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KLB is associated with alcohol drinking, and its gene product β-Klotho is necessary for FGF21 regulation of alcohol preference

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β-Klotho is necessary for FGF21 regulation of alcohol preference

Excessive alcohol consumption is a major public health problem worldwide. While drinking habits are known to be inherited, few genes have been identified that are robustly linked to alcohol drinking. We conducted a genome-wide association meta-analysis and replication study among >105,000 individuals of European ancestry, and identified β-Klotho (KLB) as a locus associated with alcohol consumption (rs11940694; \( P=9.2 \times 10^{-12}\)). β-Klotho is an obligate coreceptor for the hormone FGF21, which is secreted from the liver and implicated in macronutrient preference in man. We show that brain-specific β-Klotho knock-out mice have an increased alcohol preference and that FGF21 inhibits alcohol drinking by acting on the brain. These data suggest that a liver-brain endocrine axis may play an important role in the regulation of alcohol drinking behavior and provide a unique pharmacologic target for reducing alcohol consumption.

**Introduction**

Excessive alcohol consumption is a major public health problem worldwide causing an estimated 3.3 million deaths in 2012 (1). Much of the behavioral research associated with alcohol has focused on alcohol-dependent patients. However, the burden of alcohol-associated disease largely reflects the amount of alcohol consumption in a population, not alcohol dependence (2). It has long been recognized that small shifts in the mean of a continuously distributed behavior such as alcohol drinking can have major public health benefits (3). For example, a shift from heavy to moderate drinking could have beneficial effects on cardiovascular disease risk (4).

Alcohol drinking is a heritable complex trait (5). Genetic variants in the alcohol and aldehyde dehydrogenase gene family can result in alcohol intolerance caused by altering peripheral alcohol metabolism, and may thus influence alcohol consumption and dependence (6). However, genetic influences on brain functions affecting drinking behavior have been more difficult to detect because, as for many complex traits, the effect of individual genes is small, so large sample sizes are required to detect the genetic signal (7).

Here we report a genome-wide association (GWAS) and replication study of over 100,000 individuals of European descent. We identify a gene variant in β-Klotho (KLB) that associates with alcohol consumption. β-Klotho is a single-pass transmembrane protein that complexes with FGF receptors to form cell surface receptors for the hormones FGF19 and FGF21 (8, 9). FGF19 is induced by bile acids in the small intestine to regulate bile acid homeostasis and metabolism in the liver (9). FGF21 is induced in liver and released into the blood in response to various metabolic stresses, including high carbohydrate diets and alcohol (10-12). Notably, FGF21 was recently associated in a human GWAS study with macronutrient preference, including changes in carbohydrate, protein and fat intake (13). Moreover, FGF21 was shown to suppress sweet and alcohol preference in mice (14, 15). Our current findings suggest that the FGF21-β-Klotho signaling pathway regulates alcohol consumption in humans.

**Results**

**Association of KLB gene SNP rs11940694 with alcohol drinking in humans**

We carried out a GWAS of quantitative data on alcohol intake in 70,460 individuals (60.9% women) of European descent from 30 cohorts. We followed up the most significantly associated SNPs (six sentinel SNPs \( P<1.0 \times 10^{-8}\) from independent regions) among up to 35,438 individuals from 14 additional cohorts (Dataset S1; and Appendix 1). We analyzed both continuous data on daily alcohol intake in drinkers (as g/day, log transformed) and a dichotomous variable of heavy versus light or no drinking (Dataset S1).

Alcohol intake in drinkers across the samples was 14.0 g/day in men and 6.0 g/day in women. We performed per cohort sex-specific and combined-sex single SNP regression analyses under an additive genetic model, and conducted meta-analysis across the sex-specific strata and cohorts using an inverse variance weighted fixed effects model.

Results of the primary GWAS for log g/day alcohol are shown in Figures 1 and S1, Dataset S2. We identified five SNPs for replication at \( P<1 \times 10^{-6}\): rs11940694 in the KLB gene, rs197273 in TANK, rs7809469 in GCKR, rs350721 in ASB3 and rs10959202 in AUTS2 (Table 1, Dataset S2). In addition to rs1095202 in AUTS2 (\( P=2.9 \times 10^{-7}\)), we took forward SNP rs9435555 in AUTS2 (\( P=1.4 \times 10^{-5}\)), which was previously reported in relation to alcohol drinking (7). In both men and women the newly discovered SNPs were all significantly associated with log g/day alcohol at \( P<0.005\) (Table S1). When combining discovery and replication data, we observed genome-wide significance for SNP rs11940694 (A/G) in KLB (\( P=9.2 \times 10^{-12}\) (Table 1 and Figure S1), for which the minor allele A was associated with reduced drinking. KLB is localized on human chromosome 4p14 and encodes a transmembrane protein, β-Klotho, which is an essential component of receptors for FGF19 and FGF21 (8, 9). Rs197273 in the TRAF family member-associated NF-kappa-B activator gene (TANK) narrowly missed reaching genome-wide significance in the combined sample (Table 1, \( P=4.4 \times 10^{-8}\)). In the dichotomous analysis of the primary GWAS, SNP rs17509112 in the Caderin 13 gene (CDH13) and rs10972848 in the Tramembrae protein 82 gene (TMEM82) were significant at \( P=2.3 \times 10^{-8}\) and \( P=2.6 \times 10^{-7}\), respectively (Figure S2, Table S2 and Dataset S2), but did not reach genome wide significance in the combined analysis (Table S2).

