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

Günter Schumann[1]*†, Chunyu Liu[2-4]*, Paul F. O'Reilly[1]*, He Gao[5,6]*, Parkyong Song[7]*, Bing Xu[1], Barbara Ruggeri[1], Najaf Amin[8], Tianye Jia[1], Sara R. Preis[4], Marcelo P. Segura Lepe[5,9], Shizuo Akira[10], Caterina Barbieri[11], Sebastian E. Baumeister[12,13], Stephane Cauchi[14], Toni-Kim Clarke[15], Stefan Enroth[16], Krista Fischer[17], Jenni Hällfors[18], Sarah E. Harris[19,20], Saskia Hieber[21], Edith Hofer[22,23], Jouke-Jan Hottenga[24], Åsa Johansson[16], Peter K. Joshi[25], Niina Kaartinen[26], Jaana Laitinen[27], Rozenn N. Lemaitre[28], Anu Loukola[18,29], Jian'an Luan[30], Leo-Pekka Lyytikäinen[31], Massimo Mangino[32,33], Ani Manichaikul[34], Hamdi Mbarek[24], Yuri Milaneschi[35], Alireza Moayyeri[5,36,37], Kenneth Mukamal[38], Christopher P. Nelson[39,40], Jennifer A. Nettleton[41], Eemil Partinen[42], Rajesh Rawal[43], Antonietta Robino[44], Lynda M. Rose[45], Cinzia Sala[11], Takashi Satoh[10], Reinhold Schmidt[22], Katharina E. Schraut[25], Robert Scott[46], Albert Vernon Smith[47], John M. Starr[19,48], Alexander Teumer[12,49], Stella Trompet[50,51], André G. Uitterlinden[52,53], Cristina Venturini[32], Anne-Claire Vergnaud[5], Niek Verweij[54], Veronique Vitart[55], Dragana Vuckovic[56], Juho Wedenoja[29], Loic Yengo[14], Bing Yu[57], Weihua Zhang[5,58], Jinghua Zhao[46], Dorret I. Boomsma[24], John C. Chambers[5,58,59], Daniel I. Chasman[45,60], Toniolo Daniela[11], Eco J.C. de Geus[24], Ian J. Deary[19,61], Johan G. Eriksson[26,62-65], Tõnu Esko[17], Volker Eulenburg[66], Oscar H. Franco[53], Philippe Froguel[14,67], Christian Gieger[43], Hans J. Grabe[68], Vilmundur Gudnason[47,69], Ulf Gyllensten[16], Tamara B. Harris[70], Anna-Liisa Hartikainen[71-73], Andrew C. Heath[74], Lynne J. Hocking[75], Albert Hofman[53], Cornelia Huth[76], Marjo-Riitta Jarvelin[5,73,77,78], J. Wouter Jukema[50], Jaakko Kaprio[18,26,29], Jaspal S Kooner[58,59,79], Zoltan Kutalik[80], Jari Lahti[64,81,82], Claudia Langenberg[30], Terho Lehtimäki[31], Yongmei Liu[83], Pamela A.F. Madden[84], Nicholas G. Martin[85], Alanna C. Morrison[57], Brenda W.J.H. Penninx[35], Nicola Pirastu[25,56], Bruce M. Psaty[28,86-88], Olli T. Raitakari[89], Paul M. Ridker[45,60], Richard J. Rose[90], Jerome I. Rotter[91], Nilesh J. Samani[39,40], Helena Schmidt[92], Tim Spector[32], David J. Stott[93], David P. Strachan[94], Ioanna Tzoulaki[5,6,95], Pim van der Harst[54,96,97], Cornelia M. van Duijn[8], Pedro Marques Vidal[98], Peter Vollenweider[98], Nick Wareham[46], John B. Whitfield[85], James F. Wilson[25,55], Bruce H.R. Wolffenbuttel[99], Georgy Bakalkin[100], Evangelos Evangelou[5,95], Yun Liu[101], Kenneth Rice[102], Sylvane Desrivières[1]*, Steven A. Kliewer[7,103]*, David J. Mangelsdorf[7]*†, Christian P. Müller[21]*, Daniel Levy[2,3]*, Paul Elliott[5,6]†*

