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Peroxisome proliferator-activated receptors α and γ are linked with alcohol consumption in mice and withdrawal and dependence in humans

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Abstract

Background—Peroxisome proliferator-activated receptor (PPAR) agonists reduce voluntary ethanol consumption in rat models and are promising therapeutics in the treatment of drug addictions. We studied the effects of different classes of PPAR agonists on chronic ethanol intake and preference in mice with a genetic predisposition for high alcohol consumption and then examined human genome wide association data for polymorphisms in PPAR genes in alcohol-dependent subjects.

Methods—Two different behavioral tests were used to measure intake of 15% ethanol in C57BL/6J male mice: 24-hour two-bottle choice and limited access (3-hour) two-bottle choice, drinking in the dark. We measured the effects of pioglitazone (10 and 30 mg/kg), fenofibrate (50 and 150 mg/kg), GW0742 (10 mg/kg), tesaglitazar (1.5 mg/kg) and bezafibrate (25 and 75 mg/kg) on ethanol intake and preference. Fenofibric acid, the active metabolite of fenofibrate, was quantified in mouse plasma, liver, and brain by LC-MS/MS. Data from a human genome wide association study (GWAS) completed in the Collaborative Study on the Genetics of Alcoholism (COGA) was then used to analyze the association of single nucleotide polymorphisms (SNPs) in

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Author Contributions

Y.A.B. designed and performed experiments, analyzed data, prepared graphs/tables, and wrote the manuscript; J.M.B. and M.B. performed experiments; L.B.F. analyzed data, prepared Table 1, and edited the manuscript; G.S. designed the bioanalytical approach and edited the manuscript; A.M.G. designed experiments; L.W. analyzed data, prepared graphs, and edited the manuscript; H.J.E. designed experiments and edited the manuscript; V.H. designed experiments; T.F. and R.A.H. designed experiments and wrote the manuscript.

different PPAR genes (*PPARA, PPARD, PPARG*, and *PPARGC1A*) with two phenotypes: DSM-IV alcohol dependence (AD) and the DSM-IV criterion of withdrawal.

Results—Activation of two isoforms of PPARs, α and γ, reduced ethanol intake and preference in the two different consumption tests in mice. However, a selective PPARδ agonist or a pan agonist for all three PPAR isoforms did not decrease ethanol consumption. Fenofibric acid, the active metabolite of the PPARα agonist fenofibrate, was detected in liver, plasma, and brain after 1 or 8 days of oral treatment. The GWAS from COGA supported an association of SNPs in *PPARA* and *PPARG* with alcohol withdrawal and *PPARGC1A* with AD but found no association for *PPARD* with either phenotype.

Conclusions—We provide convergent evidence using both mouse and human data for specific PPARs in alcohol action. Reduced ethanol intake in mice and the genetic association between AD or withdrawal in humans highlight the potential for repurposing FDA-approved PPARα or PPARγ agonists for the treatment of AD.

Keywords

two-bottle choice; C57BL/6J; pioglitazone; fenofibrate; fenofibric acid; tesaglitazar; GWAS

INTRODUCTION

Peroxisome-proliferator-activated receptors (PPARs) are part of the nuclear hormone receptor superfamily. Activated PPARs translocate to the nucleus where they form a heterodimer with the nuclear hormone receptor, Retinoid × Receptor. This complex binds to PPAR response elements in the DNA to regulate transcription of many target genes. PPARs can also modify phosphorylation of proteins or inhibit activity of NF-κB and other transcription factors (Daynes and Jones, 2002). Their ability to trans-repress is thought to be the main mechanism for their anti-inflammatory actions.

There are three closely related isoforms of PPARs: PPARα, PPARδ (β) and PPARγ. Each is encoded by a different gene and has a unique tissue distribution, but all have been identified in the CNS (Schnegg and Robbins, 2011), and PPAR activity in the brain is relatively high (Kao et al., 2012). PPAR agonists have been highlighted in the treatment of several CNS diseases, including Alzheimer's, Parkinson's and Huntington's disease, schizophrenia, and ischemic brain injury (Mandrekar-Colucci et al., 2013). The PPARα agonist gemfibrozil decreased voluntary alcohol consumption in rats (Barson et al., 2009), and PPARγ agonists (pioglitazone and rosiglitazone) reduced voluntary alcohol consumption (Stopponi et al., 2011; Stopponi et al., 2013) and decreased stress-induced relapse and alcohol withdrawal symptoms in alcohol-dependent rats (Stopponi et al., 2011). PPAR agonists are also promising medications for the treatment of different drug addictions in many preclinical studies (Le Foll et al., 2013). Furthermore, expression of PPARδ and PGC-1α, the coactivator of PPARγ, is altered in brains of human alcoholics (Ponomarev et al., 2012), and PGC-1α is also altered in other neurodegenerative diseases (Austin and St-Pierre, 2012).

Given that PPAR agonists reduce alcohol consumption in rodents and have been nominated in the treatment of CNS diseases and drug addictions based on animal studies, we evaluated

the effects of selective agonists for each subtype, as well as dual and pan (triple) agonists, on ethanol intake in C57BL/6J mice using two different consumption tests. Next, we used data from a human genome wide association study (GWAS) from the Collaborative Study on the Genetics of Alcoholism (COGA), which has recruited multiplex families densely affected with AD, to analyze the association of SNPs in *PPARA, PPARD, PPARG*, and *PPARGC1A* with two phenotypes – AD and withdrawal. Using both mouse and human data, we provide overlapping evidence for a role of specific PPARs in ethanol action.

MATERIALS AND METHODS

Mice

Male C57BL/6J mice were taken from a colony maintained at The University of Texas at Austin (original breeders were purchased from Jackson Laboratories, Bar Harbor, ME). Mice were group-housed 4 or 5 to a cage. Food and water were available *ad libitum*. The vivarium was maintained on a 12:12 hour light /dark cycle with lights on at 7:00 a.m. The temperature and humidity of the room were kept constant. Baseline drinking began when the mice were 2 to 3 months old. All experiments were conducted in isolated behavioral testing rooms in the Animal Resources Center with reversed light cycle to avoid external distractions. Before beginning experiments, mice were moved to their experimental room and remained there for at least 2 weeks for adaptation to the new light cycle. All experiments were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin.

