Research Article

Altered Oligodendroglial and Neuroglial Gene Expression in Adult Rat Cerebral White Matter Following Short- and Long-Term Ethanol Exposures and Abbreviated Abstinence

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Abstract

Ethanol-mediated white matter degeneration is associated with impaired oligodendrocyte functions linked to myelin maintenance. This study examines ethanol-induced time course-dependent shifts in myelin gene expression in a chronic+binge exposure model and assesses therapeutic responses to short-term abstinence. Six-week-old male Long Evans rats were fed isocaloric liquid diets with 0% or 26% ethanol (caloric content) for 3 or 8 weeks. During the last 2 weeks of ethanol feeding, the rats were binged with 2 g/kg ethanol by intraperitoneal injection, 3x/week. In a subgroup pair-fed for 6 weeks, ethanol was tapered over 2 days and then withdrawn for 12 days. Gene expression in temporal lobe white matter was measured by qRT-PCR analysis. Chronic+binge ethanol exposures caused short-term, long-term, or step-wise changes in neuroglial gene expression such that: 1) NCAM1 mRNA levels were significantly elevated after 3 weeks ($P=0.04$); 2) PLP was reduced after 8 weeks ($P=0.003$); and 3) MAG ($P=0.008$) and RTN4 ($P=0.02$) progressively declined from 3 to 8 weeks of exposure. Abstinence did not reverse long-term ethanol effects on PLP, MAG or RTN4. Instead, it significantly reduced RPAIN ($P=0.002$) and Olig2 ($P=0.02$) relative to responses after 8 weeks of continuous ethanol exposure. In conclusion, chronic+binge ethanol exposures mainly inhibited mature myelin-associated gene expression. Short-term abstinence failed to abrogate those adverse effects of ethanol. Instead, the corresponding reductions in RPAIN and Olig2 expression suggest that during the acute phase of withdrawal or abstinence, molecular indices of white matter degeneration may worsen and that additional therapeutic measures may be required for optimum recovery.

Keywords

chronic+binge alcohol; short-term abstinence; myelin; white matter degeneration

Abbreviations: CNS: central nervous system; PDGFR- α : platelet-derived growth factor receptor- α ; GALC: galactosylceramidase; GC: group-specific component vitamin D binding protein; PROM1: prominin 1; MBP: myelin basic protein; PLP: proteolipid protein; MAG: myelin-associated glycoprotein; MOG: myelin oligodendrocyte

glycoprotein; RTN4: reticulon 4; RPAIN: RPA interacting protein; ST8Sia1: ST8 α -N-acetyl neuraminidase α -2,8-sialyltransferase 1; NCAM1: neural cell adhesion molecule 1; GSTP-1: glutathione S-transferase P-1; CSPG4: chondroitin sulfate proteoglycan 4; GFAP: glial fibrillary acidic protein; NTRK2: neurotrophic tyrosine kinase receptor, Type 2; FOXO1: forkhead Box O1; FOXO4: forkhead Box O4; Olig1: oligodendrocyte transcription factor 1; Olig2: oligodendrocyte transcription factor 2; NKX6-1: NK6 homeobox 1; PAX6: paired box 6; qRT-PCR: Quantitative Reverse Transcriptase Polymerase Chain Reaction; HPRT: hypoxanthine-guanine phosphoribosyltransferase; Act β : beta-actin; ANOVA: analysis of variance

1. Introduction

Heavy drinking over a long period of time adversely affects the central nervous system (CNS), impairing cognition and memory and causing neurodegeneration in adults [1,2]. Pathological and neuroimaging studies have shown that one of the main neurodegenerative effects of chronic alcohol exposure is white matter atrophy [3-5]. Experimental models revealed that the underlying pathologies of alcohol-induced white matter atrophy include demyelination, dysmyelination, and axonal degeneration [6]. Within the cerebrum, alcohol's targeting of the corpus callosum has been studied extensively due to corresponding deficits in inter-hemispheric communication linked to cognitive and motor deficits [2]. Magnetic resonance imaging (MRI) and postmortem studies have further demonstrated that alcohol-mediated callosal thinning worsens with advancing age [7,8], yet, with sustained abstinence, partial recovery of white matter damage [9,10] and cognitive function [11] can be achieved in adults.