1 Medical Research Council - Social, Genetic and Developmental Psychiatry Centre, Institute of Psychiatry, Psychology & Neuroscience, King's College London, United Kingdom. 2 The Framingham Heart Study, Framingham, Massachusetts 01702, USA. 3 The Population Sciences Branch, Division of Intramural Research, National Heart, Lung, and Blood Institute, Bethesda, Maryland 20824, USA. 4 Boston University School of Public Health, 715 Albany St, Boston, MA 02118, USA. 5 Department of Epidemiology and Biostatistics, School of Public Health, Imperial College London, London W2 1PG, UK. 6 MRC-PHE Centre for Environment and Health, Imperial College London, London, UK. 7 Department of Pharmacology and Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, USA 75390. 8 Genetic Epidemiology Unit, Department of Epidemiology, Erasmus MC, Rotterdam, the Netherlands. 9 Bayer Pharma AG, Müllerstr 178, 13342 Berlin, Germany. 10 Laboratory of Host Defense, World Premier International Immunology Frontier Research Center, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. 11 Division of Genetics and Cell Biology, San Raffaele Research Institute, Milano, Italy. 12 Institute for Community Medicine, University Medicine Greifswald, Greifswald, 17475, Germany. 13 Department of Epidemiology and Preventive Medicine, University of Regensburg, Germany. 14 CNRS UMR 8199, Lille Pasteur Institute, Lille 2 University, European Genomic Institute for Diabetes (EGID), Lille, France. 15 Divison of Psychiatry, University of Edinburgh, Edinburgh, UK. 16 Science for Life Laboratory, Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden. 17 Estonian Genome Center, University of Tartu, Estonia. 18 Institute for Molecular Medicine (FIMM), University of Helsinki, Helsinki, Finland. 19 Centre for Cognitive Ageing and Cognitive Epidemiology, University of Edinburgh, Edinburgh, UK, EH8 9JZ. 20 Centre for Genomic and Experimental Medicine, University of Edinburgh, Edinburgh, UK, EH4 2XU. 21 Department of Psychiatry and Psychotherapy, Friedrich-Alexander-University Erlangen-Nuremberg, Schwabachanlage 6, 91054 Erlangen, Germany. 22 Clinical Division of Neurogeriatrics, Department of Neurology, Medical University Graz, Austria. 23 Institute of Medical Informatics, Statistics and Documentation, Medical University Graz, Austria. 24 Department of Biological Psychology, Vrije Universiteit Amsterdam and EMGO Institute for Health and Care Research, Amsterdam, The Netherlands. 25 Usher Institute for Population Health Sciences and Informatics, University of Edinburgh, Teviot Place, Edinburgh, EH8 9AG, Scotland. 26 National Institute for Health and Welfare, Helsinki, Finland. 27 Finnish Institute of Occupational Health, Helsinki, Finland. 28 Cardiovascular Health Research Unit, Department of Medicine, University of Washington, Seattle, WA, USA. 29 Department of Public Health, University of Helsinki, Helsinki, Finland. 30 MRC Epidemiology Unit, University of Cambridge School of Clinical Medicine, Institute of Metabolic Science, Cambridge, CB2 0QQ, UK. 31 Department of Clinical Chemistry, Fimlab Laboratories and University of Tampere School of Medicine, Tampere 33520, Finland. 32 Department of Twin Research and Genetic Epidemiology, King's College London. 33 NIHR Biomedical Research Centre at Guy's and St. Thomas' Foundation Trust. 34 Center for Public Health Genomics and Biostatistics Section, Department of Public Health Sciences, University of Virginia, Charlottesville, Virginia 22903, USA. 35 Department of Psychiatry, EMGO Institute for Health and Care Research and Neuroscience Campus Amsterdam, VU University Medical Center/GGZ inGeest, Amsterdam, The Netherlands. 36 Institute of Health Informatics, University College London, 222 Euston Road, London NW1 2DA, UK. 37 Farr Institute of Health Informatics Research, 222 Euston Road, London NW1 2DA, UK. 38 Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA. 39 Department of Cardiovascular Sciences, University of Leicester, Glenfield Hospital, Leicester, UK. 40 National Institute for Health Research Leicester Cardiovascular Biomedical Research Unit, Glenfield Hospital, Leicester, UK. 41 Division of Epidemiology, Human Genetics, and Environmental Sciences, University of Texas Health Sciences Center, Houston, TX, USA. 42 Faculty of Medicine, University of Tartu, Estonia. 43 Department of Molecular Epidemiology, Institute of Epidemiology II, Helmholtz Zentrum München, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany. 44 Institute for Maternal and Child Health - IRCCS "Burlo Garofolo", Trieste, Italy. 45 Division of Preventive Medicine, Brigham and Women's Hospital, Boston, MA 02215, USA. 46 MRC Epidemiology Unit, University of Cambridge School of Clinical Medicine, Cambridge, UK. 47 Icelandic Heart Association, Kopavogur, Iceland. 48 Alzheimer Scotland Research Centre, University of Edinburgh, Edinburgh, UK, EH8 9JZ. 49 Interfaculty Institute for Genetics and Functional Genomics, University Medicine Greifswald, Greifswald, 17475, Germany. 50 Department of Cardiology, Leiden University Medical Center, Leiden, the Netherlands. 51 Department of Gerontology and Geriatrics, Leiden University Medical Center, Leiden, the Netherlands. 52 Department of internal Medicine, Erasmus MC, Rotterdam, the Netherlands. 53 Department of Epidemiology, Erasmus MC, Rotterdam, the Netherlands. 54 University of Groningen, University Medical Center Groningen, Department of Cardiology, 9700RB Groningen, The Netherlands. 55 MRC Human Genetics Unit, Institute for Genetics and Molecular Medicine, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, Scotland. 56 Department of Medical Sciences, University of Trieste, Italy. 57 Human Genetics Center and Div. of Epidemiology, University of Texas Health Science Center at Houston, Houston, TX, USA. 58 Department of Cardiology, Ealing Hospital, Uxbridge Road, Middlesex UB1 3HW, UK. 59 Imperial College Healthcare NHS Trust, London W12 0HS, UK. 60 Harvard Medical School, Boston, MA 02115, USA. 61 Psychology, University of Edinburgh, Edinburgh, UK, EH8 9JZ. 62 Department of General Practice and Primary health Care, University of Helsinki, Finland. 63 Helsinki University Central Hospital, Unit of General Practice, Helsinki, Finland. 64 Folkhälsan Research Centre, Helsinki, Finland. 65 Vasa Central Hospital, Vasa, Finland. 66 Institute for Biochemistry and Molecular Medicine, Friedrich-Alexander-University Erlangen-Nuremberg, Fahrstrasse 17, 91054 Erlangen, Germany. 67 Department of Genomics of Common Disease, School of Public Health, Imperial College London, London, UK. 68 Department of Psychiatry and Psychotherapy, University Medicine Greifswald, 17475 Greifswald, Germany. 69 Faculty of Medicine, University of Iceland, Reykjavik, Iceland. 70 National Institute on Aging; National Institutes of Health, Bethesda, MD, USA. 71 Department of Obstetrics and Gynecology, Oulu University Hospital, Oulu, Finland 72 Medical Research Center, University of Oulu, Oulu, Finland. 73 Unit of Primary Care, Oulu University Hospital, Oulu, Finland. 74 Department of Psychiatry, Washington University School of Medicine in St. Louis, 660 S. Euclid Ave., St. Louis, MO 63110. 75 Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK. 76 Institute of Epidemiology II, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Neuherberg, Germany. 77 Center for Life Course Epidemiology, Faculty of Medicine, P.O.Box 5000, FI-90014 University of Oulu, Finland. 78 Biocenter Oulu, P.O.Box 5000, Aapistie 5A, FI-90014 University of Oulu, Finland. 79 Faculty of Med, National Heart & Lung Institute, Cardiovascular Science, Hammersmith Campus, Hammersmith Hospital, Hammersmith Campus, Imperial College London, London W12 0NN, UK. 80 Institute of Social and Preventive Medicine, Centre Hospitalier Universitaire Vaudoise (CHUV), Lausanne 1010, Switzerland. 81 Collegium for Advanced Studies, University of Helsinki, Finland. 82 Institute of Behavioural Sciences, University of Helsinki, Finland. 83 Wake Forest School of Medicine, Department of Epidemiology & Prevention, Public Health Sciences,