Baseline drinking

Before the drinking tests (described below), mice consumed 15% ethanol for at least 3 weeks. After this period, ethanol consumption was measured for at least 4 days to ensure stable consumption. Consumption was considered stable if the intake was similar on days 1–2 and 3–4 (mice that did not drink were removed from the study). For the 24-hour two-bottle choice test, ethanol intake was then measured after saline administration for 2 days (denoted as day 2 in all graphs), and mice were grouped to provide similar levels of ethanol intake and preference based on the first 6 hours of consumption during these 2 days. Ethanol and total fluid intake are presented as g/kg/6 hours; measurements made after the next 18 hours are presented as percent of corresponding control. In the drinking in the dark test, mice were grouped to provide similar levels of ethanol intake and preference based on 3 hours of consumption during the first 2 days of saline injections (denoted as day 2 in all graphs). Ethanol and total fluid intake are presented as g/kg/3 hours. From day 3 in both drinking tests, mice were administered saline or drugs once daily and results are presented as the average from 2-day periods of consecutive drinking using different bottle positions. Overall, mice were exposed to ethanol for at least 3 weeks followed by 4 days of measured drinking before beginning the drug studies, which lasted up to 12 days. Our definition of chronic drinking is thus based on at least 5 weeks of ethanol exposure.

Drug administration

For the 24-hour two-bottle choice test, pioglitazone (10 and 30 mg/kg), fenofibrate (50 and 150 mg/kg), GW0742 (10 mg/kg), tesaglitazar (1.5 mg/kg), and bezafibrate (25 and 75

mg/kg) were administered orally by gavage (p.o.). For the limited access drinking test, 30 mg/kg pioglitazone, 150 mg/kg fenofibrate, 10 mg/kg GW0742, 1.5 mg/kg tesaglitazar, and 75 mg/kg bezafibrate were tested. Individual mice were administered a single drug at one or two different dosages and were only used in one of the ethanol drinking tests. Drugs were purchased from Sigma-Aldrich (St. Louis, MO) or Tocris Bioscience (Minneapolis, MN). All drugs were freshly prepared as suspensions in saline with 4–5 drops of Tween-80 and administered once daily in a volume 0.05 ml/10 g of body weight 60 minutes before drinking experiments. Saline containing 4–5 drops of Tween-80 was administered to control groups. Single use, sterile Becton, Dickinson and Co. gavage needles (27.5 gauge; model #305109) were used. Drug doses and routes of administration were based on previously published *in vivo* studies in rodents (Nakajima et al., 2009; Stopponi et al., 2011; Wang and Namura, 2011; Bhateja et al., 2012; Wallenius et al., 2013). If the initial dose of the drug was not effective, a higher dose was tested without exceeding the doses used in the studies above.

Tissue distribution of fenofibrate

Liver, brain, and plasma samples from C57BL/6J male mice treated for 1 or 8 days with fenofibrate (150 mg/kg; n=6 per group) were collected 2 hours after the final injection and sent to inVentiv Health Clinical Lab, Inc. (Princeton, NJ) for mass spectrometry analysis (LC-MS/MS) to measure levels of fenofibrate and its active metabolite, fenofibric acid. The parent compound, fenofibrate, was not observed at the detection limit of the bioanalytical assay. One part of plasma, brain, or liver was homogenized with 4 parts of lysate in a FastPrep (MP Biomedicals, Santa Ana, CA) homogenizer. After protein precipitation, the samples were analyzed via a Waters Acquity UPLC (Waters Corporation, Milford, MA) with a gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile on a BEH C8 2.1×50 1.7 mcm column (Waters Corporation). The internal standards were tolbutamide and warfarin. Samples were quantified by positive LC-ESI-MS/MS-Multiple Reaction Monitoring (MRM) using an API4000 (AB SCIEX, Framingham, MA) at unit/unit resolution with the heater set at 500C, spray voltage at 5000 eV, and CAD gas at 4 and data gathered via Analyst Software (AB SCIEX) and a proprietary Excel program.

Ethanol drinking – 24-hour two-bottle choice

Two drinking bottles were continuously available to individually housed mice. One contained water and the other 15% ethanol (v/v). Bottle positions were changed daily to control for position preferences. Once stable ethanol consumption was reached (see above), we measured ethanol intake after 2 days of saline injections (day 2 in graphs) and grouped mice to provide similar levels of ethanol intake and preference. We measured consumption (g/kg body weight/time) and calculated preference as the amount of ethanol consumed divided by the total amount of fluids consumed per day (a value >50% indicates a preference for ethanol). Bottles were weighed twice daily (see below for time points) for the 24-hour two-bottle choice test. Food was available *ad libitum*, and mice were weighed every 4 days beginning on day 1. Adult mouse weights are stable, and measuring weight every 4 days is adequate to ensure accuracy; furthermore, no differences in weight between groups were observed during the course of this study. Ethanol consumption was measured after 6 hours and again after the next 18 hours. Measurements made after the next 18 hours are presented

as percent of corresponding control. Measurements of ethanol intake, preference, and total fluid intake were averaged over 2-day periods with different bottle positions. Each point in the graphs (days 2, 4, 6, etc.) represents the average of 2 days of measurement. For example, day 2 is the average of days 1–2 after saline for both control and drug groups; day 4 is the average of days 3–4 after saline or drug, and day 6 is the average of days 5–6 after saline or drug. Ethanol intake, preference, and total fluid intake were also calculated after 24 hours in the two-bottle choice test.

Ethanol drinking – limited access in the dark phase (two-bottle choice drinking in the dark, DID)

This was similar to the one-bottle DID test described previously (Rhodes et al., 2005) except that 2 bottles, one containing 15% ethanol and the other water, were used (Blednov and Harris, 2008). Once stable ethanol consumptions were reached, we measured ethanol intake after 2 days of saline (day 2 in graphs) and grouped mice to provide similar levels of ethanol intake and preference. The ethanol and water bottles remained in place for 3 hours. After their removal, mice had unlimited access to 1 bottle of water. Bottle positions during 3-hour access were changed daily to avoid potential position preferences. Drinking began 3 hours after lights off. Measurements of ethanol intake, preference, and total fluid intake were averaged over 2 days with different bottle positions. Each point in the graphs (days 2, 4, 6, etc.) represents the average of 2-day periods of measurement. For example, day 2 is the average of days 1–2 after saline, and day 4 is the average of days 3–4 after either saline or drug. Separate groups of mice were used for the two different drinking tests.

Statistical Analysis

The number of mice in each group is shown in the Supplemental Tables and Figure Legends. Data are reported as the mean ± S.E.M. The statistics software program GraphPad Prism (Jandel Scientific, Costa Madre, CA) was used to perform Student's t-tests or two-way repeated measures ANOVA and Bonferroni post hoc tests.

