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Modulation of Poly ADP Ribose Polymerase (PARP) Levels and Activity by Alcohol Binge-Like Drinking in Male Mice

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Abstract

Binge drinking is a frequent pattern of ethanol consumption within Alcohol Use Disorders. Binge-like ethanol exposure increases Poly(ADP-ribose) polymerase (PARP) expression and activity. PARP enzymes have been implicated in addiction and serve multiple roles in the cell, including gene expression regulation. In this study, we examined the effects of binge-like alcohol consumption in the prefrontal cortex (PFC) of adult C57BL/6J male mice *via* a 4-day drinking-in-the-dark (DID) paradigm. The role of PARP in associated gene expression and behavioral changes was assessed by administering the PARP inhibitor ABT-888 on the last DID day. We then conducted an RNA-seq analysis of the PFC gene expression changes associated with DID-consumed ethanol or ABT-888 treatment. A separate cohort of mice was inoculated with an HSV-PARP1 vector in the PFC and subject to a DID experiment to verify whether overexpressed PARP1 increased ethanol drinking. We confirmed that alcohol increases *Parp1* gene expression and PARP activity in the PFC. RNA-seq showed significantly altered expression of 41 genes by DID-consumed ethanol, and of 48 genes by ABT-888. These results were confirmed by qPCR in 7 of the 10 genes validated, 4 of which have been previously associated with addiction. ABT-888 reduced, and overexpression of PFC PARP1 increased DID ethanol consumption. In our model, alcohol binge drinking induced specific alterations in the PFC expression of genes potentially involved in addiction. Pharmacological PARP inhibition proved effective in reversing these

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changes and preventing further alcohol consumption. Our results suggest an involvement of ethanol-induced PARP1 in reinforcing binge-like addictive behavior.

Keywords

Addiction; Alcohol Use Disorder; PARP; Epigenetics; RNA-seq

Introduction

Alcohol Use Disorder (AUD) is among the most common mental health disorders in the US, with a 29.1% lifetime prevalence (Grant et al., 2015). Binge alcohol drinking is defined as the acquisition of a blood ethanol concentration (BEC) ≥ 80 mg/dL, generally reached after 5 drinks in men and 4 drinks in women within 2 hours (“NIAAA Council Approves Definition of Binge Drinking.” *NIAAA Newsletter*, Department of Health and Human Services – National Institutes of Health, Winter 2004, 3: 3). Binge alcohol drinking contributes to more than half of the deaths associated with excessive alcohol consumption, and can lead to impairments in psychosocial functioning as well as adverse health consequences, including unintentional injuries, alcohol poisoning, gastritis, etc. (Naimi et al., 2003; Stahre et al., 2014). Accumulated data from diverse studies, including human and animal behavioral studies, brain imaging, electrophysiology, and molecular and cellular observations strongly support that the prefrontal cortex (PFC) plays a critical role in addiction (Grusser et al., 2004; Heilig et al., 2017; Wolstenholme et al., 2017). However, the molecular mechanisms underlying binge alcohol drinking as well as its biochemical effects are not fully understood.

Prior studies have focused on the role of PARP activity in cell death pathways following toxic levels of ethanol exposure (Climent et al., 2002; Cherian et al., 2008). The poly (ADP-ribose) polymerase (PARP) family is composed of 18 members. Murai *et al* (2012) reported that PARP1 is responsible for 90–95% of PARP enzymatic activity while PARP2 contributes the remaining 5–10% (Murai et al., 2012). In mouse primary cortical neuronal cultures, we found that ethanol exposure increased PARP enzymatic activity and led to gene expression changes, and that PARP inhibitors rescued some of the ethanol-induced gene expression changes (Gavin et al., 2016), indicating that PARP enzymes may act as regulators in the biochemical response to ethanol exposure (Gavin et al., 2016).

The accepted addiction cycle model presumes that alcohol drinking induces neuroadaptive changes in the brain that lead to continued alcohol seeking, therefore perpetuating the drinking cycle (Koob, 2003). We hypothesized that ethanol-induced upregulation of PARP expression and activity may lead to changes in the brain involved in increasing the alcohol drinking behavior in an animal model of binge drinking.

In support of the role of PARP enzymes in addiction, cocaine was shown to significantly increase PARP1 expression and enzymatic activity in the *nucleus accumbens*, and overexpression of PARP1 increased, and PARP inhibition decreased conditioned place preference for cocaine (Scobie et al., 2014). In this study, we show evidence that alcohol drinking increases PARP gene expression and its enzymatic activity in the PFC promoting

further alcohol consumption. PARP1 overexpression also increases voluntary alcohol intake; this effect is associated with increased expression of genes involved in binge alcohol drinking. These observed biomolecular effects of alcohol are reduced by PARP inhibition, and PARP inhibition reduces alcohol drinking.

Experimental Procedures

Drinking-in-the-Dark protocol for voluntary ethanol consumption

All animal studies were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Drinking-in-the-Dark (DID) was performed as previously published (Thiele and Navarro, 2014). Adult 12–16 week-old C57BL/6J male mice purchased from Jackson Laboratories were singly housed and allowed to acclimate to a reverse light-dark cycle (lights on: 20:00 to 8:00) for 2 weeks with *ad libitum* access to food and water. The animals were then randomly assigned to each experimental group. Following acclimation, three hours after the beginning of the dark cycle (11:00), the water bottle was replaced with a graduated bottle containing 20% ethanol in tap water for 2 hours (removed at 13:00) during the first 3 days and for 4 hours (removed at 15:00) on the 4th day. This same procedure was performed on separate cohorts of mice to assess water and saccharin drinking behavior, by replacing the water bottle with a graduated bottle containing water or 0.2% saccharin, respectively. Ethanol, water, or saccharin consumption was determined based on change in bottle volume after each session. On the fourth day, PARP inhibitor ABT-888 25 mg/kg (Selleck Chemicals#S1004) or vehicle (99.4% normal saline and 0.6% DMSO) (Veh), was administered *via* intraperitoneal injection (*i.p.*) immediately before introduction of ethanol, water, or saccharin (Fig. 1A). The time point of ABT-888 administration was chosen because the half-life of ABT-888 is 1.2–2.7 hours, with a T_{max} of 20 minutes, thereby providing the longest possible duration of ABT-888 exposure during the experiment (Donawho et al., 2007). We selected the dose of ABT-888 based on its ability to effectively eliminate PARylation (the addition of Poly-ADP Ribose groups) (Donawho et al., 2007) and its lack of off target effects (Antolin and Mestres, 2014; Jelinic and Levine, 2014). There were four treatment groups in experiments in which mice voluntarily consumed ethanol: animals that received water in the DID paradigm and were injected with vehicle (Control+Veh); animals that received water in the DID paradigm and were injected with ABT-888 (Control+ABT); animals that received ethanol in the DID paradigm and were injected with vehicle (EtOH+Veh); and animals that received ethanol in the DID paradigm and were injected with ABT-888 (EtOH+ABT). Two groups in the saccharin consumption experiment: animals that received 0.2% saccharin in the DID paradigm and were injected with vehicle (Sacch+Veh); animals that received 0.2% saccharin in the DID paradigm and were injected with ABT-888 (Sacch+ABT). Immediately following the final drinking period, mice were subject to rapid CO₂ exposure to induce unconsciousness and decapitated. Blood and brain tissue samples were obtained after decapitation: blood was quickly collected from the trunk and centrifuged at 2,500 RPM for 15 minutes to separate the serum, which was kept at –80 °C until analyzed; brain was dissected on a brain block to isolate the PFC from the two hemispheres, which were then combined, homogenized, snap frozen, and kept at –80 °C for further use. The combined