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; $P=9.2x10^{-12}$). β-Klotho is an obligate co-receptor 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.

alcohol consumption | β-Klotho | FGF21 | mouse model | human

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 $P<1.0x10^{-6}$ 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). Average 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 $P<1x10^{-6}$: rs11940694 in the *KLB* gene, rs197273 in *TANK*, rs780094 in *GCKR*, rs350721 in *ASB3* and rs10950202 in *AUTS2* (Table 1, Dataset S2). In addition to rs10950202 in *AUTS2* ($P=2.9x10^{-7}$), we took forward SNP rs6943555 in *AUTS2* ($P=1.4x10^{-4}$), 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* ($P=9.2x10^{-12}$) (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; $P=7.4x10^{-8}$). In the dichotomous analysis of the primary GWAS, SNP rs12599112 in the Cadherin 13 gene (*CDH13*) and rs10927848 in the Transmembrane protein 82 gene (*TMEM82*) were significant at $P=2.3x10^{-8}$ and $P=2.6x10^{-7}$, 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 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.

Reserved for Publication Footnotes

Table 1. Associations of single nucleotide polymorphisms* with alcohol intake (log g/day) in the genome-wide association analysis (GWAS).

SNP	Chr	Position (hg 19)	Nearest gene	Effect / other alleles	EAF#	Discovery GWAS		Replication		Combined		
						Beta (SE)	*P*-value	Beta (SE)	*P*-value	Beta (SE)	*P*-value	N
rs780094	2	27741237	*GCKR*	T/C	0.40	-0.0155 (0.0026)	$3.6x10^{-9}$	0.0035 (0.0029)	0.238	-0.0102 (0.0019)	$1.6x10^{-7}$	98,679
rs350721	2	52980427	*ASB3*	C/G	0.18	0.0206 (0.0040)	$3.2x10^{-7}$	-0.0000 (0.0042)	0.994	0.0109 (0.0029)	$1.9x10^{-4}$	100,859
rs197273	2	161894663	*TANK*	A/G	0.49	-0.0141 (0.0026)	$9.8x10^{-8}$	-0.0058 (0.0028)	0.040	-0.0103 (0.0019)	$7.4x10^{-8}$	97,631
rs11940694	4	39414993	*KLB*	A/G	0.42	-0.0137 (0.0027)	$3.2x10^{-7}$	-0.0135 (0.0030)	$5.2x10^{-6}$	-0.0136 (0.0020)	$9.2x10^{-12}$	98,477
rs6943555	7	69806023	*AUTS2*	A/T	0.29	-0.0115 (0.0030)	$1.4x10^{-4}$	-0.0070 (0.0033)	0.032	-0.0094 (0.0022)	$1.9x10^{-5}$	104,282
rs10950202	7	69930098	*AUTS2*	G/C	0.16	-0.0194 (0.0038)	$2.9x10^{-7}$	-0.0015 (0.0042)	0.720	-0.0113 (0.0028)	$5.9x10^{-5}$	105,639

* One SNP with smallest *P*-value taken forward per region
# Effect Allele Frequency, in Discovery GWAS