SNP rs11940694 is localized in intron 1 of the KLB gene. The local linkage disequilibrium (LD) structure of the KLB gene is shown in Figure S3. The minor allele frequencies of this SNP were generally high (between 0.37 and 0.44) in different ethnic groups (Table S3). We found no significant association of rs11940694 with gene expression in peripheral blood of 5,236 participants of the Framingham heart study (Table S4) (16). β-Klotho in the brain controls alcohol drinking in mice

**Significance**

Alcohol is a widely consumed drug in western societies that can lead to addiction. A small shift in consumption can have dramatic consequences on public health. We performed the largest genome-wide association meta-analysis and replication study to date (>105,000 individuals) and identified a new genetic basis for alcohol consumption during non-addictive drinking. We found a locus in the gene encoding β-Klotho (KLB) is associated with alcohol consumption. β-Klotho is an essential receptor component for the endocrine fibroblast growth factors (FGFs) 19 and 21. Using mouse models and pharmacologic administration of FGF21, we demonstrate that β-Klotho in the brain controls alcohol drinking. These findings reveal a mechanism regulating alcohol consumption in humans that may be pharmacologically tractable for reducing alcohol intake.
To examine whether β-Klotho affects alcohol drinking in mice, and whether it does so through actions in the brain, we measured alcohol intake and the alcohol preference ratio of brain-specific β-Klotho-knockout (Klb\textsuperscript{Camk2a\textminus}) mice and control floxed Klb (Klb\textsuperscript{fl/fl}) mice. We used a voluntary two-bottle drinking assay performed with water and alcohol. Since we previously showed that FGF21-transgenic mice, which express FGF21 at pharmacologic levels, have a reduced alcohol preference (14), we performed these studies while administering either recombinant FGF21 or vehicle by osmotic minipump. Alcohol preference versus water was significantly increased in vehicle-treated Klb\textsuperscript{Camk2a\textminus} compared to Klb\textsuperscript{fl/fl} mice at 16 vol. % alcohol (Fig. 2A). FGF21 suppressed alcohol preference in Klb\textsuperscript{fl/fl} mice, but not in Klb\textsuperscript{Camk2a\textminus} demonstrating that the effect of FGF21 on alcohol drinking depends on β-Klotho expressed in the brain (Fig. 2A). There was a corresponding decrease in plasma alcohol levels immediately after 16 vol. % alcohol drinking, which reflects the modulation of the drinking behavior (Fig. 2B). However, plasma FGF21 levels were comparable in Klb\textsuperscript{fl/fl} and KlbCamk2a mice administered recombinant FGF21 at the end of the experiment (Fig. 2C). Alcohol bioavailability was not different between FGF21 treated Klb\textsuperscript{fl/fl} and KlbCamk2a mice (Fig. 2D). We have previously shown that FGF21 decreases the sucrose and saccharin preference ratio in Klb\textsuperscript{fl/fl} but not KlbCamk2a mice, and has no effect on the quinine preference ratio (14). To rule out a potential perturbation of our findings as a result of the experimental procedure, we independently measured preference and consumption of 16 vol. % alcohol in Klb\textsuperscript{fl/fl} and KlbCamk2a mice without osmotic minipump implantation. Again, Klb\textsuperscript{Camk2a\textminus} mice showed significantly greater alcohol consumption and increased alcohol preference compared to Klb\textsuperscript{fl/fl} mice (Fig. 2E and F), thus replicating our findings above. Alcohol bioavailability after an intraperitoneal injection was not different between Klb\textsuperscript{fl/fl} and KlbCamk2a mice after 1 and 3 hours (Fig. 2G).

β-Klotho in brain does not regulate emotional behavior in mice

Increased alcohol drinking in humans and mice may be motivated by its reward properties or as a means to relieve anxiety.
Fig. 2. FGF21 reduces alcohol preference in mice by acting on β-Klotho in brain. (A) Alcohol preference ratios determined by two-bottle preference assays with water and the indicated ethanol concentrations for control (Klb<sup>fl/fl</sup>) and brain-specific β-Klotho knockout (KlbCamk2a) mice administered either FGF21 (0.7 mg/kg/day) or vehicle (n=10/group). (B) Plasma ethanol and (C) FGF21 concentrations at the end of the 16% ethanol step of the two-bottle assay. (D) Plasma ethanol concentrations 1 and 3 hours after i.p. injection of 2 g/kg alcohol (n=4 each group). (E) Consumption of 16% ethanol (g/kg/d) and (F) alcohol preference ratios in two-bottle preference assays performed with control (Klb<sup>fl/fl</sup>) and brain-specific β-Klotho-knockout (KlbCamk2a) mice. Alcohol preference was measured by volume of ethanol/total volume of fluid consumed (n=13/group). Values are means ± S.E.M. For (A-C, **p<0.05; ***p<0.001 for Klb<sup>fl/fl</sup> + vehicle versus Klb<sup>fl/fl</sup> + FGF21 groups; and ##p<0.01; ###p<0.001 for Klb<sup>fl/fl</sup> + FGF21 versus KlbCamk2a + FGF21 groups as determined by one-way ANOVA followed by Tukey’s post-tests. For (E, F), *p<0.05 and **p<0.01.