In the CNS, oligodendrocytes generate myelin which is needed to ensure structural and functional integrity of axons. Although myelination occurs throughout life [12,13], the largest increases occur during the early stages of development and continue through adolescence. In humans, CNS myelination is most robust during the first two decades of life [14] whereas in rodents, myelination occurs mainly during first two postnatal months [15]. Myelin is largely composed of lipids (70%), but also contains structural proteins [16]. The compositions of oligodendrocyte myelin lipids and proteins change with white matter maturation during development [17]. Myelin-associated proteins expressed by immature oligodendrocytes include nestin, vimentin, chondroitin sulfate proteoglycan 4 (CSPG4), platelet derived growth factor receptor (α -subunit, PDGFR- α), galactocerebroside/galactoceramidase (GALC), and prominin 1 (PROM1) [18, 19], whereas those expressed by mature oligodendrocytes include myelin basic protein (MBP), myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), glutathione S-transferase P-1 (GSTP-1), and galactocerebroside/galactoceramidase (GALC) [19-21].

High-level binge ethanol exposures (5 g/kg, oral gavage) have devastating effects on both neurons and oligodendrocytes, impairing proliferation and differentiation [22]. Repeated binge ethanol exposures (9.3 g/kg/day infused over 4 days) decrease survival of oligodendrocyte progenitor cells in adult rat brains [22]. Chronic (26% caloric ethanol for 8 weeks) plus binge (2 g/kg ethanol by intraperitoneal injection, 3 times/week during weeks 7 and 8) exposures inhibit expression of mature oligodendrocyte myelin-associated proteins and de novo synthesis of myelin in adult rats [23,24]. Furthermore, ultrastructural studies showed that in the chronic+binge rat model which mimics consumption patterns in humans with alcohol use disorders, frontal lobe white matter injury is due to combined pathologies in myelin (demyelination and dysmyelination) and axons (axonal degeneration) [6]. Similar observations have been made in postmortem human alcoholic brains [25]. However, abstinence following alcohol exposure can result in increased cell proliferation and formation of new neurons and oligodendrocytes, reflecting neurogenesis and myelination in adults [26,27].

Previous reports demonstrating the potential for at least partial recovery of white matter atrophy and degeneration in humans and experimental models [5,9,28], along with improvements in cognitive-executive functions in humans following periods of abstinence [11], justifies further investigation to determine the mechanisms of those therapeutic responses. To begin addressing this question, the present work utilized an adult rat chronic+binge ethanol exposure model to examine time course-dependent shifts in myelin-associated gene expression and assess therapeutic responses to short-

term abstinence. Our working hypothesis was that the progressive white matter atrophy and degeneration caused by chronic + binge ethanol exposures is mediated by impairments in the expression of myelin-associated proteins and transcription factors that are essential to myelin and axon generation, maturation, and maintenance. In addition, we proposed that the partial restoration of white matter integrity following short-term abstinence would be associated with increased expression of mature versus immature myelin-associated proteins. This work is novel because it investigates the molecular pathogenesis of alcohol-mediated white matter degeneration over time and in relation to abstinence.

2. Methods

2.1. Experimental model

Experiments using rats were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and approved by the Lifespan Institutional Animal Care and Use Committee. Six-week-old male Long Evans rats were fed with isocaloric liquid diets containing 0% or 26% ethanol by caloric content for 3 or 8 weeks ($n=6$ /group). Ethanol-fed rats were administered intraperitoneal injections of 2 g/kg ethanol 3 times per week during the last 2 weeks of ethanol feeding; controls were treated with saline. A subgroup ($n=6$) exposed to 6 weeks of ethanol was subjected to a brief period of abstinence by tapering the ethanol over 2 days, and then maintaining them on ethanol-free diets for 12 days. Since neurodegenerative effects of alcohol are detectable by 4-6 weeks of chronic ethanol feeding, we initiated abstinence after 6 weeks of ethanol exposure when the deleterious effects of ethanol would have been manifested. The short period of abstinence was used because in previous studies, a two-week interval was sufficient to increase neurogenesis [29] and improve spatial learning and memory [30] in adults.