PFC homogenates were aliquoted before use in different biochemical analyses (i.e. RNA vs. protein analysis).

Involuntary ethanol administration

In order to determine whether ABT affected ethanol metabolism, we subjected a separate cohort of mice to a fixed dose regimen of EtOH (Walter et al, 2017). Adult male C57BL/6J mice were group housed on a standard 12 light/12 dark cycle and allowed *ad libitum* access to food and water. Mice were administered ethanol 2 g/kg *i.p.* twice a day, two hours apart, for four days (EtOH), with and without ABT-888 (25 mg/kg/d) (ABT) only on the fourth day. Animals were sacrificed two hours after the final dose on the fourth day and blood was collected to perform BEC analyses.

Blood Ethanol Concentration (BEC)

An enzymatic assay using a standard curve of ethanol concentration was utilized to determine BECs (Jung and Ferard, 1978). Briefly, ethanol standard samples were prepared by mixing absolute ethanol and ultrapure water to yield a 1000 mg/dL ethanol stock solution. This solution was serially diluted to yield a set of eight standard samples in the range of 7.8–1000 mg/dL ethanol. Forty microliters of 3.75% perchloric acid was added to 10 μ L of samples (serum) and standards and centrifuged for 6 minutes at 2,000 RPM. The supernatant was retained. β -Nicotinamide adenine dinucleotide lithium salt from *Saccharomyces cerevisiae* (Sigma #N7132) was added at a final concentration of 2.5 mM, and Alcohol Dehydrogenase from *Saccharomyces cerevisiae* (Sigma #A7011) was added at a final concentration of 5 μ M to samples and standards, and incubated at 35 °C for 40 minutes. Samples and standards were read on a plate reader at 340 nm. In a representative DID experiment, BEC for the EtOH+Veh group was 90 ± 20.5 mg/dL, while the BEC for the EtOH+ABT group was 37 ± 21.9 mg/dL.

PARP1 Overexpression

Herpes Simplex Virus (HSV)-GFP (Control) and HSV-PARP1, the design of which is described in Scobie *et al.* (2014), were purchased from the Viral Gene Transfer Core of the McGovern Institute for Brain Research at MIT (Scobie et al., 2014). After the 2-week acclimation period, mice were anesthetized using ketamine 100 mg/kg and xylazine 10 mg/kg and placed in a stereotaxic equipment (Hamilton et al, 2018). Thirty-four gauge needles and Hamilton syringes were used to bilaterally infuse virus (3.33×10^5 i.u. per hemisphere, at the flow rate of 0.1 μ L/min) into the prelimbic area of the PFC (anterior/posterior: +1.9 mm; medial/lateral: ± 0.5 mm; dorsal/ventral: -2.5 mm from Bregma. These were the coordinates that best allowed us to consistently infuse the majority of virus suspension into the prelimbic area of the PFC). Animals were allowed 24 hours to recover from surgery before DID paradigm was initiated (Fig. 1B).

Western Blot

In order to verify over-expression of viral HSV-PARP1 protein, we performed Western Blot analysis. PFC proteins were extracted using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo # 78833) containing protease inhibitors. Twenty micrograms PFC

proteins were subjected to SDS–polyacrylamide gel electrophoresis followed by transfer to PVDF membranes. PVDF membranes were blocked in 3% BSA for 1 hour and then incubated with rabbit anti-PARP1 antibody in TBST (Abcam #32138) at a 1:1,000 dilution overnight. Anti-rabbit secondary antibody was then used at a 1:20,000 dilution in TBST. B-Actin was detected using mouse anti- β -actin antibody (Sigma-Aldrich #A5316) at a 1:2000 dilution in 1% nonfat dry milk overnight followed by anti-mouse secondary antibody (Cell Signaling Technology anti-mouse IgG, HRP-linked Antibody #7076) at a 1:5,000 dilution in 1% nonfat dry milk and used for normalization. Clarity Western ECL Substrate was detected using an Odyssey® Fc Imaging System and quantified using ImageJ software.

PARP Activity

PARP activity was measured following the DID paradigm in the PFC homogenates obtained as described above, using the Trevigen PARP Universal Colorimetric Assay Kit (#4677-096-K) according to the manufacturer's instructions and our prior publication (Gavin et al., 2016). Briefly, PFC protein extracts were prepared by sonicating thawed PFC homogenates on high power (0.5 s on/off) in EBC lysis buffer with protease inhibitors for 5 minutes. Twenty micrograms of protein per well were used by diluting samples in a mix of 30% EBC lysis buffer and 70% 1X PARP Buffer. Values represent OD reading of samples minus blank (30% EBC lysis buffer and 70% 1X PARP buffer) and expressed as fold over the DID Control+Veh condition after normalization to a standard curve created using recombinant PARP.