Collaborative Study on the Genetics of Alcoholism (COGA)

Alcoholic probands were recruited from alcohol treatment programs through 6 sites (Begleiter et al., 1995; Foroud et al., 2000); institutional review boards at all sites approved the study. The probands and family members were administered the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) (Bucholz et al., 1994; Hesselbrock et al., 1999). Individuals less than 18 years of age were administered an adolescent version. When multiple interviews were available from an individual, data from the SSAGA with the maximum total number of endorsed DSM-IV AD criteria were utilized, and only for individuals who had ever reported consuming alcohol.

Two phenotypes were analyzed in the COGA data set: (1) DSM-IV AD and (2) the DSM-IV criterion of withdrawal. Individuals younger than 15 years of age at their most recent interview who were alcohol dependent were excluded for this phenotype, as well as individuals less than 23 years old who did not meet DSM-IV criteria. This was to exclude young children and adolescents not representative of an adult sample as well as individuals

not past the primary age of risk. All individuals with SSAGA data for the withdrawal criterion were included in the analysis.

To maximize contribution of genetic variants to the risk of AD and to reduce heterogeneity, a subsample of COGA families was selected for a family-based association study. These families consisted of primarily self-reported European American. Families were prioritized based on (i) largest number of alcohol dependent family members with DNA, (ii) the largest number of family members with DNA and electrophysiological data, and (iii) the largest number of family members with DNA. The final sample consisted of 118 large European American families, with 2,322 individuals with available DNA (Kang et al., 2012; Wang et al., 2013). Genotyping was performed at the Genome Technology Access Center at Washington University School of Medicine in St. Louis using the Illumina Human OmniExpress array 12.VI as well as at the Center for Inherited Diseases using the Illumina 1M array. Further genotyping details, including SNP and sample cleaning, are available in Wang et al. (2013). The average number of genotyped individuals in a family was 20, and there was an average 5.9 members meeting criteria for DSM-IV AD. A total of 684 individuals were classified as alcohol dependent and 964 as unaffected, and 327 individuals endorsed the withdrawal criterion, while 1,459 did not.

Only SNPs having a minor allele frequency of 5% or greater that were within 10 kb of *PPARA, PPARD, PPARG, and PPARGC1A* were considered in the analysis. The Genome-Wide Association Analyses with Family Data package (Chen and Yang, 2010) implementing a log additive model was used for analysis of AD. The generalized disequilibrium test (Chen et al., 2009), employing data from all discordant relative pairs, was used for analysis of the withdrawal criterion. To account for secular trends, sex and birth cohort defined by year of birth (<1930, 1930–1949, 1950–1969, ≥1970), were used as covariates. In regions of interest, imputed SNPs were analyzed to further evaluate the evidence for association. SNPs were imputed to 1000 Genomes (EUR, August 2010 release) using BEAGLE 3.3.1 (Browning and Browning, 2009) as described in Wang et al. (2013).

RESULTS

In the two-bottle choice test (continuous access to ethanol and water), the PPARγ agonist pioglitazone (Sakamoto et al., 2000) reduced ethanol intake and preference (without changing total fluid intake) during the first 6 hours at the highest dose tested (30 mg/kg) (Figure 1A; Supplemental Table 1; Supplemental Figure 1 A,B,C). This effect was not seen after the next 18 hours of ethanol consumption (Supplemental Figure 2 A,B,C; Supplemental Table 2). The PPARα agonist, fenofibrate (Willson et al., 2000), reduced ethanol intake and preference after the first 6 hours at the highest dose tested (150 mg/kg) without changing total fluid intake (Figure 1B; Supplemental Table 1; Supplemental Figure 1 D,E,F). In contrast to pioglitazone, the fenofibrate effect was long-lasting and observed for 24 hours after administration (Supplemental Figure 2 D,E,F; Supplemental Table 2). The PPARδ agonist, GW0742 (Sznaidman et al., 2003), did not change ethanol intake at any time point (Figure 1C; Supplemental Figure 1 G,H,I; Supplemental Figure 2 G,H,I; Supplemental Tables 1, 2). A dual PPARα and γ agonist, tesaglitazar (Cronet et al., 2001; Ljung et al., 2002), produced a strong, long-lasting reduction of ethanol intake and

preference (Figure 1D; Supplemental Figure 1 J,K; Supplemental Figure 2 J,K; Supplemental Tables 1, 2). However, this drug increased total fluid intake, especially after the first 6 hours (Supplemental Figure 1 L; Supplemental Figure 2 L; Supplemental Tables 1, 2). Finally, the pan agonist bezafibrate (which activates PPARα/γ/δ) (Willson et al., 2000), modestly reduced preference (not intake) after the first 6 hours at the highest dose tested (75 mg/kg) (Figure 1 E; Supplemental Figure 1 M,N,O; Supplemental Figure 2 M,N,O; Supplemental Tables 1, 2). The effects of the PPAR agonists on ethanol intake, preference, and total fluid intake after 24 hours were also calculated (Supplemental Figure 3; Supplemental Table 3). Fenofibrate and tesaglitazar reduced ethanol intake and preference after 24 hours in the two-bottle choice test, as reported for the other time points above. The effectiveness of pioglitazone after 24 hours was weaker compared to its initial effects after 6 hours. Bezafibrate (75 mg/kg) reduced ethanol preference, but not intake, after 6 hours. As expected, no effects of bezafibrate were observed after 24 hours.

In a 'binge' model of limited access to ethanol, pioglitazone had no effect (Figure 2 A; Supplemental Figure 4 A,B,C; Supplemental Table 4), but fenofibrate strongly reduced ethanol intake and preference without changing total fluid intake (Figure 2 B; Supplemental Figure 4 D,E,F; Supplemental Table 4). GW0742 did not change ethanol intake (Figure 2 C; Supplemental Figure 4 G,H,I; Supplemental Table 4). Tesaglitazar profoundly reduced ethanol intake and preference and also increased total fluid intake (Figure 2 D; Supplemental Figure 4 J,K,L; Supplemental Table 4). Bezafibrate (75 mg/kg) modestly reduced ethanol intake and preference without changing total fluid intake (Figure 2 E; Supplemental Figure 4 M,N,O; Supplemental Table 4). Thus, in both tests, activation of α and γ PPARs (but not δ) reduced alcohol intake and preference in mice genetically predisposed to drink high levels of alcohol. No changes in body weight were observed in control (saline) or drug treatment groups in either drinking test (data not shown).

We collected plasma, liver, and brain samples from mice treated for 1 or 8 days with 150 mg/kg of fenofibrate and measured tissue levels of fenofibric acid, the active metabolite, by LC-MS/MS. Liver, plasma, and brain levels were very high, high, and low, respectively (Table 1), and maximal levels in brain were reached after a single injection.