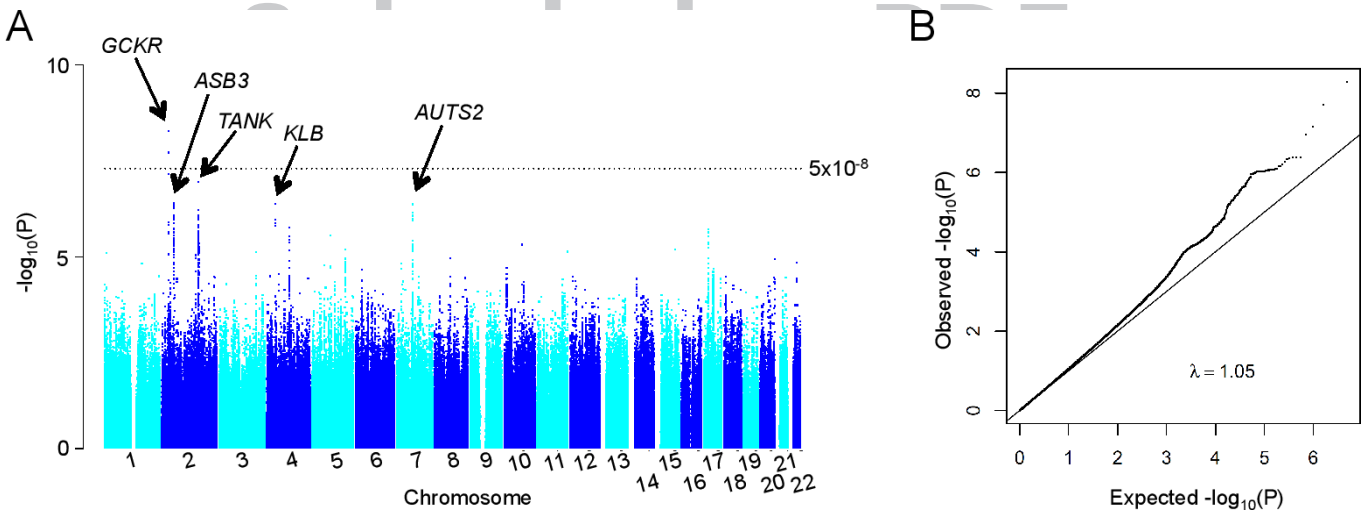

Fig. 1. . Genome-wide association results of log g/day alcohol in AlcGen and CHARGE+ consortia. **(A)** Manhattan plot showing the significance of the association (-$\log_{10}$ transformed *P* value on the y axis) for each SNP at chromosomal position shown on the x axis. The dotted line represents the genome-wide significance level at $P=5x10^{-8}$. The genes that were followed up are labelled. **(B)** Quantile-quantile plot comparing the expected *P* value on the x axis and the observed *P* value on the y axis (both were -log10 transformed).

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^{Camk2a}) mice and control floxed *Klb* ($Klb^{fl/fl}$) 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^{Camk2a} compared to $Klb^{fl/fl}$ mice at 16 vol. % alcohol (Fig. 2A). FGF21 suppressed alcohol preference in $Klb^{fl/fl}$ mice, but not in Klb^{Camk2a} 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 $Klb^{fl/fl}$ and *KlbCamk2a* mice administered recombinant FGF21 at the end of the experiment (Fig. 2C). Alcohol bioavailability was not different between FGF21 treated $Klb^{fl/fl}$ and *KlbCamk2a* mice (Fig. 2D). We have previously shown that FGF21 decreases the sucrose and saccharin preference ratio in $Klb^{fl/fl}$ 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 $Klb^{fl/fl}$ and Klb^{Camk2a} mice without osmotic minipump implantation. Again, Klb^{Camk2a} mice showed significantly greater alcohol consumption and increased alcohol preference compared to $Klb^{fl/fl}$ mice (Fig. 2E and F), thus replicating our findings above. Alcohol bioavailability after an intraperitoneal injection was not different between $Klb^{fl/fl}$ and Klb^{Camk2a} 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

