Genome-wide association and genetic functional studies identify *autism susceptibility candidate 2* gene (*AUTS2*) in the regulation of alcohol consumption

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Alcohol consumption is a moderately heritable trait, but the genetic basis in humans is largely unknown, despite its clinical and societal importance. We report a genome-wide association study meta-analysis of ~2.5 million directly genotyped or imputed SNPs with alcohol consumption (gram per day per kilogram body weight) among 12 population-based samples of European ancestry, comprising 26,316 individuals, with replication genotyping in an additional 21,185 individuals. SNP rs6943555 in *autism susceptibility candidate 2* gene (*AUTS2*) was associated with alcohol consumption at genome-wide significance ($P = 4 \times 10^{-8}$ to $P = 4 \times 10^{-9}$). We found a genotype-specific expression of *AUTS2* in 96 human prefrontal cortex samples ($P = 0.026$) and significant ($P < 0.017$) differences in expression of *AUTS2* in whole-brain extracts of mice selected for differences in voluntary alcohol consumption. Downregulation of an *AUTS2* homolog caused reduced alcohol sensitivity in *Drosophila* ($P < 0.001$). Our finding of a regulator of alcohol consumption adds knowledge to our understanding of genetic mechanisms influencing alcohol drinking behavior.

genome-wide analysis | epidemiologic | transcriptional expression analysis

Alcohol drinking accounts for 9% of the disease burden in developed countries and is linked to more than 60 diseases, including cancers, cardiovascular diseases, liver cirrhosis, neuropsychiatric disorders, injuries, and fetal alcohol syndrome (1). The burden of alcohol-associated disease is largely caused by the level of alcohol consumption in a population, not alcohol dependence (2). Although much of the population variance of alcohol drinking is nongenetic, reflecting large societal, lifestyle, and behavioral influences, there is also an important genetic component (3). Heritability of alcohol drinking is estimated to be ~40% (4), and its genetic component gradually grows in importance as individuals age (3, 5). Alcohol drinking behavior, as well as alcohol addiction, has complex, non-Mendelian inheritance patterns, indicating an involvement of multiple genes (5). Accordingly, any single gene contributes only to a limited extent to the phenotypes observed in alcohol consumption (6). In contrast to alcohol addiction, which has been investigated in numerous genetic studies, including recent genome-wide association studies (GWAS) analyses (7–9), few genes regulating alcohol consumption in humans have been described—with the notable exception of alcohol dehydrogenase (3, 5, 10). This may, to some extent, reflect the complexity of the phenotype, because the genetic and environmental determinants of alcohol drinking behavior may vary over the lifespan, and there may be substantial heterogeneity of intake and measurement across different populations and studies.

Here, we combine discovery through GWAS with functional genetic studies to identify genetic mechanisms associated with alcohol drinking behavior. We first analyzed GWAS data on daily alcohol intake from 26,316 individuals in 12 populations of European ancestry (Tables S1 and S2A), both for all persons and for 21,607 alcohol drinkers after exclusion of 4,709 nondrinkers;

this is because abstainers may not drink for cultural, health, or social reasons, and this group may include former problem drinkers (11). We then carried out functional genetic studies in both humans and animal models (Fig. S1).

