adapted from Swanson, LW (2004) Brain Maps: Structure of the Rat Bain, 3rd ed., New York, Elsevier). Largest extent of lesions in the group are shown in black with smallest lesions shown as white insets (e.g. at -5.00). Right column micrographs show a medium-sized lesion in the NVHL group vs. a SHAM rat.

Figure 3. Acquisition of concurrent instrumental self-administration of oral alcohol and iv nicotine. (**A**) Active alcohol (ETOH) lever hits presenting access to 0.1 ml 2s/10e solution were greater in NVHL rats (**), (**B**) with no group differences in Time Out (T.O.) responding on the alcohol lever. On the nicotine (NIC) lever, (**C**) active hits delivering 0.015 mg/kg nicotine were greater in NVHL rats (**), accompanied by greater across-sessions growth of lever pressing in NVHLs (** day x lesion). (**D**) T.O. responding on the NIC lever was also greater in NVHLs overall and in term of lesion specific growth but with less statistical strength (*). (**E**) Blank lever hits were also elevated in NVHLs overall (*) but with less strength than at alcohol and nicotine levers, and with no day x lesion interaction. All bars reflect means $\pm$ SEMS with asterisks above bars representing simple t-test comparisons by lesion status on that day. All asterisks represent degree of statistical significance (*$p<0.05$; **$p<0.01$; ***$p< 0.001$).

Figure 4. Linear correlations between active ETOH lever pressing (cups presenting 0.1ml of 2s/10e solution) and tail blood alcohol levels collected 30 +/-10 minutes after sessions on

Fridays of week 1 and 2 (sessions 5 and 10). After session 5 (**A**) adequate samples were drawn from n=25 rats yielding a significant liner correlation ($F_{(1,24)}$=16.8, $p<0.001$), and after session 10 (**B**) all 31 rats yielded a significant correlation ($F_{(1,30)}$=6.9, $p<0.05$).

Figure 5. Re-examination of acquisition responding as relative fractions of activity on alcohol vs. nicotine vs. blank levers. The fractions of total pressing evolved highly significantly on all 3 levers (***day), settling into 6:3:1 ratios (ETOH: NIC: BLANK) by day 5. There was no overall group difference in preference for lever, although NVHLs showed a significant time progression of responding preference on the nicotine lever (*day x lesion). All bars reflect means $\pm$ SEMS; (*$p<0.05$; ***$p< 0.001$).

Figure 6. Extinction from nicotine only with continuation of alcohol access. NVHL rats continued to press more frequently on the ETOH lever (*) in terms of both active (**A**) and total (active + T.O.) hits (**B**). Meanwhile, overall responding extinguished on the NIC lever (***day) but with NVHL rats continuing to press for nicotine (**). All bars reflect means $\pm$ SEMS; (*$p<0.05$; **$p<0.01$; ***$p< 0.001$).

Figure 7. Extinction from nicotine and alcohol access. (**A**) Total hits on the ETOH lever declined relatively precipitously (***day) compared to (**B**) on the NIC lever (*day) which had already been largely extinguished. NVHLs continuing to press more overall on both ETOH and NIC levers (*). All bars reflect means $\pm$ SEMS; (*$p<0.05$; ***$p< 0.001$).

Figure 8. Nicotine and alcohol reinstatement sessions. No lesion based differences emerged on the blank lever, regardless of saline (SAL) vs. nicotine (NIC) pre-injection condition vs. return of alcohol access with nicotine pre-injections (NIC). Pressing on the ETOH lever increased strongly (***day) with return of alcohol access but not differentially so by lesion.

NVHLs persisted in showing increased activity on the NIC lever (***lesion) regardless of

reinstatement condition. All bars reflect means $\pm$ SEMS; (***p< 0.001).