RNA-Seq Sample Preparation and Analysis

Control+Veh, EtOH+Veh, and Control+ABT groups were used in the RNA-seq experiments (n=4 per group). The miRNeasy Mini Kit (Qiagen # 217004) was used to extract RNA from the PFC samples collected as described above and the so-obtained extracts were subjected to RNA-seq analysis. Next-generation mRNA sequencing was performed using QuantSeq3mRNA-Seq library preparation kit (Lexogen #015) according to the manufacturer's instructions. Single-end 75 bp sequencing was performed on a high throughput Illumina NextSeq 500. Raw sequence reads were mapped to the mouse genome (mm 10) and gene annotation file Mus_musculus GRCm38 using TopHat (Galaxy Version 2.1.0) (Trapnell et al., 2009). Transcript abundance (FPKM: fragments per kilobase per million mapped reads) was quantified using Cufflinks (Trapnell et al., 2012). We conducted pairwise comparisons between either Control+Veh vs. Control+ABT samples or Control +Veh vs. EtOH+Veh samples, and calculated statistical values using Cuffdiff. Q-Values (i.e. p-values adjusted for False Discovery Rate) less than 0.05 were considered as statistically significant. In addition, these two gene lists were subjected to an overrepresentation analysis for biological pathways using Enrichr for KEGG, and DAVID for gene ontology (Chen et al., 2013). Enrichr is an integrative web-based program for the knowledge-based enrichment of genome-wide sequencing data, and incorporates terms from an increasing list of gene sets libraries. We applied the Enrichr algorithm against the KEGG gene set library. DAVID is a data-mining platform that combines functionally descriptive data with intuitive graphical displays. Gene expression data will be deposited to the Gene Expression Omnibus (GEO) upon publication of this manuscript.

RNA extraction and real-time qRT-PCR

PFC tissue obtained as described above was homogenized in TRIzol reagent to extract RNA using the published protocol (Invitrogen #15596026). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems #4268814). cDNA quantification was accomplished using a PikoReal Real-Time PCR and Maxima® SYBR Green/ROX qPCR Master Mix (Thermo #K0221). Primers were designed to span at least one intron–exon boundary to minimize DNA contamination. Expression of target genes was normalized to *Hprt1* (see Table 1 for primer sequences).

Mouse Primary Neuron Culture

We performed mouse primary cortical neuron culture as described in our prior publications (Gavin et al., 2016; Gavin et al., 2015), in order to reliably and directly validate PARP involvement in expression changes of target genes elicited from the RNA-seq outputs. Briefly, plates were coated overnight with PDL 0.1 mg/mL (Sigma #P1149). Fetal cortices from E18 C57BL/6 mice (Charles River) were dissected and meninges removed using Whatman® qualitative filter papers (Grade 3; VWR #28456-065). Neurons were initially dissociated using enzymatic methods, papain (Sigma #P4762) and Dnase (Sigma #D5025), then mechanically dissociated. Neurons were cultured in serum-free medium comprised of: neurobasal media (Gibco #21103049), fungizone (Gibco #15290018), gentamicin (Corning #30-005-CR), D+glucose (Sigma #G8769), B27 supplement (Thermo #17504044), glutamax (Gibco #35050061). On the third day of culture, neurons were treated with 2.5 μ M cytosine β -D-arabinofuranoside (Sigma #C1768) to remove dividing cells, followed by a 100% media change 24 hours later. Cells were transduced on the seventh day in culture with HSV-PARP1 or HSV-GFP and harvested after six hours.

Statistics

Data were analyzed with Student's t-test, one-way analysis of variance (ANOVA), or repeated measures ANOVA with post-hoc Bonferroni or Tukey tests as appropriate. All statistical analyses were performed using GraphPad Prism version 7 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) or SPSS (IBM Corp. released 2016, IBM SPSS Statistics for Windows, version 24.0, Armonk, NY).

Results

Effects of ethanol on *Parp1* gene expression and PARP activity.

We have previously reported that ethanol increases PARP activity in cultured cortical neurons (Gavin et al., 2016). In the present study, we determined the *in vivo* effects of voluntary alcohol intake on the PFC of male C57BL/6J mice on *Parp1* expression. *Parp1* mRNA levels measured *via* qRT-PCR were significantly increased by DID ethanol drinking (Fig. 2A; $t_{13}=4.652$, $p=0.0005$, $n=7-8$ per group). We then tested the effect of ethanol drinking in the DID paradigm as well as the effects of an inhibitor of PARP activity ABT-888, alone or in combination, on PARP activity. A two-way ANOVA analysis showed a significant ethanol drinking $\times$ treatment (ABT-888) interaction ($F_{1,16}=8.71$, $p=0.009$, $n=4-6$ per group); Tukey post-hoc revealed that PARP activity was higher in the EtOH+Veh group

compared to the Control+Veh group, $p<0.05$, and to the EtOH+ABT group, $p=0.02$. (Fig. 2B). ABT-888 was not found to affect locomotor activity (Supplementary Fig. S1).

Pharmacological inhibition of PARP activity reduces voluntary ethanol consumption

In order to test the hypothesis of a possible role of PARP in maintaining drinking behavior, mice were exposed to separate iterations of the DID paradigm described above using different cohorts: one cohort of mice underwent DID with bottles containing ethanol, a separate cohort underwent DID with bottles containing water, and a third cohort underwent DID with bottles containing 0.2% saccharin; on the fourth day, before the beginning of the last drinking session (please note this is 4-hour long, as opposed to the 2-hour long drinking sessions of the first three days), animals in each cohort were split in two groups and treated with ABT-888 or vehicle. ABT-888 did not affect water (Fig. 3A, two-way repeated measures ANOVA $F_{1,84}=3.14$, $p=0.080$, $n=11-12$ per group) or saccharin (Fig. 3B, two-way repeated measures ANOVA Treatment: $F_{1,32}=0.08$, $p=0.77$, $n=5-6$ per group) consumption. However, animals injected with ABT-888 drank significantly less ethanol than animals injected with the vehicle (Fig. 3C, two-way repeated measures ANOVA, $F_{1,84}=8.23$, $p=0.005$; Bonferroni post-hoc $p<0.01$, $n=11-12$ per group). To determine if the differences in ethanol drinking behavior could be accounted for by an effect of ABT on ethanol metabolism we measured the BEC of mice administered a fixed dose of ethanol *i.p.* with and without ABT (involuntary ethanol administration). There was no significant difference in BEC between mice administered the same dose of ethanol together with vehicle vs. ethanol with ABT (Mean $\pm$ SEM: 285 $\pm$ 37.6 mg/dL vs. 222 $\pm$ 50.5 mg/dL; $t_8=1.01$, $p=0.34$, $n=5$ per group).