Results

The geometric mean of alcohol intake among drinkers varied across the samples from 0.09 to 0.24 g/d per kg in males and from 0.02 to 0.16 g/d per kg in females (Table S1). In age-adjusted single SNP regression analyses (additive genetic model) of the contributing cohorts, adjustment using genomic control (12) for inflation because of interindividual relatedness or population stratification was modest ($\lambda = 1.00–1.05$) (Table S2B). In the metaanalyses across cohorts, quantile–quantile plots also showed good adherence to expectation ($\lambda = 1.03$ for the analyses that included nondrinkers and $\lambda = 1.02$ for the analyses among drinkers) (Fig. S2 A and B). We identified the top-ranking SNP from each of the six GWAS metaanalyses (i.e., for males and females combined and for each sex considered separately among drinkers and nondrinkers and among drinkers only). This identified SNPs in or near *Ras protein-specific guanine nucleotide-releasing factor 2* (*RASGRF2*), *OTU domain-containing protein 3* (*OTUD3*), *chromodomain protein on Y chromosome-like* (*CDYL*), *syndecan-binding protein 2* (*SDCBP2*), *neuropilin- and tolloid-like 1* (*NETO1*), and *carboxypeptidase A6* (*CPA6*) (Tables 1 and 2, Fig. S2 C–H, and Table S3 A and B). To identify further plausible independent association signals to take forward to replication, we applied a procedure in each analysis whereby we removed all SNPs within 200 kb of the top-ranked SNP and then identified the most significant remaining association as the second-ranked SNP. We reapplied this procedure to further identify the third-ranked associated SNP. Among these second- and third-ranked SNPs, we identified four that were intragenic, lying within *LHFP-like protein 3* (*LHFPL3*), *muscleblind-like protein 2* (*MBNL2*), *GLIS family zinc finger protein 3* (*GLIS3*) (Table S3C), and *autism susceptibility candidate 2* (*AUTS2*) (Fig. S2E). Only one of these genes, namely *AUTS2*, has previously been implicated in neurobehavioral disorders (13). We, therefore, additionally selected rs6943555, the top-ranking SNP in *AUTS2*, for replication (Tables 1 and 2).

Results of the replication analyses (Table S4) for the seven selected SNPs for men and women combined are shown in Table 2. Allowing for 28 tests [SNPs (7) × men/women (2) × quantile/log (2)], we set significance level for replication at 1.8×10^{-3}; we recognize that this is conservative, because the tests are not independent (we did not include an extra degree of freedom for men and women combined, because this is based on a combination of the results for men and women considered separately). Of the seven SNPs tested, only rs6943555 in *AUTS2* attained statistical significance in the replication analyses according to the above criterion: $P = 1.2 \times 10^{-3}$ and $P = 6.9 \times 10^{-6}$ for men and women combined quantile transformation and log-transformed analyses, respectively (Table 2). In addition, rs6943555 attained genome-wide significance overall: $P = 4.2 \times 10^{-8}$ and $P = 4.1 \times 10^{-9}$ (Table 2).

Table 1.   SNPs selected for replication genotyping: genomic context, reference (minor)/alternative allele, and frequency of reference (minor) allele

SNP	Chr	Chr band	Nearest gene (bp)	Context	Reference (minor) allele/alternative allele	Frequency of reference (minor) allele (%)
rs16823039	1	p36.13	*OTUD3* (24,351)	Intergenic	C/T	11
rs26907	5	q14.1	*RASGRF2* (0)	Intronic/promoter*	A/G	17
rs2985678	6	p25.1	*CDYL* (4,645)	Downstream	T/C	28
rs6943555	7	q11.22	*AUTS2* (0)	Intronic	A/T	24
rs4500065	8	q13.2	*CPA6* (40,136)	Intergenic	C/G	12
rs8090940	18	q22.3	*NETO1* (68,467)	Intergenic	A/G	29
rs6104890	20	p13	*SDCBP2* (612)	Intronic	T/C	16

Chr, chromosome. Reference (minor) allele and frequency of reference (minor) allele estimated in four cohorts, Cohorte Lausannoise (COLAUS), the Rotterdam Study (ERGO), Northern Finland Birth Cohort (NFBC), and Turin (>4,000 samples in each cohort; Table S1). Chromosome and position (in Build 36) of SNPs to the nearest gene.
*Dependent on the isoform of *RASGRF2*.

In the replication cohorts among drinkers, the minor ancestral allele at rs6943555 is associated with 5.5% lower alcohol consumption (Table 2). Regional association and forest plots for rs6943555 are shown in Fig. S3 *A–C*. Additional analyses in population-based samples with categorical rather than continuous data on alcohol consumption or in alcohol dependence samples did not yield any significant findings (Table S5 *A–C*).

Results for men and women considered separately are given in Table S3 *A and B*. Although there was a suggestive signal for rs26907 in *RASGRF2* in the initial meta-analysis among drinkers (log transformed) in men ($P = 1.0 \times 10^{-07}$), this did not achieve statistical significance in the replication analysis after Bonferroni correction ($P = 2.4 \times 10^{-02}$).