We sought to link our novel results in mice to human alcoholism using data from COGA. We selected two phenotypes, DSM-IV AD and alcohol withdrawal, that were related to preference measured in the mouse model. AD is characterized by excessive intake on a regular basis, while withdrawal reflects negative consequences from drastic reductions in alcohol intake. A total of 43 SNPs in *PPARA* were genotyped; four provided evidence of association with withdrawal ($5.1 \times 10^{-3} < p < 0.04$; Figure 3A) while none were associated with AD ($p > 0.15$; data not shown). A total of 107 SNPs were tested in *PPARG*; five SNPs provided evidence of association with withdrawal ($9.5 \times 10^{-3} < p < 0.05$; Figure 3B) and 1 with AD ($p = 0.03$; data not shown). None of the 30 SNPs in *PPARD* supported an association with either AD ($p > 0.22$) or withdrawal ($p > 0.38$) (Supplemental Figure 5). We extended our studies of *PPARG* to include the gene for its transcriptional coactivator, *PPARGC1A*. We tested 46 SNPs and 3 provided support for an association with AD ($8.6 \times 10^{-3} < p < 0.04$) but none with withdrawal ($p > 0.07$) (Figure 3C). In all regions with evidence supporting association ($p < 0.05$), imputed SNPs were analyzed. The imputed SNPs in

PPARGC1A provided additional evidence supporting the association with AD ($p<0.001$; Figure 3C).

DISCUSSION

Our results show that activation of PPARα and PPARγ (but not PPARδ) reduces ethanol intake and preference in both chronic voluntary and limited access 'binge' drinking models in mice with a genetic predisposition for high ethanol consumption. A PPARα agonist reduced ethanol consumption in rats (Barson et al., 2009), and PPARγ agonists reduced ethanol drinking, stress-induced relapse, and withdrawal in alcohol-preferring rats (Stopponi et al., 2011). These effects were not due to changes in blood alcohol levels and were prevented by injection of a selective PPARγ antagonist into the lateral cerebroventricle, showing the importance of central PPARs in mediating reduced alcohol drinking (Stopponi et al., 2011).

The ability of PPAR ligands to trans-repress or inhibit the activity of transcription factors like NF-κB is thought to be the main mechanism for their anti-inflammatory actions. Both PPAR agonists and NF-κB inhibitors reduce ethanol intake and preference in mice. For example, an inhibitor of NF-κB (caffeic acid phenylethyl ester) reduced ethanol intake and preference in C57BL/6J mice (Harris and Blednov, 2013). A selective inhibitor of IKKβ, which regulates NF-κB activation, reduced ethanol consumption and preference in these mice (Truitt et al., 2013). Furthermore, genes with NF-κB elements were generally upregulated in post-mortem brains from human alcoholics (Okvist et al., 2007). *NFKB1*, which encodes a 105 kDa Rel-family protein whose full-length form inhibits transcription and is cleaved into the 50 kDa DNA-binding subunit of NF-κB, has been associated with AD (Edenberg et al., 2008). NF-κB regulates the development and function of both innate and adaptive immunity (Boersma and Meffert, 2008), and NF-κB and its signaling pathways have become a focal point for intense drug discovery efforts (Gupta et al., 2010; Karin et al., 2004). NF-κB is a point of convergence for many extracellular signals that activate gene expression and plays a key role in inflammation and disease (Gamble et al., 2012; Schmid and Birbach, 2008). Considering evidence for the neuroimmune system in regulating ethanol drinking (Harris and Blednov, 2013; Mayfield et al., 2013) and the role of PPARs in reducing inflammation, PPAR agonists may reduce drinking via their anti-inflammatory mechanisms. This might be expected if the drinking models used here induce sufficient immune activation. Altered expression of immune-related genes was observed in liver and prefrontal cortex from C57BL/6J mice after chronic ethanol treatment (Osterndorff-Kahanek et al., 2013). Changes were greatest in liver compared to prefrontal cortex and differed depending on the ethanol treatment paradigm. Systemic injection of PPAR agonists also induced changes in the expression of immune-related genes in the liver but did not produce prominent changes in neuroimmune pathways in C57BL/6J mice (Ferguson et al., 2014). NF-κB targets were not downregulated in liver or brain following PPAR agonist treatment, but it should be noted that these mice were ethanol naive (Ferguson et al., 2014).

The selective PPAR effects that we observed in mice are supported by human genomic data, suggesting a potential genomic link between PPARs and AD. Variations in *PPARA* and *PPARG* were modestly associated with withdrawal in humans while no evidence of

association for either phenotype was demonstrated for variants in *PPARD*. *PPARGC1A*, which codes for PGC-1α, a coactivator for PPARγ transcriptional activity, was associated with AD. PGC-1α increases mitochondria (and peroxisome) generation while decreasing buildup of reactive oxygen species, allowing for positive effects of oxidative metabolism (Austin and St-Pierre, 2012). PGC-1α expression is highly inducible by physiological cues and decreased expression is associated with aging and other neurodegenerative diseases (Austin and St-Pierre, 2012) and schizophrenia (Jiang et al., 2013).

PPAR agonists may have limited ability to reach the brain in rodents (Dasgupta et al., 2007; Weil et al., 1988). We show that although brain levels of the active metabolite of fenofibrate are lower than those in liver and plasma, fenofibric acid does reach mouse brain 2 hours after a single oral treatment. The brain levels attained are likely high enough to activate PPARα but not other PPARs (Willson et al., 2000). Fenofibric acid reaches near maximal levels in brain, liver, and plasma after a single injection, and we also show that the effect of fenofibrate on ethanol consumption does not increase with repeated injections. As mentioned previously, all PPAR isoforms are expressed in the CNS and the overall PPAR activity in the brain is high. The effects of pioglitazone and rosiglitazone on ethanol drinking are blocked by a selective PPARγ antagonist injected into the lateral cerebroventricle, indicating a direct action of PPAR agonists in rat brain (Stopponi et al., 2011). In addition, systemic administration of PPAR agonists produces CNS effects, including improvement of cognitive function (Bhateja et al., 2012), attenuation of hyperactivity induced by early ethanol exposure (Marche et al., 2011), improvement of reduced motor activity following MPTP treatment (Kreisler et al., 2010), and neuroprotection (Bordet et al., 2006). These studies clearly demonstrate that PPAR agonists act in the brain, and Mandrekar-Colucci et al. (2013) highlight the use of PPAR agonists in neurological diseases.