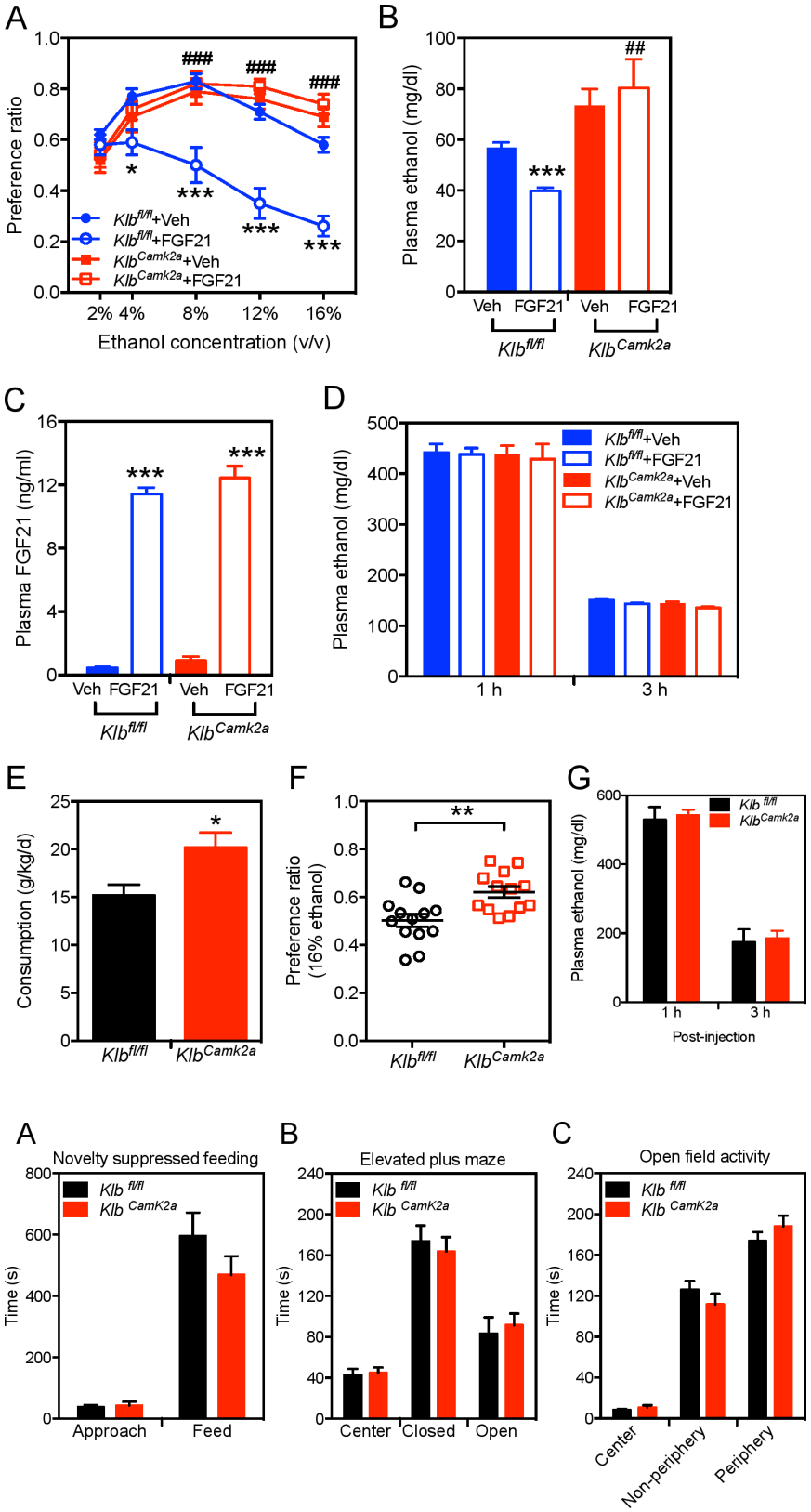

Fig. 2. FGF21 reduces alcohol preference in mice by acting on β-Klotho in brain. **(A)** Alcohol preference ratios determined by two-bottle preference assays with water and the indicated ethanol concentrations for control (*Klb*$^{fl/fl}$) and brain-specific β-Klotho knockout (*KlbCamk2a*) mice administered 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 preferences assays performed with control (*Klb*$^{fl/fl}$) and brain-specific β-Klotho-knockout (*KlbCamk2a*) mice. Alcohol preference was measured by volume of ethanol/total volume of fluid consumed (n=13/group). **(G)** Plasma ethanol concentrations 1 and 3 hours after i.p. injection of 2 g/kg alcohol (n=5/group). Values are means ±S.E.M. For **(A-C),** *p<0.05; ***p<0.001 for *Klb*$^{fl/fl}$ + vehicle versus *Klb*$^{fl/fl}$ + FGF21 groups; and $^{\#\#}$p<0.01; $^{\#\#\#}$p<0.001 for *Klb*$^{fl/fl}$ + FGF21 versus *Klb*^{Camk2a} + 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*$^{fl/fl}$) and brain-specific β-Klotho-knockout (*Klb*^{Camk2a}) 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*$^{fl/fl}$ and *Klb*^{Camk2a} mice in behavioral paradigms measuring anxiety, including novelty suppressed feeding (Fig. 3A), elevated plus maze (Fig. 3B),

and open field activity tests (Fig. 3C). However, we did not find differences between $Klb^{fl/fl}$ and Klb^{Camk2a} mice in any of these anxiety measures or in general locomotor activity. Our finding of increased alcohol preference in Klb^{Camk2a} mice may thus be caused by alteration of alcohol-associated reward mechanisms. While this notion is consistent with our previous results showing *Klb* expression in areas important for alcohol reinforcement, specifically the nucleus accumbens and the ventral tegmental area (14), additional studies will be required to determine precisely where in the brain and how β-Klotho affects alcohol drinking.

Discussion

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.

FGF21 is induced in liver by simple sugars through a mechanism involving the transcription factor carbohydrate response element binding protein (10, 11, 15, 21, 22). FGF21 in turn acts on brain to suppress sweet preference (14, 15). Thus, FGF21 is part of a liver-brain feedback loop that limits the consumption of simple sugars. Notably, FGF21 is also strongly induced in liver by alcohol and contributes to alcohol-induced adipose tissue lipolysis in a mouse model of chronic-binge alcohol consumption (12). Our present data suggest the existence of an analogous feedback loop wherein liver-derived FGF21 acts on brain to limit the consumption of alcohol. However, additional studies will be required to establish the existence of this FGF21 pathway in vivo.