SNPs rs7590720 and rs1344694 downstream of the *peroxisomal trans-2-enoyl-CoA reductase* (*PECR*) gene have previously been reported to attain genome-wide significance in alcohol dependence (9). In our analyses of alcohol consumption, P values for these two SNPs were ≥0.5 in all analyses. We also examined association data within ±50 kb of 121 candidate autosomal genes for addictions (alcoholism, other addictions, and disorders of mood and anxiety) listed in a recent review (14). The SNP with the lowest P value for each of the 121 genes is shown in Table S6.

On the basis of our GWAS and replication findings, we selected *AUTS2* for further functional genetic characterization in both humans and animal models. We analyzed gene expression of *AUTS2* in silico using BioGPS (http://biogps.gnf.org/#goto= welcome) and found widespread expression in numerous human tissues, including several brain regions (Fig. S3D). In quantitative PCR experiments of ex vivo human tissue, we detected expression of *AUTS2* in the brain regions most implicated in reinforcement mechanisms, the frontal cortex, caudate putamen (including the nucleus accumbens), amygdala (15) and to a lesser extent, liver (Fig. 1A). We then conducted genotype-specific quantitative PCR analyses of 96 prefrontal cortex samples from human brain. We detected increased expression of *AUTS2* in carriers of the minor A allele of rs6943555 compared with T allele ($P = 0.026$) (Fig. 1B).

We did not identify nonsynonymous genetic variants after sequencing the exons most proximal to rs6943555 in 200 individuals (*Materials and Methods*).

We further tested the role of *AUTS2* in animal models of alcohol reinforcement. Transcriptional expression analysis of *AUTS2* in whole-brain extracts of seven mouse models, known to differ markedly in voluntary alcohol consumption (16), revealed significant expression differences ($P < 0.017$ after Bonferroni correction for three probe sets) for two of three probe sets:

Table 2.   *P* values in primary GWAS meta-analysis, replication samples, and overall and effect sizes per reference (minor) allele in replication cohorts for men and women combined: quantile transformation (includes nondrinkers) and log transformation (drinkers only)

SNP	Nearest gene	GWAS	*P* values Replication	*P* values Overall	Effect (95% CI)*
Quantile transformation					
rs16823039	*OTUD*	6.9×10^{-01}	4.6×10^{-01}	4.0×10^{-01}	−0.0028 (−0.0103, 0.0046)
rs26907	*RASGRF2*	6.4×10^{-02}	5.5×10^{-02}	7.9×10^{-03}	−0.0104 (−0.0209, 0.0002)
rs2985678	*CDYL*	1.3×10^{-03}	5.0×10^{-01}	1.3×10^{-01}	0.0025 (−0.0047, 0.0097)
rs6943555	*AUTS2*	8.9×10^{-06}	1.2×10^{-03}	4.2×10^{-08}	−0.0126 (−0.0281, 0.0030)
rs4500065	*CPA6*	9.9×10^{-07}	5.5×10^{-01}	5.0×10^{-04}	0.0023 (−0.0051, 0.0096)
rs8090940	*NETO1*	2.5×10^{-05}	4.9×10^{-01}	6.2×10^{-04}	0.0025 (−0.0046, 0.0096)
rs6104890	*SDCBP2*	1.3×10^{-02}	5.4×10^{-01}	6.0×10^{-02}	−0.0058 (−0.0242, 0.0126)
Log transformation					
rs16823039	*OTUD*	5.3×10^{-02}	5.1×10^{-01}	1.0×10^{-01}	−0.7 (−2.7, 1.4)
rs26907	*RASGRF2*	7.4×10^{-04}	8.7×10^{-02}	2.2×10^{-04}	−2.6 (−5.5, 0.4)
rs2985678	*CDYL*	1.1×10^{-06}	5.7×10^{-01}	8.4×10^{-03}	0.6 (−1.4, 2.6)
rs6943555	*AUTS2*	1.1×10^{-04}	6.9×10^{-06}	4.1×10^{-09}	−5.5 (−7.8, −3.1)
rs4500065	*CPA6*	5.3×10^{-04}	2.4×10^{-01}	2.3×10^{-03}	1.2 (−0.8, 3.2)
rs8090940	*NETO1*	4.6×10^{-03}	4.9×10^{-01}	1.4×10^{-02}	0.7 (−1.3, 2.7)
rs6104890	*SDCBP2*	5.8×10^{-02}	2.2×10^{-01}	3.0×10^{-01}	−3.1 (−7.9,1.9)