Overexpression of PARP1 in the PFC is sufficient to increase voluntary ethanol consumption

In order to more directly assess the role of PARP1 overexpression in the PFC on ethanol consumption, we injected HSV-PARP1 or HSV-GFP (control) virus into the PFC. Gene overexpression by HSV vectors occurs 12 hours after transduction, peaks at 3–5 days post-injection, and dissipates 10 days post-surgery (Penrod et al., 2015). We conducted the experiment at the peak of HSV transduction as we subjected mice to the DID protocol 24 hours after stereotaxic injection of the virus and we carried out the DID experiment during the following 4 days. Overexpression of PARP1 mRNA and protein expression was validated by real time RT-PCR and immunoblot, respectively. Fig. 4A shows that *Parp1* mRNA was significantly upregulated in the PFC of HSV-PARP1-transduced mice compared to HSV-GFP-transduced mice (unpaired t-test, $t_{10}=3.727$, $p=0.0039$, $n=6$ per group); we also tested *Parp2* mRNA expression under the same conditions to test whether increased *Parp1* expression would lead to a compensatory down-regulation of *Parp2*. We found that *Parp2* mRNA expression was not affected by *Parp1* overexpression. We also verified that PARP1 protein was indeed overexpressed by Western blot (unpaired t-test, $t_5=2.849$, $p=0.0359$, $n=3-4$ per group) (Fig. 4B, C). Finally, we validated PFC transduction of the virus by GFP immunohistochemistry (Supplementary Fig. S2). Overexpression of PFC PARP1 significantly increased cumulative ethanol consumption across the four-day paradigm by mixed model ANOVA (HSV-GFP vs. HSV-PARP1; $F_{3,39}=24.2$, $p=0.0005$, $n=8-9$ per group) (Fig. 4D). Pairwise comparisons on individual days using independent Student's t-test

showed significant increase in alcohol consumption on days 2 ($p<0.02$) and 3 ($p<0.051$), but not 4. The lack of an effect on day 4 drinking may be due to the fact that overexpression mediated by HSV peaks at days 2 and 3 (i.e. 3–4 days after inoculation, which occurred on day 0) and decreases thereafter (Penrod et al., 2015). Alternatively, the lack of increase in ethanol drinking on day 4 may be due the fact that, while on days 2–3 mice drink alcohol for 2 h, on day 4 they drink for 4 h; this longer drinking time might allow the HSV-GFP group to catch up on the alcohol intake. Water consumption was not affected by PARP1 overexpression (repeated measures ANOVA, $F_{1,38}=0.94$, $p=0.337$, $n=5-6$ per group) (Fig. 4E). These results strongly support a role of PARP1 overexpression in the PFC in binge-like alcohol consumption in mice.

Effects of ethanol drinking and PARP inhibition on gene expression by RNA-seq analysis and validation by qPCR

Based on the findings that ethanol treatment increased *Parp1* mRNA levels and PARP enzymatic activity while PARP inhibition reduced ethanol drinking, we endeavored to identify genes whose expression was regulated by ethanol drinking and/or PARP inhibition as a first step towards the identification of possible targets for treatment development. We carried out RNA-seq analyses comparing Control+Veh to EtOH+Veh samples and Control+Veh to Control+ABT samples. We found that EtOH significantly altered the expression of 41 genes; ABT significantly altered the expression of 48 genes; 9 genes were altered by both EtOH and ABT, including *Ccl2*, *Nr4a1*, *Mmp14*, *Elfn2*, *Pbx3*, *Gpr88*, *Lrrc10b*, *Penk*, *Drd1*. The 89 genes regulated by ethanol drinking and/or PARP inhibition are listed in Supplementary Table S1.

In order to understand the higher order functional significance of the differentially expressed genes, KEGG pathway and gene ontology (GO) analyses were performed. Using Enrichr, the top KEGG pathways identified for DID EtOH were “Morphine addiction,” “Chemokine signaling pathway,” “TNF signaling pathway,” and “Alcoholism,” although only “Morphine addiction” reached statistical significance. The top KEGG pathways impacted by ABT treatment were “cAMP signaling pathway,” “Dopaminergic synapse,” “Amphetamine addiction,” and “Alcoholism” (Table 2). Using DAVID, the top GO categories for EtOH were “Extracellular space,” “Locomotor behavior,” “Neuronal cell body,” and “Axon,” while the top GO categories for ABT were “Neuronal cell body,” “Perinuclear region of cytoplasm,” and “Axon terminus” (Table 3). These results strongly indicate that alcohol-induced PARP activity is associated with the expression of neuronal and addictive-behavior related genes *in vivo*.

The majority of the genes regulated by ethanol drinking showed increased expression (34 out of 41); conversely, the majority of the genes regulated by PARP inhibition (ABT) were down-regulated (30 out of 48). In total, 60 genes out of 89 were either up-regulated by ethanol or down-regulated by ABT, including four genes that were both upregulated by ethanol drinking and down-regulated by PARP inhibition (*Gpr88*, *Lrrc10b*, *Penk*, and *Drd1*) and for this reason were selected for validation. In addition, we selected for validation five genes upregulated by ethanol (*Adora2a*, *Rgs9*, *Syndig11*, *Tac1*, and *Pde10a*) and one

downregulated by ABT (*Spock3*). In total we selected for validation 10 genes in a new cohort of animals exposed to ethanol drinking (Fig. 5A) or injected with ABT (Fig. 5B).

As shown in Fig. 5A, we confirmed that EtOH+Veh increased mRNA expression of *Lrrc10b* ($t_{16}=2.87$, $p=0.011$, $n=8-10$ per group), *Penk* ($t_{17}=2.58$, $p=0.019$, $n=9-10$ per group), *Drd1* ($t_{17}=3.36$, $p=0.004$, $n=9-10$ per group), *Adora2a* ($t_{13}=2.58$, $p=0.019$, $n=7-8$ per group), *Rgs9* ($t_{14}=2.70$, $p=0.017$, $n=8$ per group), and *Tac1* ($t_{15}=3.21$, $p=0.006$, $n=8-9$ per group), but not of *Gpr88*, *Pde10a*, *Spock3*, or *Syndig11*.