On the day of sacrifice, body weights, brain weights, and blood alcohol concentrations were measured (Supplementary Figure 1). Upon sacrifice, the temporal lobes were harvested, frozen on dry ice, and stored at -80°C . Although the neurotoxic and degenerative effects of alcohol occur prominently in the prefrontal cortex, temporal lobe, hippocampus, and cerebral white matter [2], our studies focused on its adverse effects in temporal white matter as an extension of previous work using this model in which we demonstrated alcohol-induced temporal lobe white matter atrophy with myelin loss, aberrant expression of mature glial and neuronal proteins, and sphingolipid and phospholipid profiles [23,28].

2.2. Targeted Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) Arrays

Total RNA extracted from fresh frozen tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) was reverse transcribed using the AMV 1st Strand cDNA

Synthesis Kit (Roche, Indianapolis, IL). The resulting cDNA templates were used to measure mRNA transcripts encoding 18 neuroglial and 6 transcription factor genes by qPCR in a Roche Lightcycler 480 System [23]. Primer pairs were designed using Primer 3 software (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Relative mRNA abundance was calculated using the $2^{-\Delta\Delta C_t}$ method with results normalized to internal controls (Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and Beta-actin (Act β)).

2.3. Statistical analysis

Results are presented as mean $\pm$ standard deviation. Inter-group comparisons were made by one-way or two-way analysis of variance (ANOVA) with Tukey post hoc tests (GraphPad Prism 6, San Diego, CA, USA). Each experimental group included 6 rats. Post hoc test significant differences ($P \leq 0.05$) and trend effects ($0.05 \leq P \leq 0.1$) are shown in the graphs.

3. Results

3.1. Glial neuronal markers investigated

We used a custom targeted PCR array to measure mRNA transcripts encoding structural and transcription factor proteins expressed in immature or mature oligodendroglia. The rationale for selecting the gene targets was based on known shifts in molecular markers of oligodendroglia that occur with maturation. Oligodendrocytes arise from oligodendrocyte precursor cells (OPCs) that express high levels of nestin, vimentin, chondroitin sulfate proteoglycan 4 (CSPG4), platelet derived growth factor receptor (α -subunit, PDGFR- α), galactocerebroside/galactoceramide (GALC), and prominin 1 (PROM1) [18, 19]. Differentiation of OPCs into immature oligodendrocytes are regulated by transcription factors Olig1, Olig2, NKX6-1, and PAX6 [18, 31]. Immature oligodendrocytes progress into mature oligodendrocytes that contain myelin specific proteins such as myelin basic protein (MBP), myelin associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), and glutathione S-transferase P-1 (GSTP-1) [19-21]. In addition, reticulon 4 (RTN4), RPA interacting protein (RPAIN), and ST8 alpha-N-acetyl neuraminide alpha-2,8-sialyltransferase 1 (ST8Sia1) have been used as a marker of oligodendrocytes and myelin sheaths [31-33]. NCAM1 and NTRK2 are neuronal markers that mediate neuronal development, survival, proliferation, differentiation, and plasticity [34, 35]. GFAP is an intermediate filament protein used as astrocyte marker. FOXO-1 and FOXO-4 are transcription factors that target insulin signaling and regulate oligodendrocyte precursor development and myelination [36, 37].

3.1.1. Short- versus long-term ethanol exposure and abbreviated abstinence effects on immature oligodendroglial gene expression: For immature oligodendroglia we measured mRNA levels of nestin,

vimentin, platelet-derived growth factor receptor-alpha (PDGFR- α), galactosylceramidase (GALC), group-specific component vitamin D binding protein (GC), and prominin 1 (PROM1). Two-way ANOVA tests demonstrated ethanol exposure $\times$ time interactive trend effects for Nestin ($P=0.08$) and a trend effect for duration of ethanol exposure on GC expression ($P=0.07$). In addition, one-way ANOVA with Tukey multiple comparisons of 8-week control, 8-week ethanol, and abstinence groups demonstrated that short-term abstinence had no significant or trend effects on immature oligodendroglia.