Adjusted for sex by stratification in metaanalyses. CI, confidence interval.
*Effect sizes per reference (minor) allele are percentile rank change (quantile transformation) and percentage change (log transformation).
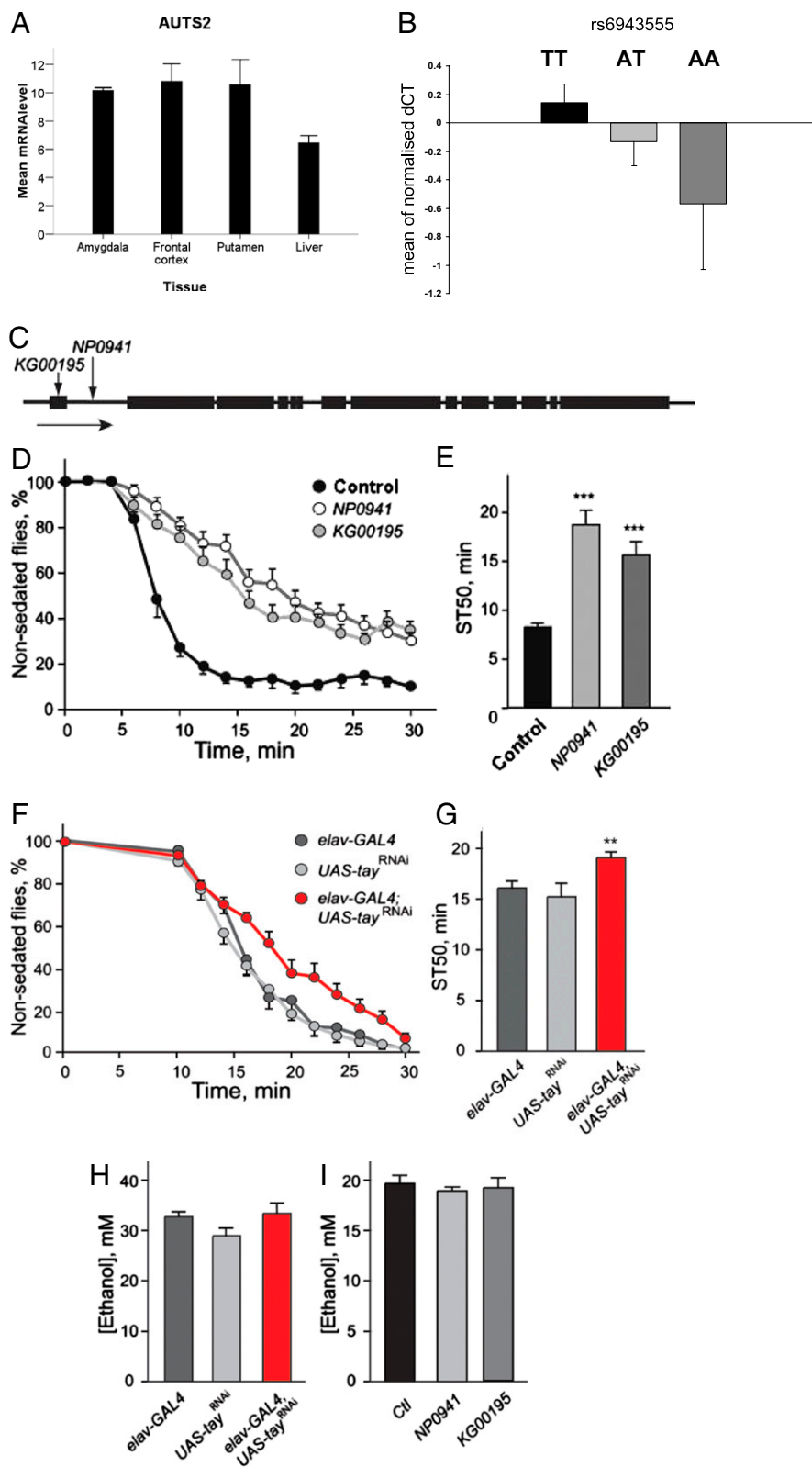
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Fig. 1. Functional genetic analysis of *AUTS2*. (*A*) Tissue-specific quantitative mRNA expression. Mean mRNA level (SE) of *AUTS2*, represented by $100/\delta$ Ct, across three independent experiments in human amygdala, frontal cortex, putamen, and liver. Mean Ct values for the housekeeping gene (*GAPDH*) were similar across tissues: 21.97, 22.42, 21.64, and 19.93, respectively. (*B*) Genotype-specific quantitative mRNA expression in human prefrontal cortex. Generalized linear model for *AUTS2* mRNA levels. Increased *AUTS2* mRNA expression observed in carriers of the minor A allele of rs6943555 relative to the major T allele; gene dose effect was significant at $P = 0.026$. Regression estimates and SEs are shown for the predictors in the model; negative normalized ΔCt values indicate higher expression levels. (*C*) Line diagram representing *tay* (*dAUTS2*) gene region of *Drosophila melanogaster*. Exons are shown as boxes and positions of P-element insertions by vertical arrows. Direction of transcription is to the right (arrow; http://flybase.org). (*D* and *E*) Sensitivity to ethanol sedation of flies with *dAUTS2/tay* P-element insertion. P-element insertion mutants *KG00195* and *NP0941*, in the 5′-UTR and first intron of *tay*, respectively, exhibit strongly reduced sensitivity to ethanol sedation relative to the control strain ($F_{2,23} = 20.7$; ***$P < 0.001$; $n = 8$). (*F* and *G*) Sensitivity to ethanol sedation of flies expressing *dAUTS2/tay* RNAi in neurons. Flies harboring both the neuronal GAL4 driver *elav-GAL4^{c155}* and the Gal4-responsive *UAS-tay^{RNAi}* construct exhibited significantly reduced sensitivity to ethanol sedation compared with control flies harboring either transgene alone ($F_{2,23} = 7.4$; **$P = 0.004$; $n = 8$). (*H* and *I*) Ethanol absorption of flies with reduced *dAUTS2/tay*. Internal ethanol concentrations after ethanol exposure for (*H*) *tay* RNAi experiment and (*I*) *tay* mutants. Neuronal expression of *dAUTS2* RNAi did not alter ethanol absorption ($F_{2,23} = 2.55$; $P = 0.1$; $n = 8$), and mutants in *dAUTS2/tay* did not show any significant difference from controls ($F_{2,11} = 0.252$; $P = 0.78$; $n = 4$).