As shown in Fig. 5B, we confirmed that PARP inhibition (Control+ABT) decreased *Gpr88* ($t_{11}=2.55$, $p=0.027$, $n=5-8$ per group), *Lrrc10b* ($t_{12}=2.59$, $p=0.024$, $n=6-8$ per group), *Penk* ($t_{15}=3.88$, $p=0.002$, $n=8-9$ per group), *Spock3* genes ($t_{13}=2.97$, $p=0.011$, $n=7-8$ per group), but not *Drd1*, *Tac1*, *Pde10a*, or *Syndig11*. We also found that Control+ABT reduced the expression of *Adora2a* ($t_{12}=2.40$, $p=0.034$, $n=7$ per group) and *Rgs9* ($t_{13}=3.53$, $p=0.004$, $n=7-8$ per group).

In summary, our validation studies confirmed RNA-seq results for seven out of 10 genes under both experimental conditions, EtOH+Veh and Control+ABT. Additionally, the qPCR validations experiments identified four genes that are upregulated by EtOH+Veh and down-regulated by Control+ABT, namely *Adora2a*, *Rgs9*, *Lrrc10b*, and *Penk* (Fig. 5). However, qRT-PCR validation experiments did not confirm the RNA-seq results for *Gpr88* and *Drd1*: *Gpr88* was indeed downregulated by ABT, but was not upregulated by EtOH, while the opposite is true for *Drd1*. Consequently, unlike for other validated genes, *Gpr88* and *Drd1* do not show a “reversion” effect.

PARP1 overexpression induces *Adora2a*, *Rgs9*, and *Penk* gene expression changes in primary cultures of cortical neurons

In order to test whether PARP1 overexpression is causally linked to increased expression of *Adora2a*, *Rgs9*, and *Penk* (*Lrrc10b* was not sufficiently expressed in these cells to be measured reliably), we infected E18 primary cortical neuron cultures with the HSV-PARP1 or the HSV-GFP (control) viral vectors. As shown in Fig. 6A, neurons infected with HSV-PARP1 expressed higher *Parp1* mRNA levels than neurons infected with HSV-GFP. Furthermore, the mRNA levels of *Adora2a* ($t_{10}=3.640$, $p=0.005$; $n=6$ wells per group), *Rgs9* ($t_{10}=3.935$, $p=0.003$; $n=6$ wells per group), and *Penk* ($t_{10}=3.413$, $p=0.007$; $n=6$ wells per group) were significantly higher in neurons infected with HSV-PARP1 compared to control HSV-GFP infected neurons (Fig. 6).

Discussion

In the present study, we showed evidence that *Parp1* mRNA is upregulated by alcohol drinking, and that the inhibition of PARP activity decreases alcohol drinking, while overexpressing PARP1 in the PFC increases alcohol consumption. We showed that *Parp1* was induced in the PFC after exposure to alcohol in the DID experiment, DID ethanol consumption was reduced using the PARP inhibitor ABT-888 and increased by viral PARP1 overexpression. Taken together, these results strongly suggest that ethanol-induced PARP activity and expression is implicated in ethanol drinking behavior.

It is well known that ethanol stimulates PARP enzymatic activity and PARP1 expression (Gavin et al., 2016; Tajuddin, N. F. et al., 2013; Cherian et al., 2008; Tajuddin, N. et al., 2014; Jagtap and Szabo, 2005; Menk et al., 2010; von Haefen et al., 2011; Climent et al., 2002; Collins et al., 2014; Ieraci and Herrera, 2018). However, PARP's effects on molecular biological pathways in response to ethanol remain to be fully elucidated. High ethanol doses also can cause cell death *via* the PARP-specific death pathway "*parthanatos*," though this finding is inconsistent across studies (Tajuddin, N. et al., 2018; Tajuddin, N. et al., 2014; Ieraci and Herrera, 2018). Most prior studies investigated the association between ethanol-induced increases in PARP enzymatic activity and gene expression in the context of inflammation. Rats administered moderate or high doses of ethanol had increased PARP gene expression and enzymatic activity in hippocampal-entorhinal slices, which were associated with increased expression of phospholipases associated with neuroinflammation, and increased expression of the neuro-immune agonist high mobility group box 1 (HMGB1) (Tajuddin, N. et al., 2018). The pro-inflammatory effects of PARP in the CNS are consistent with studies in peripheral tissues in which it was shown that PARP1 knockout mice are resistant to LPS-induced endotoxic shock and have lower tumor necrosis factor (TNF)- α and nitric oxide production (Mandir et al., 2000; Conde et al., 2001; Carrillo et al., 2004; Jog et al., 2009). The association between PARP and the innate immune system may partly explain the reduction in ethanol consumption following PARP inhibition treatment reported here, as several studies indicate that suppressing the immune response to ethanol decreases voluntary consumption (Warden et al., 2018b; Warden et al., 2018a; Ronan et al., 2018; Blednov et al., 2017; Truitt et al., 2016). However, we did not observe modulation of the *Hmgbl* gene by either ethanol drinking or PARP inhibition in our RNA-seq study. The TNF signaling pathway was an enriched KEGG pathway in our RNA-seq analysis. The three genes in this pathway found to be altered in our RNA-seq analysis were *Mmp14*, *Ccl2*, and *Fos*. *Mmp14*, a matrix metalloproteinase that digests the extracellular matrix, was increased by both EtOH and ABT treatment, *Ccl2* is a chemokine that we found reduced by both alcohol and PARP inhibition, and *Fos* is a transcription factor that was reduced by PARP inhibitor treatment.

RNA-seq and qPCR validation after DID and ABT-888 *in vivo* experiments and PARP1 overexpression *in vitro* experiments strongly suggest a role of PARP1 in the regulation of *Lrrc10b*, *Rgs9*, *Adora2a*, and *Penk*. LRRC proteins play critical roles in organizing neural connectivity, axon guidance, synapse formation and interactions with cytoskeletal proteins (Ng and Xavier, 2011; de Wit et al., 2011). However, little is known about the function of *Lrrc10b*. Neurons have elaborate cytoskeletal structures, and alterations in these genes are often seen in response to changes in neuronal spine density, which is profoundly affected by drugs of abuse (Spiga et al., 2014). All drugs of abuse, including alcohol, either directly or indirectly modulate adenosine signaling in neurons through *Adora2a*'s direct interactions with dopamine, glutamate, and cannabinoid receptors (Hack and Christie, 2003; Brown and Short, 2008). As such, *Adora2a* receptor antagonists have been proposed as treatments for drug addiction (Ferre et al., 2007), indicating the need for further research into these treatments in response to alcohol abuse. *Penk* and *Rgs9* are two opioid-related genes impacted by both ethanol and PARP inhibition. *Penk* encodes for proenkephalin, the precursor of enkephalin peptides, which are ligands for δ and μ opioid receptors (Al-Hasani and Bruchas, 2011) and *Rgs9* negatively regulates G-protein signaling, reducing the