Furthermore, there are examples of PPAR activation affecting brain function via their systemic metabolic effects. Oleoylethanolamide is an endogenous lipid mediator that is released when fat enters the small intestine, and it induces satiety via PPARα in the gut (Fu et al., 2003). Administration of oleoylethanolamide improves memory retention in rats by acting as a PPARα agonist and facilitating memory consolidation through noradrenergic activation of the basolateral amygdala, a mechanism involved in memory enhancement (Campolongo et al., 2009). Also, this lipid mediator restores gut-stimulated dopamine release in a PPARα-dependent manner and eliminates motivation deficits in mice consuming a high-fat diet (Tellez et al., 2013). Thus, lipid/PPAR signaling in the periphery may regulate central behaviors.

Some of the behavioral effects that we observed might be attributed to the systemic effects of PPAR agonists on metabolism. For example, PPAR agonists can affect alcohol and acetaldehyde dehydrogenase mRNAs in the liver (Ferguson et al., 2014), which could increase acetaldehyde and potentially reduce alcohol consumption. However, given the evidence for central action of PPAR agonists on ethanol drinking (Stopponi et. al., 2011), their ability to alter neuronal gene expression in mouse brain following systemic injection (Ferguson et al., 2014), their role in many CNS effects and diseases, and our results showing that the active metabolite of fenofibrate rapidly reaches mouse brain, the effects on ethanol drinking observed in this study are likely mediated via central mechanisms.

PPAR agonists with fewer side effects are being sought, and PPARα agonists are widely used and better tolerated than PPARγ agonists (Cheatham, 2010; Mandrekar-Colucci et al., 2013). The clinical usefulness of α agonists, together with our findings demonstrating the ability of fenofibrate to reduce alcohol consumption in mice and the human genomic link between *PPARA* and withdrawal, highlight a potential role for PPARα agonists in treating alcoholism. Given that there are only three FDA-approved drugs for AD (disulfiram, naltrexone, and acamprosate) with limited efficacy, improved targets for medication development remain a primary goal of alcohol research. Although research has typically focused on traditional sites involved in synaptic transmission, evidence suggests that PPAR and other signaling pathways in brain may be unexplored targets for medication development to reduce excessive alcohol consumption and prevent relapse.

Overall, PPAR agonists are beneficial for treating several key problems of AD: i) excessive consumption as demonstrated here and by previous studies (Barson et al., 2009; Stopponi et al., 2011; Stopponi et al., 2013), ii) ethanol-induced liver injury (Enomoto et al., 2003), iii) neurodegeneration (Mandrekar-Colucci et al., 2013), and iv) nicotine use (Mascia et al., 2011; Panlilio et al., 2012). We provide novel support for the efficacy of selective PPAR agonists in two mouse models of excessive alcohol consumption and found that human polymorphisms in specific *PPAR* genes may be associated with alcohol withdrawal or AD, demonstrating convergent evidence for PPARs in alcohol action in mice and humans. In particular, the evidence of association in humans is strongest for *PPARGC1A* and AD. Our proof of principle approach combines both mouse and human data to systematically evaluate and nominate specific PPARs. An overall similar approach recently showed that SNPs of *FKBP5* were associated with alcohol withdrawal in humans, and Fkbp5 knockout mice also showed greater withdrawal severity (Huang et al., 2014). Our results provide support for the first human genetic link between PPARs and alcohol-related phenotypes and suggest that further studies are warranted to evaluate repurposing PPAR agonists for treating AD. Some of these drugs are already FDA approved and some have been nominated for treating addictions in preclinical studies. The study by Mason et al. (2014) provides an example of a clinical trial showing the potential of repurposing gabapentin, a widely prescribed calcium channel/GABA medication, for treating AD. Clinical studies showing favorable drug safety profiles and effectiveness in treating AD and relapse-dependent symptoms will benefit pharmacotherapies and offer patients more treatment options. We propose that behavioral evaluation of drug targets in animals, followed by analysis of genetic variants in humans, may be an effective strategy for advancing therapeutics for AD and other polygenic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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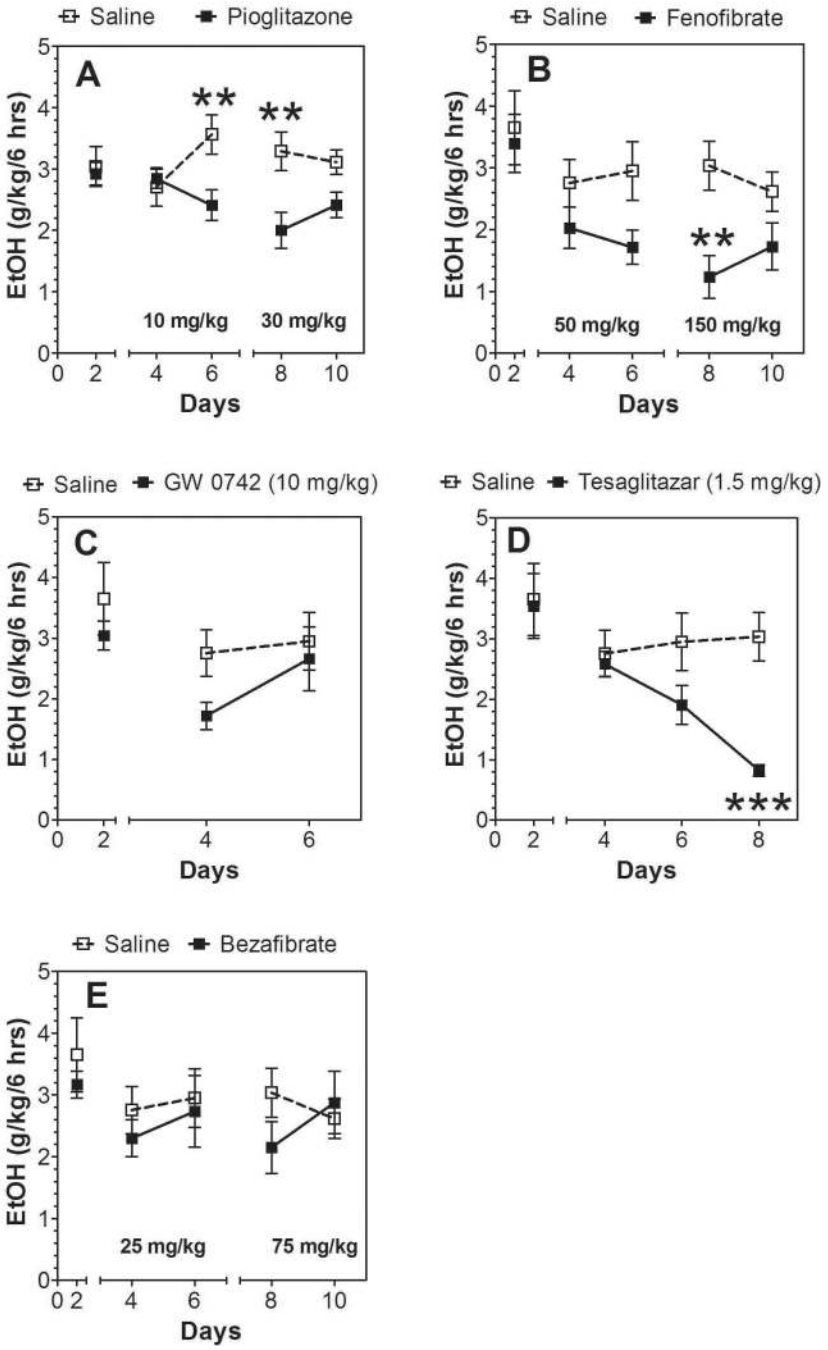