$P = 0.005$, $P = 0.019$, and $P = 0.001$ (Table S7). Murine *AUTS2* maps to a Quantitative Trait Locus (QTL) for alcohol preference detected on chromosome 5 of high alcohol-preferring (HAP1)/low alcohol-preferring (LAP1) mice which were found to have highly significant expression differences of *AUTS2* (Table S7) (16).

Behavioral characterization of two different *Drosophila* insertion mutants in the *AUTS2* homolog *tay* (Fig. 1*C*) showed reduced alcohol sensitivity ($P < 0.001$) (Fig. 1 *D* and *E*). Pan-neuronal down-regulation of *tay* by RNA interference resulted in a similar phenotype (Fig. 1 *F* and *G*). These alterations in be-

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havior were not caused by decreased ethanol absorption, because internal ethanol concentrations in mutant flies after ethanol exposure were similar to controls (Fig. 1 *H* and *I*).

Discussion

In this study, we identified at genome-wide significance an association of *AUTS2* with alcohol intake, and we used functional genetic studies ex vivo and in animal models to characterize and further validate the signal from GWAS. This has the advantage of providing deeper biological insights than from use of GWAS data alone. The approach may be particularly suited for phenotypes such as alcohol drinking behavior for which the genetic and environmental determinants may vary over the lifespan (3) and where there may be substantial heterogeneity of both intake and measurement across the very large population samples needed for GWAS.