3.1.2. Short- versus long-term ethanol exposure and abbreviated abstinence effects on mature oligodendroglial gene expression: For mature myelinating oligodendroglia, we measured myelin basic protein (MBP), proteolipid protein 1 (PLP-1), myelin-associated glycoprotein 1 (MAG-1), myelin oligodendrocyte glycoprotein 1 (MOG-1), reticulon 4 (RTN4), RPA interacting protein (RPAIN), and ST8 alpha-N-acetyl neuraminide alpha-2,8-sialyltransferase 1 (ST8Sia1). Two-way ANOVA tests demonstrated that the duration of ethanol exposure had significant inhibitory effects on MAG ($P=0.008$) and RTN4 ($P=0.02$) and a trend effect on RPAIN ($P=0.07$) expression. In addition, significant ethanol and time interactive effects were observed for PLP and MOG expression ($P=0.04$). Long term (8 weeks) ethanol exposure significantly reduced PLP expression ($P=0.003$) and had a trend decrease in MOG expression ($P=0.07$) relative to controls (Figures 1A and 1C). Also, it is noteworthy that the mean levels of MBP expression increased with age and decreased with ethanol exposure over the course of the experiment. However, the inter-group statistical differences failed to reach significance due to the large standard deviations (Figure 1E). Short-term abstinence failed to normalize ethanol mediated reductions in PLP and MOG expression and decreased RPAIN expression relative to control ($P=0.002$) and ethanol groups ($P=0.004$) (Figures 1A, 1C, and 1D). ST8Sia1 expression was similar in control and ethanol brains over time and remained unaltered with short-term abstinence (Figure 1G).

3.1.3. Short- versus long-term ethanol exposure and abbreviated abstinence effects on neuroglial gene expression: For neuroglial markers, we measured mRNA levels of neural cell adhesion molecule 1 (NCAM1), glutathione S-transferase P-1 (GSTP1), chondroitin sulfate proteoglycan 4 (CSPG4), glial fibrillary acidic protein (GFAP), and neurotrophic tyrosine kinase receptor, Type 2 (NTRK2). Ethanol exposure had significant stimulatory effects on NCAM1 expression at the 3-week time point ($P=0.04$), while mRNA expression remained unchanged in control, ethanol, and abstinence groups at the 8-week time point (Figure 2A). Furthermore, two-way ANOVA tests revealed significant ethanol $\times$ time interactive effects ($P=0.04$) and a trend effect for ethanol exposure ($P=0.06$). The mean levels of GSTP1 and NTRK2 decreased with ethanol exposure

Figure 1: Short- versus long-term ethanol exposure and abbreviated abstinence effects on mature oligodendroglial gene expression. RNA extracted from control, ethanol-exposed, and ethanol-abstinent temporal lobes were reverse transcribed, and cDNAs were used to measure oligodendroglial gene expression by qPCR analysis. Gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method and results were normalized to Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and Beta-actin ($\text{Act}\beta$). Graphs represent mRNA levels for (A) PLP, (B) MAG, (C) MOG, (D) RPAIN, (E) MBP, (F) RTN4, and (G) ST8Sia1. Inter-group comparisons were made by one way and two-way analysis of variance (ANOVA) with Tukey post hoc tests.

and time; however, the statistical analysis failed to reach significance due to large variations within the group (Figures 2B and 2C). Ethanol or abstinence had no significant or trend effects on CSPG4 and GFAP expression (Figures 2D and 2E).

3.1.4. Short- versus long-term ethanol exposure and abbreviated abstinence effects on transcription factor gene expression: Glial transcription factors include Forkhead Box O1 and O4 (FOXO1, FOXO4), oligodendrocyte transcription factor 1 and 2 (Olig1,

Figure 2: Short- versus long-term ethanol exposure and abbreviated abstinence effects on neuroglial gene expression. mRNA levels of (A) NCAM1, (B) GSTP1, (C) NTRK2, (D) CSPG4, and, (E) GFAP were measured in temporal lobes of control, ethanol-exposed, and ethanol-abstinent rats by qPCR analysis. Gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method and results were normalized to Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and Beta-actin (Act β). Inter-group comparisons were made by one-way and two-way analysis of variance (ANOVA) with Tukey post hoc tests.