downstream signaling of opioid receptors (Traynor, 2012). There is a great deal of evidence implicating opioid receptors in alcoholism, with opioid antagonists, such as naltrexone, being an effective treatment for AUD (Sawicka and Tracy, 2017). Secondly, changes in *Penk* expression are seen in both alcoholic patients and rodent alcohol models (Sarkisyan et al., 2015; Alaux-Cantin et al., 2013; de Gortari et al., 2000; Zhang et al., 2013). In the striatum, *Rgs9* is upregulated in response to cocaine administration (Rahman et al., 2003; Thomas et al., 1998). *Rgs9* knockout attenuates opioid seeking behavior of mice (Gaspari et al., 2017), and blocking the *Adora2a* receptor has been shown to attenuate goal-directed learning and methamphetamine-induced behaviors (Li et al., 2018; Wright et al., 2016). Therefore, PARP inhibitor treatment may be able to decrease alcohol drinking behavior *via* modulating downstream signaling of the opioid pathway by decreasing *Rgs9* expression or adenosine signaling and goal-directed learning by decreasing *Adora2a* expression.

Some limitations of the present study need to be pointed out. First, the results were obtained only in a limited sub-population of animals, namely C57BL/6J male mice. Although the choice of studying alcohol consumption behavior in a high-drinking mouse genotype is backed by previous literature, additional work needs to be done to extrapolate our findings to a wider and more diverse animal population, that would include female subjects as well as different mouse strains with different intrinsic drinking behaviors. The experiments presented in this study were carried out only in male mice for the following scientific reasons: (I) recent evidence suggests PARP-1 inhibition is a sex-specific effect, as showed by the more pronounced neuroprotective effects of post-ischemia PARP inhibition male mice (Chen et al 2020), and other sex-specific organ effects for PARP have been observed (Calvo et al 2016, Quillinan et al 2014); (II) men appear to have higher PARP1 activity, and in rodents these sex-specific effects are evident in the liver, the site of ethanol metabolism (Zaremba et al 2011); (III) there are sex differences in the escalation of alcohol consumption during the DID paradigm (Sneddon et al 2019). All together these observations strongly suggest that the relationship between alcohol drinking and PARP needs to be investigated in females using approaches different from the ones used in males and will therefore be explored in a future study tailored specifically to female mouse behavior.

A second limitation the authors acknowledge for this study is the strict focus on the PFC as the sole brain region object of our investigation. Our goal was to expand our previous findings in cultured cortical neurons obtained from the same strain of mice used in the present study (Gavin et al., 2016) and to test an entirely novel pathway that ultimately leads to the modification of neuronal DNA and assembly of chromatin, including epigenetic enzymatic activity (such as PARP enzymes), expression levels, and targeted downstream outcomes. Although generalizability to other regions is an important objective, a priority at the outset was “replicability”, best performed in an anatomically and biochemically parsimonious model, where reliable genetic tools are readily available. Furthermore, the use of PFC outputs from RNA-seq allowed us to directly validate the effect of PARP on mice cortical neurons in primary neuron cultures experiments. The mPFC plays an essential role in addiction by exerting executive control over approach/avoidant behavior in relation to environmental stimuli. It has been implicated in binge alcohol drinking in clinical (Kvamme et al., 2015) and rodent studies (Linsenhardt & Lapish, 2015). Human imaging studies indicate alcoholics have heightened PFC responses to cues associated with alcohol (Grusser

et al., 2004). Glutamate released from prelimbic mPFC neurons into the NAc core promotes drug seeking behavior (Peters, et al., 2009). The importance of PFC neuronal activity in AUD is also indicated by the fact that the mechanism of action of many AUD medications is through their effects on neurotransmission in the PFC (Gass & Olive, 2008). All this evidence suggests that the PFC is clearly implicated in addictive behaviors, but the examination of other relevant brain areas is needed to better understand the scope and potential applications of the present findings.

The data presented in this manuscript indicates a contribution of PARP1 to binge drinking behaviors. Alcohol consumption increases PARP transcription and activity and PARP1 overexpression increases alcohol consumption. On the other hand, pharmacological inhibition of PARP activity decreases alcohol consumption. Notwithstanding the limitations discussed above, these results collectively point at a primary role of PARP1 in gene expression changes associated with binge alcohol consumption, possibly suggesting the facilitation of a more drinking-inclined phenotype, at least in the male animal subjects we studied.

However, further studies are needed in order to understand whether the same results can be replicated in a wider population, including female animals, and different mouse strains. Future studies should also focus on the biomolecular changes induced by binge alcohol drinking in brain anatomic areas other than the PFC, as well as on the potential use of different and perhaps more specific and orally available PARP1 inhibitors as pharmacological tools to obtain further insights in the relationship between binge alcohol and PARP activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ABT	ABT-888
Adora2a	Adenosine A2a Receptor
AUD	Alcohol Use Disorder
BEC	Blood Ethanol Concentration
BSA	Bovine Serum Albumin
Ccl2	C-C Motif Chemokine Ligand 2

CNS	Central Nervous System
DAVID	Database for Annotation, Visualization, and Integrated Discovery
DID	Drinking-in-the-Dark
Drd1	Dopamine Receptor D1
Elfn2	Extracellular Leucine Rich Repeat and Fibronectin Type III Domain Containing 2
EtOH	Ethanol
GO	Gene Ontology
Gpr88	G Protein-Coupled Receptor 88
HMGB1	High Mobility Group Box 1
Hprt1	Hypoxanthine Phosphoribosyltransferase 1
HSV-GFP	Herpes Simplex Virus-Green Fluorescent Protein
HSV-PARP1	Herpes Simplex Virus-Poly-ADP Ribose Polymerase-1
i.p.	Intraperitoneal
i.u.	Infectious Units
KEGG	Kyoto Encyclopedia of Genes and Genomes
Lrrc10b	Leucine-Rich Repeat-Containing Protein 10B
Mmp14	Matrix Metalloproteinase 14
NGS	Normal Goat Serum
Nr4a1	Nuclear Receptor Subfamily 4 Group A Member 1
PARP	Poly-ADP Ribose Polymerase
PBST	Phosphate Buffered Saline (PBS)/Tween
Pbx3	PBX Homeobox 3
Pde10a	Phosphodiesterase 10A
Penk	Proenkephalin
PFC	Prefrontal Cortex
PVDF	Polyvinylidene Fluoride
qRT-PCR	Quantitative Reverse Transcriptase-Polymerase Chain Reaction
Rgs9	Regulator of G protein signaling 9