In murine brain, there is evidence that FGF21 suppresses sweet preference through effects on the paraventricular nucleus in the hypothalamus (15). Among its actions in the hypothalamus, FGF21 induces corticotropin-releasing hormone (18, 19), which is a strong modulator of alcohol consumption (23). Notably, β-Klotho is also present in mesolimbic regions of the brain that regulate reward behavior, including the ventral tegmental area and nucleus accumbens, and FGF21 administration reduced tissue levels of dopamine and its metabolites in the nucleus accumbens (14). Thus, FGF21 may act coordinately on multiple brain regions to regulate the consumption of both simple sugars and alcohol.

In closing, our data linking β-Klotho to alcohol consumption together with previous GWAS data linking FGF21 to macronutrient preference raise the intriguing possibility of a liver-brain endocrine axis that plays an important role in the regulation of complex adaptive behaviors, including alcohol drinking. While our findings support an important role for the *KLB* gene in the regulation of alcohol drinking, we cannot rule out the possibility that *KLB* rs11940694 acts by affecting neighboring genes. Therefore additional genetic and mechanistic studies are warranted. Finally, it will be important to follow up on our findings in more severe forms of alcohol drinking, since our results suggest that this pathway could be targeted pharmacologically for reducing the desire for alcohol.

Methods

Alcohol phenotypes

Alcohol intake in grams of alcohol per day was estimated by each cohort based on information about drinking frequency and type of alcohol consumed. For cohorts that collected data in 'drinks per week', standard ethanol contents in different types of alcohol drinks were provided as guidance to convert the data to 'grams per week', which was further divided by 7 to give intake as 'grams per day'. Adjustment was made if cohort-specific drink sizes differed from the standard. For cohorts that collected alcohol use in grams of ethanol per week, the numbers were divided by 7 directly into 'grams per day'. Cohorts with only a categorical response to the question for drinks per week used mid-points of each category for the calculation. All non-drinkers (individuals reporting zero drinks per week) were removed from the analysis. The 'grams per day' variable was then $\log_{10}$ transformed prior to the analysis. Sex-specific residuals were derived by regressing alcohol in $\log_{10}$ (grams per day) in a linear model on age, age-square, weight, and if applicable, study site and principal components to account for population structure. The sex-specific residuals were pooled and used as the main phenotype for subsequent analyses.

Dichotomous alcohol phenotype was created based on categorization of 'drinks per week' variable. Heavy drinking was defined as >=21 drinks per week in men, or >=14 drinks per week in women. Light (or zero) drinking was defined if male participants had <=14 drinks per week, or female participants had <=7 drinks per week. Drinkers having >14 to <21 drinks for men, or >7 to <14 drinks for women were excluded. Where information was available, current non-drinker who was former drinker of >14 drinker per week in men, and >7 drinks per week in women, as well as current non-drinker who was a former drinker of unknown amount were excluded; whereas current non-drinkers who were former drinkers of <=14 for men or <=7 for women were included. Further exclusion was made if there were missing data on alcohol consumption or on the covariates.

The analyses only included participants of European origin and were performed in accordance with the principles expressed in the Declaration of Helsinki. Each cohort's study protocol was reviewed and approved by their respective institutional review board and informed consent was obtained from all study subjects.

Discovery GWAS in AlcGen and CHARGE+ and replication analyses

Genotyping methods are summarized in Dataset S1B, S1C and S1F. SNPs were excluded if: HWE $P < 1\text{x}10^{-6}$ or based on cohort-specific criteria; MAF < 1%; imputation information score < 0.5; if results were only available from 2 or fewer cohorts, or total N < 10,000. Population structure was accounted for within cohorts via principal components analysis (PCA). Linkage disequilibrium (LD) score regression (24) was conducted on the GWAS summary results to examine the degree of inflation in test statistics, and genomic control correction was considered unnecessary (λ_{GC}=1.06 and intercept=1.00; λ=0.99 to 1.06 for individual cohorts, Dataset S1B and S1C). SNPs were taken forward for replication from discovery GWAS if they passed the above criteria and if they had $P < 1\text{x}10^{-6}$ (one SNP with the smallest *P* taken forward in each region, except for *AUTS2* for which two SNPs were taken forward based on previous results (7)). Meta-analyses were performed by METAL (25) or R (v3.2.2).

Gene expression profiling in Framingham study

In the Framingham study, gene expression profiling was undertaken for the blood samples of a total of 5,626 participants from the Offspring (N=2,446) at examination eight and the Third Generation (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 from the total 1.2 million core probe sets. Quality control procedures for transcripts have been described previously. All data used herein are available online in dbGaP (http://www.ncbi.nlm.nih.gov/gap; accession number phs000007).

The *cis*-expression quantitative trait loci analysis in the Framingham study

To investigate possible effects of rs11940694 in *KLB* on gene expression, we performed *cis*-eQTL analysis. The SNP in *KLB* was used as the independent variable in association analysis with the transcript of *KLB* measured using whole blood samples in the FHS (n=5,236). Affymetrix probe 2724308 was used to represent the *KLB* overall transcript levels. Age, sex, BMI, batch effects and blood cell differentials were included as covariates in the association analysis. Linear mixed model was used to account for familial correlation in association analysis.