Olig2), NK6 Homeobox 1 (NKX6-1), and Paired box 6 (PAX6). FOXO1 expression was increased trend-wise by ethanol exposures at the 3-week time point relative to control brains ($P=0.1$). However, expression reduced trend-wise over time in the ethanol group and remained at low levels after abstinence ($P=0.1$) (Figure 3A). Two-way ANOVA tests detected significant ethanol $\times$ time interactive effects for FOXO1 expression in the temporal lobe ($P=0.01$). FOXO4 was similarly expressed in control, ethanol, and abstinence groups and remained unaltered over time (Figure 3B). Significant stimulatory ethanol exposure ($P=0.02$) and duration effects ($P=0.05$) were observed for Olig2 expression. In contrast, short-term abstinence significantly decreased Olig2 mRNA levels relative to 8-week ethanol exposed brains ($P=0.01$) (Figure 3D). A similar expression pattern was observed for Olig1 in control and ethanol samples, but

the statistical analysis failed due to a large variation in the 8-week ethanol exposed group (Figure 3C). NKX6-1 and PAX6 expression remained unchanged after short- or long-term ethanol exposures or abbreviated abstinence (Figures 3E and 3F).

4. Discussion

Herein, we examined time-dependent effects of chronic + binge ethanol exposures and short-term abstinence on white matter myelin, neuroglial, and transcription factor gene expression in the temporal lobes of adult male Long Evans rats. The chronic ethanol exposures were initiated during late adolescence (6 weeks of age) and bingeing was performed when the rats were adults at either 8 to 9 weeks (short-term exposure) or 11 to 14 weeks (long-term exposure) of age. Ethanol abstinence began after 6 weeks of chronic and 2 weeks of binge ethanol exposures

Figure 3: Short- versus long-term ethanol exposure and abbreviated abstinence effects on transcription factor gene expression. mRNA levels of (A) FOXO-1, (B) FOXO-4, (C) Olig1, (D) Olig2, (E) NKX6-1, and (F) PAX6 were measured in temporal lobes of control, ethanol-exposed, and ethanol-abstinent rats by qPCR analysis. Gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method and results were normalized to Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and Beta-actin (Act β). Inter-group comparisons were made by one-way and two-way analysis of variance (ANOVA) with Tukey post hoc tests.

and lasted for 12 days. At the endpoints, the animals were either 9 (young adult) or 14 (adult) weeks of age. This design allowed us to study ethanol's detrimental effects on the brain in its late developmental and adult stages and whether these adverse effects could be reversed by short-term abstinence. Histopathological studies revealed that ethanol caused progressive time course-dependent reductions in white matter myelin resembling human alcohol related brain disease [3,28,38]. Short-term abstinence resulted in partial recovery of myelin suggesting similarities with alcohol abstinence effects in alcoholics [10,28,39].

In an earlier study, we demonstrated that long-term chronic + binge ethanol exposures alter ethanol metabolism in young adult rat brains by significantly increasing the activity of alcohol dehydrogenase

(ADH) and aldehyde dehydrogenase (ALDH) in favor of acetaldehyde build-up [40]. Furthermore, alcohol increased oxidative stress marked by increased cellular injury (keratin 18), protein oxidation (protein carbonyl), and lipid peroxidation (8-epimer of Prostaglandin F 2α) [41]. In contrast to studies using immature (young) rats, the present work showed that chronic + binge ethanol exposures and abbreviated abstinence in adult rats has only modest effects on the expression of immature oligodendrocyte genes. Nestin and GC were the two immature oligodendroglial genes inhibited by ethanol. Nestin is an intermediate filament protein that promotes the disassembly of phosphorylated vimentin during progenitor cell division [42,43] and is necessary for proper survival and self-renewal of neural progenitor cells [44]. GC encodes vitamin D binding protein which promotes proliferation and differentiation of neural stem cells

into OPCs and oligodendrocytes via vitamin D receptor signaling [42,45]. The inhibitory effects of alcohol on nestin and GC suggest that ethanol adversely affects oligodendrocyte survival, maturation and function.