Although the function of *AUTS2* is not known, it is developmentally regulated and a highly conserved neuronal nuclear protein (17), first described in the context of autism (13) and mental retardation (18). More recently, it has been associated with Attention Deficit Hyperactivity Disorder (ADHD) (19), which is associated with increased alcohol intake (20). *AUTS2* is expressed in striatal dopaminergic neurons (17) involved in reward mechanisms and frontocortical glutamatergic and GABAergic neurons (17) influencing alcohol sensitivity and impulsivity (21). This neuronal expression pattern is consistent with our finding of genotype-specific differential expression of *AUTS2* in human postmortem prefrontal cortex and suggests a role for this gene in primary reinforcement (22). It also provides a possible mechanism linking *AUTS2* with impulsivity, relevant to both ADHD (19) and alcohol reinforcement.

In our behavioral animal models, we provide corroborative functional evidence for the involvement of *AUTS2* in alcohol drinking behavior. The findings in mouse models selected for high vs. low alcohol consumption—especially the observation that *AUTS2* maps to a QTL on chromosome 5 (16) in HAP1 vs. LAP1 mice—support the involvement of this gene in mechanisms of alcohol reinforcement. In the case of HAP1/LAP1 mice, this may include an involvement of *AUTS2* in regulating alcohol preference (16) as well as impulsivity (23). In contrast, down-regulation of *AUTS2* homolog *tay* in *Drosophila* results in reduced sensitivity to the effects of alcohol, pointing to *AUTS2*-mediated regulation of the level of response to alcohol. Although the percent homology shared by mammalian AUTS2 and *Drosophila* tay proteins is low, a neurological role for *Drosophila* tay has been described (24). A low level of response to alcohol has been identified as a risk factor for alcohol dependence in both human and animal studies (25, 26). Thus, our functional genetic experiments provide evidence for the involvement of *AUTS2* in alcohol drinking behavior across three different species. Our results point to different mechanisms by which *AUTS2* may influence alcohol consumption, which might vary depending on the species investigated and its neuro-developmental level as well as gene expression patterns in different brain regions.

In summary, our approach combining signals from GWAS with functional genetic studies identifies *AUTS2* in the regulation of alcohol intake in both humans and animal models. Our findings indicate the potential importance of common genetic variants influencing levels of alcohol consumption in the general population and may lead to a better understanding of mechanisms underlying alcohol drinking behavior.

Materials and Methods

Alcohol Intake Data. Quantitative information on alcohol consumption among the 21,607 drinkers was obtained from study-specific questionnaires. It was converted into grams per day intake using standard conversion factors and divided by body weight (kilograms). In the analyses that included nondrinkers, we ranked individuals according to intake (grams per day per kilogram) and performed the data analyses using the resultant study-specific quantiles. Individuals were ranked 1–*N* within each population sample according to intake. We calculated, for each percentage rank, the quantile value under a unit normal distribution, which was then treated as a quan-

titative variable in subsequent analyses. Where intake was tied, each individual was randomly assigned a relative rank, and the mean of their quantile-transformed values was used. For example, if there were *M* nondrinkers in the cohort, the ranks 1–*M* were randomly assigned (without replacement) to each of the nondrinkers. In the analyses of drinkers only, we used log transformation to normalize the data.

GWAS Meta-Analysis. For GWAS, genotyping was done on a variety of platforms (Affymetrix 500K, Illumina HumanHap 300, Illumina 317K, Illumina 370K, and Perlegen 600K); rs6943555 in *AUTS2* was directly genotyped on the Affymetrix 500K platform. Imputation of nongenotyped SNPs in the HapMap CEU v21a or v22 was carried out within each study using MaCH (27) or IMPUTE (28, 29) (Table S2A). SNPs were excluded if imputation quality score assessed by r2.hat (MaCH) or .info (IMPUTE) was <0.5 or minor allele frequency was <1%. We carried out analyses for men and women combined (stratified by cohort and sex) and for men and women considered separately. We performed age-adjusted single SNP regression analyses under an additive genetic model using SNPTEST, PLINK, and GENABEL (Table S2A). Cohort- and sex-specific effect estimates were oriented to the forward strand of the human genome reference sequence and adjusted for inflation caused by interindividual relatedness or population stratification using genomic control (12). We then conducted meta analysis across cohorts using an inverse variance weighted fixed effects model.