Mouse studies

All mouse experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center. Male littermates (2 to 4-month-old) maintained on a 12 hr light/dark cycle with *ad libitum* access to chow diet (Harlan Teklad TD2916) were used for all experiments. The *Klb* gene was deleted from brain by crossing $Klb^{fl/fl}$ mice with *Camk2a*-Cre mice on a mixed C57BL/6J;129/Sv background as described (26).

Alcohol drinking in mice

For voluntary two-bottle preference experiments, male mice (n=9-13 per group) were given access to two bottles, one containing water and the other containing 2-16% ethanol (vol/vol) in water. After acclimation to the two-bottle paradigm, mice were exposed to each concentration of ethanol for 4 days. Total fluid intake (water + ethanol-containing water), food intake and body weight were measured each day. Alcohol consumption (g) was calculated based on EtOH density (0.789 g/ml). To obtain accurate alcohol intake that corrected for individual differences in littermate size, alcohol consumption was normalized by body weight per day for each mouse. As a measure of relative alcohol preference, the preference ratio was calculated at each alcohol concentration by dividing total consumed alcohol solution (ml) by total fluid volume. Two-bottle preference assays were also performed with sucrose (0.5 and 5%) and quinine (2 and 20 mg/dl) solutions. For all

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). Mice were single caged following mini-pump surgery, which was conducted under isoflurane anesthesia and 24 hour buprenorphine analgesia. Mice were allowed to recover from mini-pump surgery for 4 days prior to alcohol drinking tests. After experiments, mice were sacrificed by decapitation and plasma was collected using EDTA or heparin after centrifugation for 15 minutes at 3000 rpm. Plasma FGF21 concentrations were measured using the Biovendor FGF21 ELISA Kit according to manufacturer's protocol.

Plasma ethanol concentration and clearance

For alcohol bioavailability tests, mice (n=4-5 per group) were injected i.p. with alcohol (2.0 g/kg, 20% w/vol) in saline, and tail vein blood was collected after 1 and 3 hours. Plasma alcohol concentrations were measured using the EnzyChrom™ Ethanol Assay Kit.

Emotional behavior in mice

For open field activity assays, naïve mice were placed in an open arena (44 x 44 cm, with the center defined as the middle 14 x 14 cm and the periphery defined as the area 5 cm from the wall), and the amount 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 fasted for 12 hours 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 the two groups was performed by unpaired two-tailed 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.

Acknowledgments

Funding sources and acknowledgments for contributing authors and consortia can be found in the supplementary information (Appendix 2). Part of this work used computing resources provided by the MRC-funded UK MEDical Bioinformatics Partnership Programme (UK MED-BIO) (MR/L01632X/1). **Author contributions** G.S., P.E., D.L., C.P.M., D.J.M. and S.A.K. designed the study, acquired and analyzed data, and wrote the manuscript; P.S. performed animal experiments, acquired and analyzed data, and contributed to writing the manuscript; C.L., P.F.O'R., H.G. and E.E. analyzed GWAS data and contributed to writing the manuscript; B.X., B.R. and S.D. carried out functional analyses and contributed to writing the manuscript; G.B. and Yu.L. acquired and analyzed epigenetic data; N.A., T.J., S.R.P., M.P.S.L., and K.R. analyzed GWAS data. The following authors contributed to the primary GWAS and replication by participating in (i) study concept/design: D.I. B., J.C.C., D.I.C, T.D., I.J.D., E.J.C. deG., J.G.E., T.E., O.H.F., P.F., C.G., H.J.G., V.G., U.G., T.B.H., A.-L.H., A.C.H., A.H., C.H., M.-R.J., J.W.J., J.K., J.S.K., J.L., C.L., T.L., D.L., Y.L., P.A.F.M., N.G.M., A.C.M., J.A.N., B.W.J.H.P., N.P., B.M.P., O.T.R., P.M.R., R.J.R., J.I.R., N.J.S., H.S., R.S., T.S., Ta.S., J.M.S., D.J.S., D.P.S., S.T., I.T., P.vdH., C.M.vD., P.V., N.W., J.F.W., B.H.R.W.; (ii) data acquisition: S.E.B., D.I.B., J.C.C., D.I.C., I.J.D., E.J.C.deG., U.G., T.E., O.H.F., P.F., V.G., S.E.H., T.B.H., A.-L.H., A.C.H., L.J.H., A.H., C.H., M.-R.J., J.W.J., N.K., J.K., J.S.K., J.L., C.L., T.L., D.L., Yo.L., P.A.F.M., N.G.M., A.C.M., K.M., J.A.N., B.W.J.H.P., B.M.P., O.T.R., P.M.R., R.J.R., J.I.R., C.S., N.J.S., H.S., R.S., J.M.S., D.J.S., D.P.S., I.T., P.vdH., C.M.vD., A.G.U., C.V., V.V., P.V., N.W., J.B.W., J.F.W. B.H.R.W; (iii) data analysis: N.A., S.A., C.B., S.E.B., S.C., T.-K.C., S.E., K.F., C.G., J.H, S.H., S.E.H., A.C.H., E.H., J.-J.H., Å.J., P.K.J., Z.K., J,-A.L., R.N.L., C.L., Cl.L., Yo.L., A.L., J.L., Jari L., L.-P.L., M.M., A.M., N.G.M., H.M., Y.M., Ani.M., C.P.N., J.A.N., E.P., N.P., S.R.P., R.R., A.R., L.M.R., K.E.S., R.S., Rh.S., A.V.S., D.P.S., A.T., S.T., A.-C.V., N.V., P.M.-V., V.V., D.V., J.W., J.B.W., L.Y., B.Y., W.Z., J.Z. **Conflict of interest statement:** Dr. Psaty serves on the DSMB for a clinical trial funded by the manufacturer (Zoll LifeCor) and on the Steering Committee of the Yale Open Data Access project funded by Johnson & Johnson. Dr. Mangelsdorf serves on the scientific advisory board of Metacrine. The other authors report no competing financial interests.