Figure 1. Effects of PPAR agonists on ethanol intake after the first 6 hours in the 24-hour two-bottle choice test in C57BL/6J male mice

After at least 3 weeks of 15% ethanol consumption and after stable intake was reached, ethanol (EtOH) consumption was measured (g/kg/6 hours) after 2 days of saline administration (day 2 in graph) and mice were grouped to provide similar levels of ethanol intake and preference. Beginning on day 3, saline or drug was administered and intake averaged over 2-day periods using different bottle positions (see Methods for details). A. Pioglitazone (n=13) B. Fenofibrate (n=6) C. GW0742 (n=6) D. Tesaglitazar (n=6) E. Bezafibrate (n=6). Data were analyzed by two-way repeated measures ANOVA followed by

Bonferroni's test for multiple comparisons. *p<0.05; **p<0.01; ***p<0.001 compared to control.

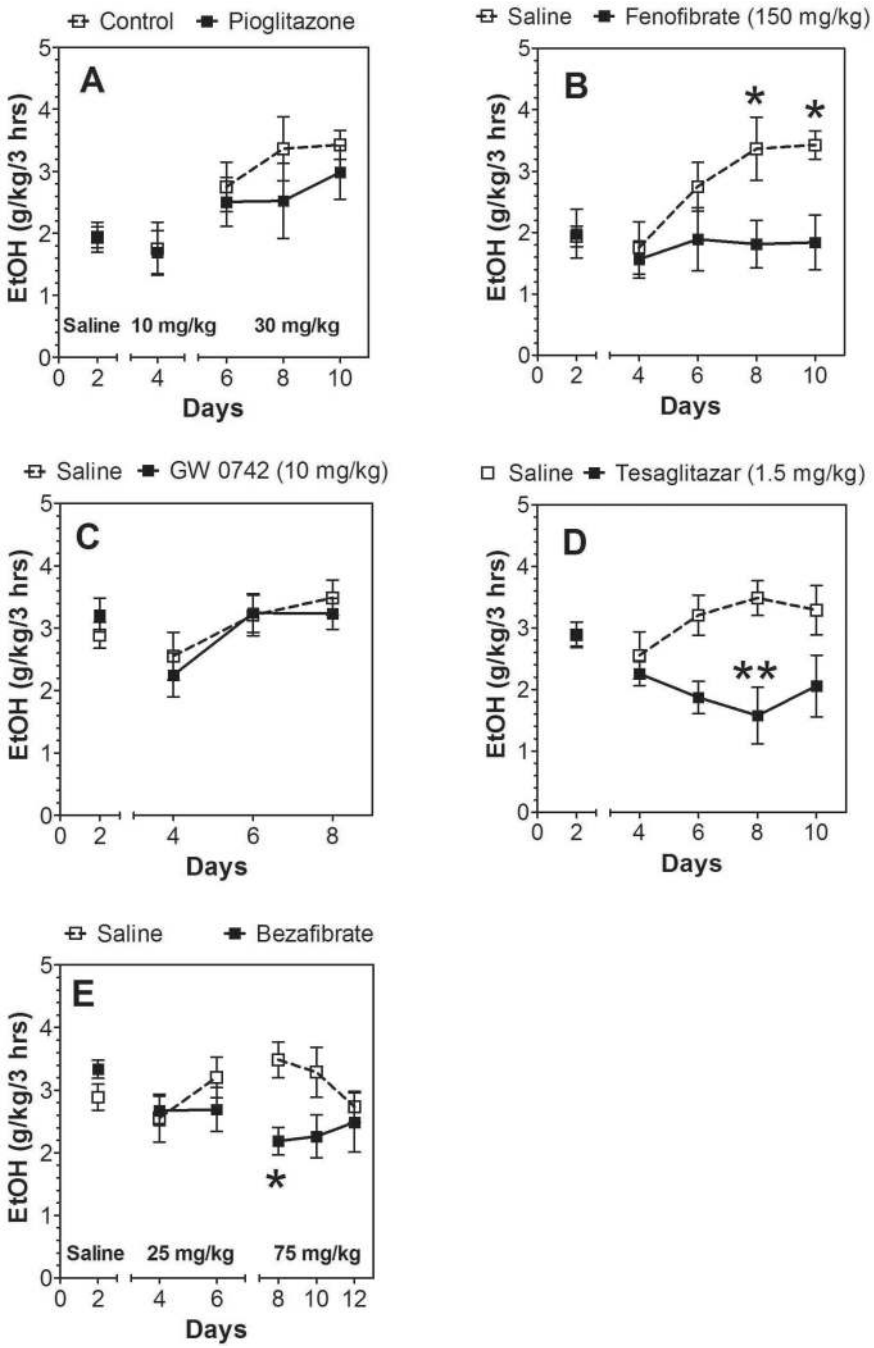
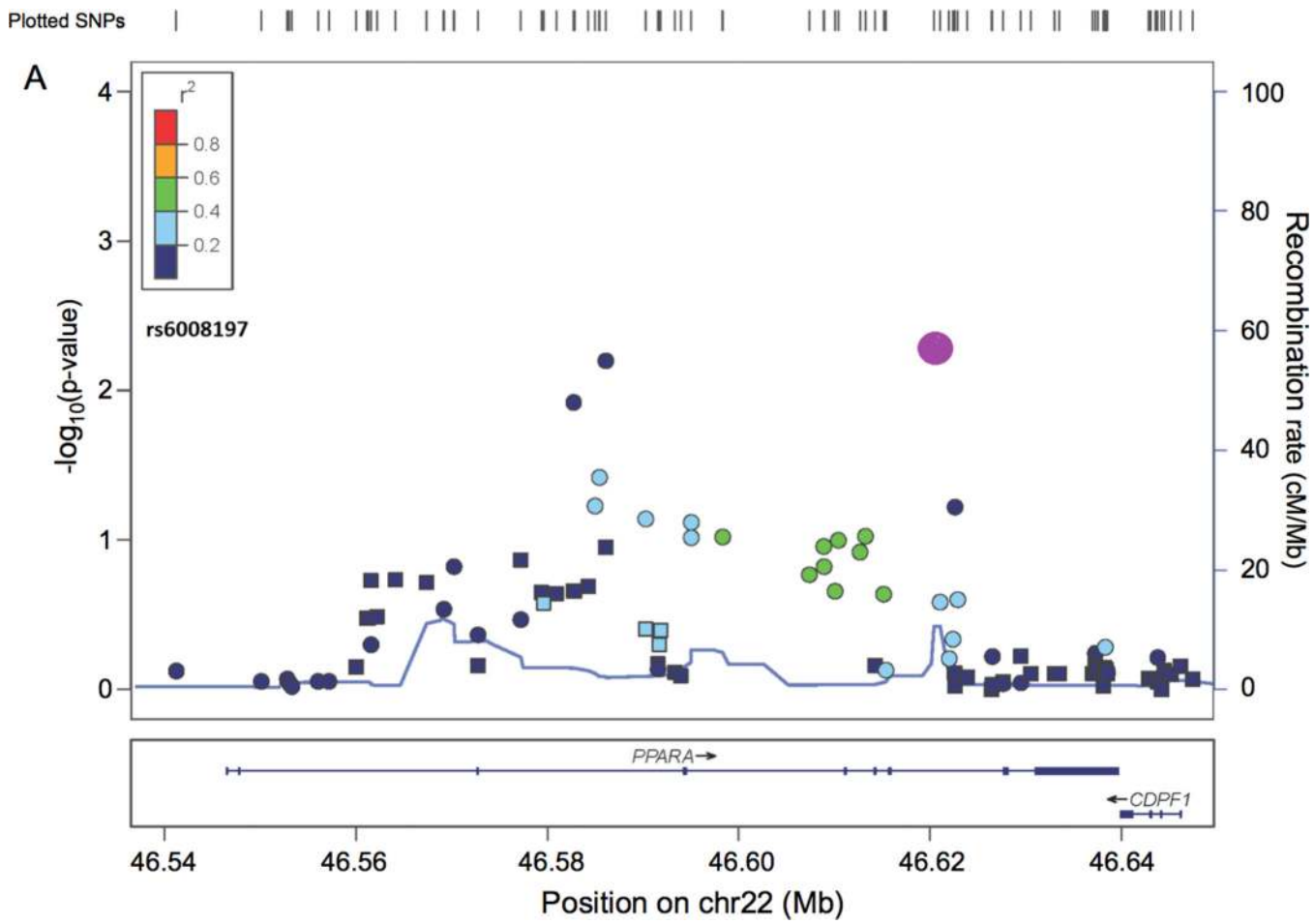
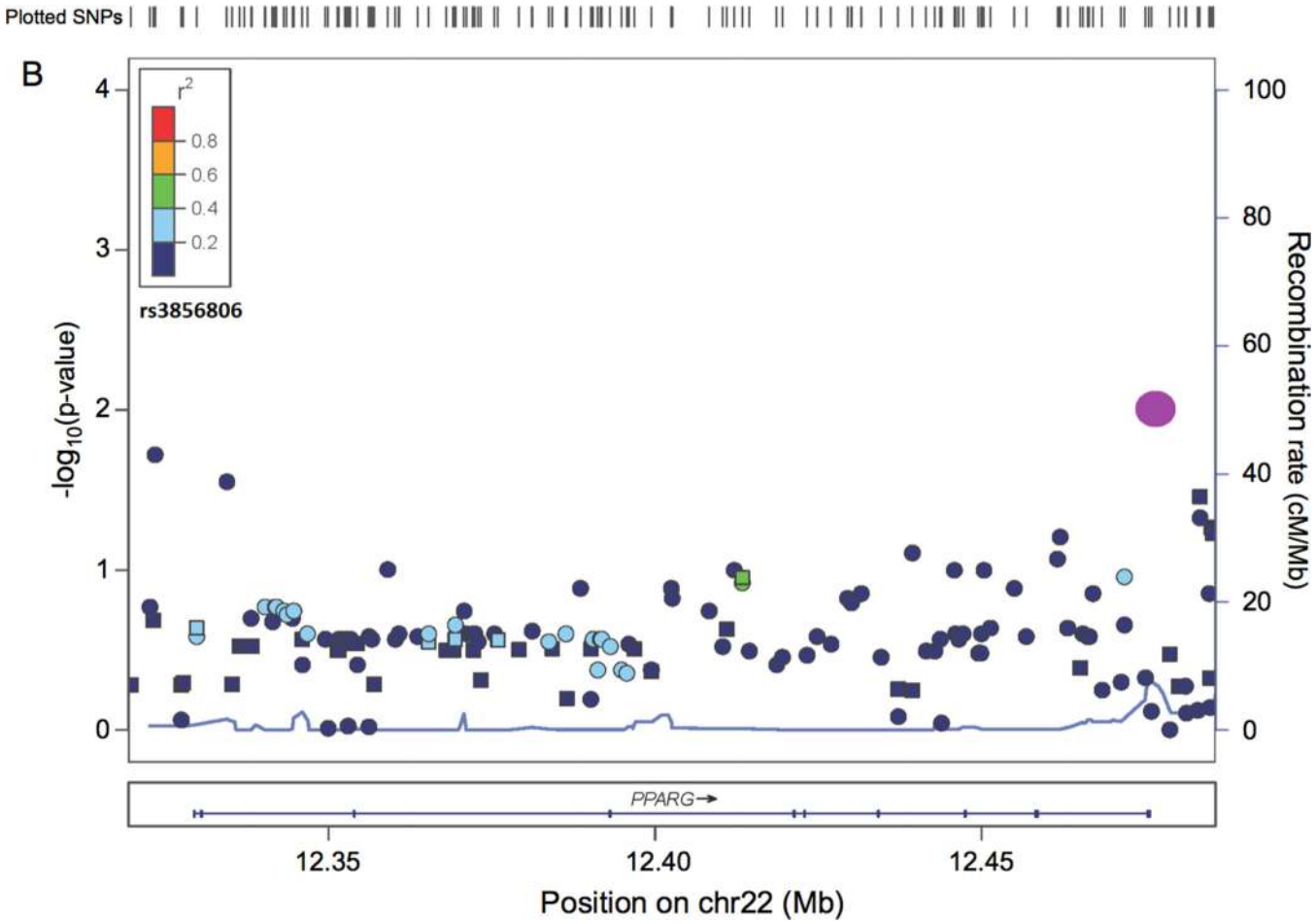