Chronic + binge ethanol exposures had progressive inhibitory effects on the mature oligodendrocyte genes. MAG and RTN4 are potent inhibitors of neurite outgrowth and promote axonal regeneration and plasticity in the adult CNS [46,47]. Ethanol mediated reductions in MAG mRNA may reflect deficits in glia-axon interactions that are necessary for maintenance and structural integrity of myelinated axons [47]. PLP-1 is the major structural protein of CNS myelin responsible for myelin sheath compaction needed for close apposition of the cytoplasmic and extracellular surfaces of glial plasma membranes [48, 49]. Therefore, the inhibitory effects of alcohol on PLP could compromise the structural and functional integrity of myelin sheaths.

MOG, a minor protein component of myelin, is exclusively expressed on the surface myelin sheath and oligodendrocyte membranes [50]. Since MOG's expression coincides with the onset of myelination, it may represent a marker of oligodendrocyte differentiation and maturation [51]. Correspondingly, our finding that ethanol inhibits MOG may indicate that chronic + binge exposures delay oligodendroglial maturation, which could impact the integrity of myelin homeostasis.

RPA is a single stranded DNA-binding protein involved in DNA replication, repair, and recombination [52]. RPA interacting protein (RPAIN) facilitates import of RPA complex into nucleus and is highly expressed in mature myelinated axons in the CNS [53]. The reductions in RPAIN expression that occurred after a brief interval of abstinence suggest that withdrawal-associated cellular and molecular responses do not rapidly abrogate ethanol's adverse effects on the integrity of CNS myelinated axons and instead may exacerbate pre-existing injury, perhaps due to persistence of metabolic dysfunction.

With respect to neuroglial genes, since NCAM1 mediates neuronal adhesion and neurite outgrowth [34], the elevated expression detected after short-term ethanol exposure likely reflect increased neuritic sprouting responses. However, those effects could be positive and associated with regeneration and repair, or negative and related to oxidative stress. We favor the latter in light of ethanol's simultaneous inhibitory effects on NTRK2 and GSTP1 which respectively function as positive regulator of synapse formation and plasticity [35], and inhibitor of Cdk5 signaling through p25/p35, a driver of neurodegeneration [54].

Transcription factor responses to chronic + binge ethanol exposures varied with duration of exposure and withdrawal, and in ways that could have impaired metabolic, maturation, or homeostatic functions of

oligodendrocytes. Ethanol had a stimulatory trend effect on FOXO1 after 3 weeks of exposure, but an inhibitory trend effect after 8 weeks of exposure. FOXO-1 transcription factor modulates expression of insulin and insulin-like growth factor regulated target genes involved in glucose and lipid metabolism and favoring states of insulin resistance [36,37,55]. Therefore, with longer durations of ethanol exposure, FOXO1 inhibition may represent a compensatory protective response. The finding that ethanol stimulated Olig2 expression supports the concept that oligodendroglial progenitor cells can be activated in the adult brain [56], perhaps in response to oligodendrocyte death. However, taken together with the impairments in the expression of mature oligodendroglial proteins discussed earlier, the up-regulation of Olig2 would have served to expand a population of immature oligodendroglia that lacked capacity to generate mature compact myelin. The short-term abstinence inhibited Olig1 and Olig2 expression. The mechanism by which abstinence inhibited Olig1 and Olig2 expression is uncertain, but it may correspond to one of the "switches" that halts proliferation and enables maturation of oligodendroglial cells.

5. Conclusions

This study demonstrated that short- and long-term chronic + binge ethanol exposures alter expression of myelin-associated genes and transcription factors such that the aggregate effects are to inhibit maturation and related functions of oligodendrocytes while enabling proliferation of immature oligodendroglia. The net effect would have been to compromise the structural and functional integrity of myelin and myelinated axons in the adult brain. The generally larger effect size associated with longer durations of ethanol exposure reinforce the concept that alcohol-related white matter injury/degeneration is progressive. The findings with respect to the effects of short-term abstinence were mixed, but at least in several instances, they were consistent with partial recovery responses. Future studies will evaluate recovery responses following longer periods of ethanol abstinence.

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