SDS	Sodium Dodecyl Sulfate
Spock3	SPARC (Osteonectin), Cwcv And Kazal Like Domains Proteoglycan 3
Syndig11	Synapse Differentiation Inducing 1 Like
Tac1	Tachykinin Precursor 1
TBST	Tris-Buffered Saline (TBS) and Polysorbate 20
TNF	Tumor Necrosis Factor
Veh	Vehicle

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Highlights

- Alcohol consumption in binge-like DID paradigm induces PARP expression and PARP activity in the PFC of C57BL/6J male mouse
- Virus-induced PARP1 overexpression in the PFC increases voluntary alcohol consumption in C57BL/6J male mice
- PARP inhibitor ABT-888 decreases voluntary alcohol consumption in C57BL/6J male mice
- RNA-seq of DID mice PFC samples identified genes upregulated by EtOH involved in addiction behavior and neuronal function
- Four genes selected based on RNA-seq results were validated via qRT-PCR as upregulated by EtOH and downregulated by ABT-888

Figure 1. General experimental design for *in vivo* experiments.

Acclimation time: mice were individually housed in a reversed light/dark cycle (lights on at 8 pm, off at 8 am). **(A)** DID day 1–3: in the EtOH group, water bottles were replaced with graduated bottles containing 20% ethanol solution (or water, or 0.2% saccharin in experiments aimed at assessing ABT-888 effect on water or saccharin drinking, respectively) for 2 hours (11 am – 1 pm) during the dark cycle; water bottles were replaced with graduated bottles containing water in the Control group at the same time. DID day 4: in the EtOH group, water bottles were replaced with bottles containing 20% ethanol solution (or water, or 0.2% saccharin in experiments aimed at assessing ABT-888 effect on water or saccharin drinking, respectively) for 4 hours (11 am – 3 pm) during the dark cycle; water bottles were replaced with graduated bottles containing water in the Control group at the same time; in experiments where the effect of PARP inhibition on alcohol consumption was studied, the PARP inhibitor ABT-888 was injected *i.p.* immediately before the beginning of alcohol drinking on day 4. All mice were sacrificed immediately after the final drinking period. **(B)** Day 0: HSV-GFP (control) or HSV-PARP1 viral vector was inoculated in the PFC *via* stereotaxic surgery, in order to assess the effects of PARP1 overexpression on ethanol consumption in the DID paradigm; mice were left in their cages to recover from surgery for 24 hours before the start of the DID experiment, which was conducted as described above; no ABT-888 treatment was performed in the HSV-GFP- and HSV-PARP1-inoculated mice.

Figure 2. Effects of ethanol and/or the PARP inhibitor ABT-888 on *Parp1* gene expression and PARP enzymatic activity in the PFC.

(**A**) Adult C57BL/6J male mice underwent the DID protocol described in “Methods”. *Parp1* mRNA determined by qPCR (n=7–8 per group). (**B**) Adult C57BL/6J male mice underwent the DID protocol described in “Methods”. On the fourth day, PARP inhibitor ABT-888 (ABT; 25 mg/kg) or vehicle (Veh) were administered *i.p.* immediately before introduction of ethanol or water bottle. At the end of the drinking protocol mice were euthanized and the PFC was dissected. PARP activity determined by the Trevigen PARP Universal Colorimetric Assay Kit (n=4–6 per group). * $p < 0.05$, *** $p < 0.001$.

Figure 3. PARP inhibition by ABT-888 (ABT) significantly decreased the amount of ethanol (EtOH) consumed in the DID paradigm.

Mice were subjected to DID paradigm with ABT-888 (ABT) 25 mg/kg *i.p.* administered immediately before the final drinking period on the fourth day. **(A)** Mice were subjected to the DID paradigm, with bottles containing water only. ABT treatment (Control+ABT) did not significantly affect water consumption compared to Control+Veh (n=11–12 per group). **(B)** A separate cohort of mice was subjected to the same DID paradigm with bottles containing saccharin solution. ABT treatment (Sacch+ABT) did not have a significant effect on the amount of saccharin solution consumed compared to Sacch+Veh (n=5–6 per group). **(C)** In a third cohort of mice, ABT treatment decreased EtOH consumption (EtOH+ABT) compared to vehicle control (EtOH+Veh) (n=11–12 per group). * $p < 0.05$.

Figure 4. PARP1 overexpression in the PFC increased ethanol consumption.

Mice were stereotactically injected with HSV-PARP1 or control HSV-GFP virus (3.33×10^5 i.u. per hemisphere). Animals were allowed 24 hours to recover from surgery before DID paradigm was initiated. Immediately following the final drinking period brain tissues were sectioned using a brain block and snap frozen for mRNA and protein analysis. **(A)** *Parp1* mRNA overexpression and the lack of compensatory changes in *Parp2* were determined by qRT-PCR (n=6 per group). **(B)** HSV-PARP1 associated PARP1 protein overexpression was determined by immunoblot compared to HSV-GFP control (n=3–4 per group). **(C)** Representative immunoblot showing increased PARP1 expression in HSV-PARP1 transduced samples (P) compared with HSV-GFP transduced samples (G). **(D)** Viral overexpression of PARP1 significantly increased ethanol consumption during the DID paradigm as determined by two-way repeated measures ANOVA (n=8–9 per group). **(E)** There was no significant difference in water consumption between HSV-PARP1 and HSV-GFP transduced animals (n=5–6 per group). * $p < 0.05$; ** $p < 0.01$; *** $p = 0.0506$.