Fig. 3. Behavior tests in brain-specific β-Klotho knockout mice. Results from (A) novelty suppressed feeding, (B) elevated plus maze and (C) open field activity assays performed with control (Klb<sup>fl/fl</sup>) and brain-specific β-Klotho-knockout (KlbCamk2a) mice (n=15 each group). Values are the time (seconds) spent for each step of the assay.

and stress (17). In mice, FGF21 increases corticotropin-releasing hormone expression in hypothalamus, circulating glucocorticoid concentrations and sympathetic outflow (18-20), which are linked to heightened anxiety. We therefore tested Klb<sup>ββ</sup> and KlbCamk2a mice in behavioral paradigms measuring anxiety, including novelty suppressed feeding (Fig. 3A), elevated plus maze (Fig. 3B),...
Here we report that in a GWAS performed in over 100,000 individuals, SNP rs11940694 in KLB associates with alcohol consumption in non-addicts. We further show that mice lacking β-Klotho in the brain have increased alcohol consumption and are refractory to the inhibitory effect of FGF21 on alcohol consumption. These findings reveal a previously unrecognized brain pathway regulating alcohol consumption in humans that may prove pharmacologically tractable for suppressing alcohol drinking.

**Discussion**

In the animal model, β-Klotho acts by affecting neighboring genes. Thereby, altering the expression of FGF21, a hormone involved in the regulation of alcohol consumption. The absence of KLB results in a decreased expression of β-Klotho in the ventral tegmental area, a brain region involved in alcohol reward behavior.

**Methods**

**Alcohol phenotypes**

Alcohol intake in grams of alcohol per day was estimated by each study based on information about drinking frequency and type of alcohol consumed. For cohorts that collected data in ‘drinks per week’, standard etoh drink sizes were used to estimate daily alcohol intake. For cohorts that collected data in grams of alcohol per week, the number of drinks was calculated based on the weight of alcohol and its density.

**Gene expression profiling in Framingham study**

In the Framingham study, gene expression profiling was undertaken on the blood samples of a total of 5,626 participants from the Offspring (N=2,446) and Heirs (N=3,180) at examination two. Fasting peripheral whole blood samples (2.5ml) were collected in Paxgene Tubes (PreAnalytIX, Hombrechtikon, Switzerland). RNA expression profiling was conducted using the Affymetrix Human Exon Array ST 1.0 (Affymetrix Inc, Santa Clara, CA) for samples that passed RNA quality control. The expression values for ~18,000 transcripts were obtained for replication from discovery GWAS if they passed the above criteria and if they had P<0.10. One SNP with the smallest P value was chosen for replication in each region, except for AUTS2 for which two SNPs were chosen for replication based on previous results (7).

Meta-analyses were performed by METAL (25) or R (v3.2).

**Gene expression profiling in human studies**

In the human studies, gene expression profiling was performed on human tissues, such as brain tissue, to identify genes that are associated with alcohol consumption. The expression levels of these genes were then used to predict alcohol consumption in the population.
experiments, the positions of the two bottles were changed every two days to exclude position effects.

**Mouse experiments with FGF21**

For FGF21 administration studies, recombinant human FGF21 protein provided by Novo Nordisk was administered at a dose of 0.7 mg/kg/day by subcutaneous osmotic mini-pumps (Alzet 1004). C57/BL6J mice were housed in a climate-controlled vivarium with controlled lighting (12 h/12 h light/dark cycle) with food and water available ad libitum. All studies were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Mice were anesthetized with isoflurane before cannulation. Following a 2-hour recovery period, mice were divided into treatment groups and treated intraperitoneally with FGF21 (160 mg/kg body weight) every 2 days for 7 days. Body weights were recorded weekly, and food intake was measured. For elevated plus maze activity assays, mice were placed in the center of a plus maze with 2 dark enclosed arms and 2 open arms. Mice were allowed to move freely around the maze, and the total duration of time spent in the center versus along the walls and total distance traveled were measured. For elevated plus maze activity assays, mice were placed in the center of a plus maze with 2 dark enclosed arms and 2 open arms. Mice were allowed to move freely around the maze, and the total duration of time in each arm and the frequency to enter both the closed and open arms was measured. For novelty suppression of feeding assays, mice were fasted for 12 hours and were placed in a novel environment and the time to approach and eat a known food was measured.

**Statistical analysis**

All data are expressed as means ± S.E.M. Statistical analysis between groups was performed using Student’s t-test using Excel or GraphPad Prism (GraphPad Software, Inc.). For multiple comparisons, one-way analysis of variance (ANOVA) with post-hoc Tukey was done using SPSS.