Figure 2. Effects of PPAR agonists on ethanol intake during limited access (3-hour) two-bottle choice Drinking in the Dark test in C57BL/6J male mice

After at least 3 weeks of 15% ethanol consumption and after stable intake was reached, ethanol (EtOH) consumption was measured (g/kg/3 hours) after 2 days of saline administration (day 2 in graph) and mice were grouped to provide similar levels of ethanol intake and preference. Beginning on day 3, saline or drug was administered and intake averaged over 2-day periods using different bottle positions (see Methods for details). A. Pioglitazone B. Fenofibrate C. GW0742 D. Tesaglitazar E. Bezafibrate. Data were analyzed by Student's t-test or two-way repeated measures ANOVA followed by Bonferroni's test for

multiple comparisons. *p<0.05; **p<0.01; ***p<0.001 compared to control. (n=6 for all groups)





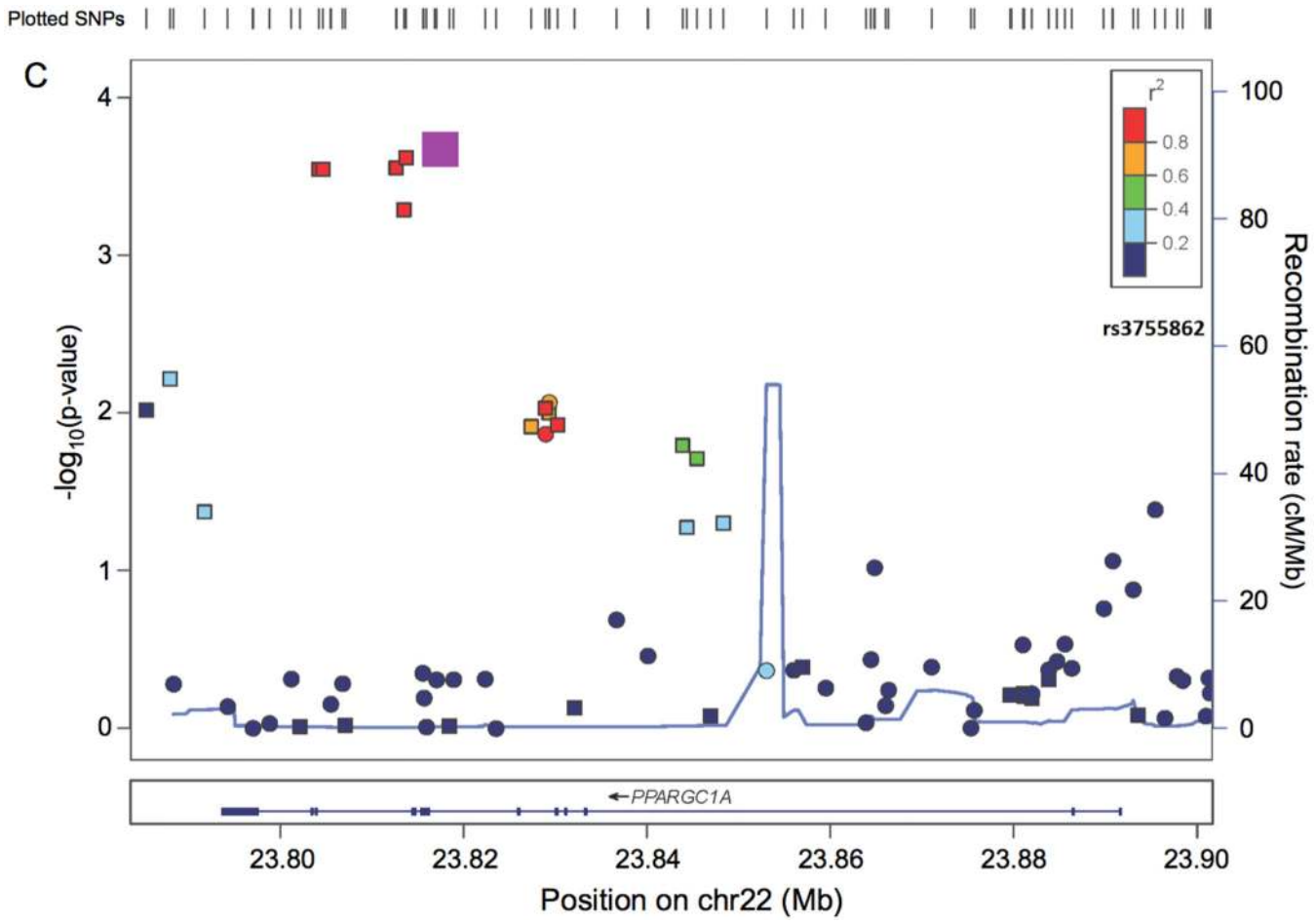


Figure 3. Association results from the Collaborative Study on the Genetics of Alcoholism (COGA)

A. *PPARA* and withdrawal B. *PPARG* and withdrawal C. *PPARGC1A* and AD. Y-axis denotes the $-\log_{10}$ (p-value) for association. X-axis is the physical position on the chromosome (Mb). The most significantly associated SNP is denoted with a purple symbol, and the SNP name is shown below the color scale. The extent of linkage disequilibrium (LD, as measured by r^2) between each SNP and the most significantly associated SNP with the lowest p-value within the gene is indicated by the color scale. Larger values of r^2 indicate greater LD. Association results with genotyped SNPs are shown as a circle while association results with imputed SNPs are shown as a square.

Table 1

Fenofibric acid concentrations in mouse brain, plasma, and liver

Fenofibric acid concentrations are shown in tissues after either 1 or 8 days of oral fenofibrate injection (150 mg/kg; n=6 per group). Tissues were harvested 2 hours after the final injection and analyzed by LC-MS/MS. Numbers represent the mean with the standard error in parentheses.

	1 day	**8 days**
Brain (μg/g)	1.22 (0.266)	0.881 (0.189)
Plasma (μg/mL)	37.2 (6.63)	58.2 (13.7)
Liver (μg/g)	103 (7.17)	131 (9.79)