Figure 5. Validation of 10 genes identified through RNA-seq in the PFC of mice exposed to ethanol through the DID paradigm or administered PARP inhibitor ABT-888 (ABT). Immediately following the final ethanol drinking period or 4 hours after ABT administration, mice were decapitated and brain tissues were sectioned for mRNA analysis. **(A)** DID ethanol exposure (EtOH+Veh) significantly increased the expression of *Lrrc10b* (n=8–10 per group), *Penk* (n=9–10 per group), *Drd1* (n=9–10 per group), *Adora2a* (n=7–8 per group), *Rgs9* (n=8 per group), and *Tac1* (n=8–9 per group) genes. **(B)** PARP inhibitor (Control+ABT) significantly reduced the expression of *Gpr88* (n=5–8 per group), *Lrrc10b* (n=6–8 per group), *Penk* (n=8–9 per group), *Adora2a* (n=7 per group), *Rgs9* (n=7–8 per group) and *Spock3* (n=7–8 per group) genes compared to vehicle controls (Control+Veh). * $p < 0.05$; ** $p < 0.01$, as determined by t-test.

Figure 6. Over-expression of PARP1 increases *Adora2a*, *Rgs9*, and *Penk* in mouse E18 Primary Cortical Neuron Cultures.

(**A**) Viral expression of *Parp1* mRNA expression was detected six hours after HSV-PARP1 transduction (n=6 wells per group). (**B**) HSV-PARP1 increased *Adora2a*, *Rgs9*, and *Penk* mRNA expression in cortical neuron cultures compared with HSV-GFP control (n=6 wells per group). ** $p < 0.01$, as determined by t-test.

Table 1.

mRNA primer sets used in this study.

Genes	5' Primer	3' Primer
<i>Adora2a</i>	TTCCATCTTCAGCCTCTTGG	ATGGGTACCACGTCCTCAA
<i>Gpr88</i>	CTGGACCACAGATGCTGCT	CTGGCCAACTCTTCACACCT
<i>Drd1</i>	GCTCCTGATGGAACACCATT	GCTTAGCCCTCACGTTCTTG
<i>Hprt1</i>	GCCGAGGATTTGAAAAAGT	ACAGAGGGCCACAATGTGAT
<i>Lrrc10b</i>	CCCAGTTCTCCACCCTGATA	TCTGGCCCTCACCTCCTATT
<i>Rgs9</i>	TTCGTGTTGTGGTCGTGAAT	TGAGATTCTTGGGGTCTTGC
<i>Parp1</i>	AACTTGCTGGCATCCTGTC	TGCACTTTTGACACCATGT
<i>Parp2</i>	GTGACTTGTTCTGGTGACCT	AGGCTTCAAAGTTTCCTCTT
<i>Pde10a</i>	GGGCAGAGATGTCGAAGAAG	GGTGCCTTCTGTGGAGTCAT
<i>Penk</i>	TCCTGCCTCCTGGCTACAGT	TGCAGGAGATCCTTGCAGGT
<i>Spock3</i>	AGACAGCAAGACCCACCTTG	GCACAATCTGCAACACCATT
<i>Syndig11</i>	GTGTCCTACGGGGTTCAAGA	GAGTGTGGCCAGGAAGAGAG
<i>Tac1</i>	GTGTCACGTGGCTCTACAGG	GCCACGAGGATTTTCATGTT

Table 2.
KEGG Pathway analysis of RNA-seq data.

Genes with increased mRNA expression in response to either ethanol (DID EtOH+Veh) or the PARP inhibitor ABT-888 (DID Control+ABT) compared to control (DID Control+Veh) are in bold and those with decreased expression are in regular font style.

KEGG Identity	ABT-888			EtOH		
	Count	Adjusted <i>P</i> value	Genes	Count	Adjusted <i>P</i> value	Genes
Morphine addiction	3	0.021	<i>Drd1</i> , <i>Gng4</i> , <i>Pde4a</i>	3	0.0463	<i>Pde10e</i> , <i>Gng7</i> , <i>Drd1</i>
Alcoholism	4	0.0195	<i>Drd1</i> , <i>Gng4</i> , <i>Ppp1cb</i> , <i>Ppp1r1b</i>	3	0.0892	<i>Adora2a</i> , <i>Gng7</i> , <i>Drd1</i>
Cocaine addiction	2	0.0439	<i>Ppp1r1b</i> , <i>Drd1</i>	2	0.0892	<i>Drd1</i> , <i>Rgs9</i>
Chemokine signaling pathway	3	0.0642	<i>Gng4</i> , <i>Prkcd</i> , <i>Ccl2</i>	3	0.0892	<i>Gng7</i> , <i>Ccl2</i> , <i>Rasgrp2</i>
Dopaminergic synapse	5	0.0007	<i>Fos</i> , <i>Drd1</i> , <i>Gng4</i> , <i>Ppp1cb</i> , <i>Ppp1r1b</i>	2	0.194	<i>Gng7</i> , <i>Drd1</i>
TNF signaling pathway	3	0.0235	<i>Mmp14</i> , <i>Ccl2</i> , <i>Fos</i>	3	0.1924	<i>Mmp14</i> , <i>Ccl2</i>
cAMP signaling pathway	6	0.0007	<i>Fos</i> , <i>Rapgef3</i> , <i>Drd1</i> , <i>Pde4a</i> , <i>Ppp1cb</i> , <i>Ppp1r1b</i>	2	0.2258	<i>Adora2a</i> , <i>Drd1</i>
Circadian entrainment	3	0.021	<i>Fos</i> , <i>Gng4</i> , <i>Per2</i>	1	0.2258	<i>Bhlhe40</i>
Amphetamine addiction	4	0.0007	<i>Fos</i> , <i>Drd1</i> , <i>Ppp1cb</i> , <i>Ppp1r1b</i>	1	0.2573	<i>Drd1</i>

Table 3.

Enriched Gene Ontology (GO) categories based on RNA-seq.

GO term	ABT-888		Alcohol	
	Count	<i>P</i> value	Count	<i>P</i> value
Neuronal cell body (GO:0043025)	8	0.0001	7	0.0004
Perinuclear region of cytoplasm (GO:0048471)	8	0.0003	-	-
Cytoplasm (GO:0005737)	24	0.0006	-	-
Nucleus (GO:0005634)	22	0.0014	-	-
Adult walking behavior (GO:0007628)	-	-	3	0.0027
Extracellular space (GO:0005615)	-	-	9	0.005
Axon terminus (GO:0043679)	4	0.0006	3	0.011
Locomotory behavior (GO:0007626)	3	0.0238	5	<0.00005
Axon (GO:0030424)	4	0.0343	6	0.0005
Response to hypoxia (GO:0001666)	5	0.0008	3	0.0478
Behavioral fear response (GO:0001662)	2	0.0889	3	0.0027