1. Anonymous (2014) *Global status report on alcohol and health* (World Health Organization).
2. Rehm J, *et al.* (2009) Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders. *Lancet* 373(9682):2223-2233.
3. Rose G (1981) Strategy of prevention: lessons from cardiovascular disease. *Br Med J (Clin Res Ed)* 282(6279):1847-1851.
4. Hines LM & Rimm EB (2001) Moderate alcohol consumption and coronary heart disease: a review. *Postgrad Med J* 77(914):747-752.
5. Heath AC, Meyer J, Eaves LJ, & Martin NG (1991) The inheritance of alcohol consumption patterns in a general population twin sample: I. Multidimensional scaling of quantity/frequency data. *J Stud Alcohol* 52(4):345-352.
6. Bierut LJ, *et al.* (2012) ADH1B is associated with alcohol dependence and alcohol consumption in populations of European and African ancestry. *Mol Psychiatr* 17(4):445-450.
7. Schumann G, *et al.* (2011) Genome-wide association and genetic functional studies identify autism susceptibility candidate 2 gene (AUTS2) in the regulation of alcohol consumption. *Proc Natl Acad Sci USA* 108(17):7119-7124.
8. Fisher FM & Maratos-Flier E (2016) Understanding the Physiology of FGF21. *Annu Rev Physiol* 78:223-241.
9. Owen BM, Mangelsdorf DJ, & Kliewer SA (2015) Tissue-specific actions of the metabolic hormones FGF15/19 and FGF21. *Trends in endocrinology and metabolism: TEM* 26(1):22-29.
10. Dushay JR, *et al.* (2015) Fructose ingestion acutely stimulates circulating FGF21 levels in humans. *Molecular metabolism* 4(1):51-57.
11. Sanchez J, Palou A, & Pico C (2009) Response to carbohydrate and fat refeeding in the expression of genes involved in nutrient partitioning and metabolism: striking effects on fibroblast growth factor-21 induction. *Endocrinology* 150(12):5341-5350.
12. Zhao C, *et al.* (2015) FGF21 mediates alcohol-induced adipose tissue lipolysis by activation of systemic release of catecholamine in mice. *Journal of lipid research* 56(8):1481-1491.
13. Chu AY, *et al.* (2013) Novel locus including FGF21 is associated with dietary macronutrient intake. *Human molecular genetics* 22(9):1895-1902.
14. Talukdar S, *et al.* (2016) FGF21 Regulates Sweet and Alcohol Preference. *Cell Metab* 23(2):344-349.
15. von Holstein-Rathlou S, *et al.* (2016) FGF21 Mediates Endocrine Control of Simple Sugar Intake and Sweet Taste Preference by the Liver. *Cell Metab* 23(2):335-343.
16. Splansky GL, *et al.* (2007) The Third Generation Cohort of the National Heart, Lung, and Blood Institute's Framingham Heart Study: design, recruitment, and initial examination. *Am J Epidemiol* 165(11):1328-1335.
17. Muller CP & Schumann G (2011) Drugs as instruments: a new framework for non-addictive psychoactive drug use. *Behav Brain Sci* 34(6):293-310.
18. Liang Q, *et al.* (2014) FGF21 maintains glucose homeostasis by mediating the cross talk between liver and brain during prolonged fasting. *Diabetes* 63(12):4064-4075.
19. Owen BM, *et al.* (2014) FGF21 acts centrally to induce sympathetic nerve activity, energy expenditure, and weight loss. *Cell Metab* 20(4):670-677.
20. Douris N, *et al.* (2015) Central Fibroblast Growth Factor 21 Browns White Fat via Sympathetic Action in Male Mice. *Endocrinology*:en20142001.
21. Iizuka K, Takeda J, & Horikawa Y (2009) Glucose induces FGF21 mRNA expression through ChREBP activation in rat hepatocytes. *FEBS Lett* 583(17):2882-2886.
22. Uebanso T, *et al.* (2011) Paradoxical regulation of human FGF21 by both fasting and feeding signals: is FGF21 a nutritional adaptation factor? *PLoS One* 6(8):e22976.
23. Heilig M & Koob GF (2007) A key role for corticotropin-releasing factor in alcohol dependence. *Trends Neurosci* 30(8):399-406.
24. Bulik-Sullivan BK, *et al.* (2015) LD Score regression distinguishes confounding from polygenicity in genome-wide association studies. *Nat Genet* 47(3):291-295.
25. Willer CJ, Li Y, & Abecasis GR (2010) METAL: fast and efficient meta-analysis of genomewide association scans. *Bioinformatics* 26(17):2190-2191.
26. Bookout AL, *et al.* (2013) FGF21 regulates metabolism and circadian behavior by acting on the nervous system. *Nature Med* 19(9):1147-1152.