Replication Analyses. For replication, we chose top-ranking SNPs, which were selected on the basis of (*i*) association test results from the GWAS meta-analyses and (*ii*) biological plausibility. First, we chose the SNP with lowest *P* value from independent regions of association in the GWAS metaanalyses for all persons, drinkers only, men and women combined, and men and women considered separately. Then, for each of these six analyses, we looked for intragenic SNPs among the second- and third-ranking regions for which we sought information on biological relevance of the genes for neurobehavioral traits as a further basis for inclusion in the replication studies. We carried out direct genotyping or in silico replication of the selected SNPs in seven independent samples with continuous data on alcohol consumption (Table S4).

Twin Studies. One of each monozygous twin pair was included in the Australian Semi-Structured Assessment for Genetics of Alcoholism (SSAGA). In the Finnish Twin Cohort (FTC), we used mean alcohol consumption if both twins were drinkers and the value of the drinking twin if the cotwin had missing data or was an abstainer. Both twins in the Twins U.K. sample were used with family structure taken into account in the association model.

Fine Sequencing of *AUTS2* Proximal Exons. Sequencing of *AUTS2* exons most proximal to rs6943555 was done in 200 individuals from the Data from an Epidemiological Study on the Insulin Resistance syndrome (DESIR) study using an ABI 3730xl DNA Analyzer. It revealed two rare noncoding mutations (3,815: T/del; 4,123: C/T) in exon 4 of the predicted long isoform and three rare noncoding mutations (112: C/G; 113: A/G; 331: A/G) in exon 5 in the short isoform of *AUTS2*.

Human Brain Tissue and Genotype-Specific Expression. *Postmortem sample.* Brains from suicide victims (17 male and 10 female) and control individuals (41 male and 28 female), a total sample of 96 individuals, were obtained at autopsy at the Department of Forensic Medicine, Semmelweis University Medical School (Budapest) (30). The brains were microdissected and stored in the Human Brain Tissue Bank, Budapest. Medical, psychiatric, and drug histories of suicides were obtained through chart review and interviews with the attending physician/psychiatrist and family members. Control participants did not have psychiatric illness or alcohol or drug abuse during the last 10 y. Causes of death in control subjects were acute cardiac failure or traffic accident. After removal from the skull, the brains were rapidly frozen on dry ice and stored at −70 °C until microdissection (2 d to 2 mo later). At time of dissection, the brain samples were sliced into 1- to 1.5-mm coronal sections at 0–10 °C. Cortical areas were cut from the sections using a fine microdissecting (Graefe's) knife or microdissecting needles; the dorsomedial prefrontal cortex (Brodmann area 9) was dissected just dorsal to the frontopolar area, including the most anterior portions of the superior and middle frontal gyri. The samples were stored at −80 °C until further use. Tissue harvesting occurred after written informed consent was obtained from next of kin and with local ethics committee (Semmelweis University) approval.
Sample preparation. Total RNA and DNA were extracted from brain tissue using TRIzol according to manufacturer's protocol (Invitrogen). Before cDNA synthesis, the RNA samples were prepared using the RNeasy Mini Kit (Qiagen) and treated with RQ1 RNase-free DNase (Promega) following the manufacturer's

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instructions to ensure no DNA contamination. The concentration of extracted total RNA and DNA was determined by measuring absorbance at 260 and 280 nm with a spectrophotometer. RNA quality was analyzed with the Agilent 2100 Bioanalyzer; 100 ng RNA was used for cDNA synthesis performed using the Invitrogen SuperScript III first-strand synthesis kit according to the manufacturer's instructions with a mix of random hexamers and Oligo(dT).

Genotyping. SNP rs6943555 was genotyped by single-base extension using SNaPShot chemistry (Applied Biosystems). Initial PCR amplification was performed using HotStar Taq DNA polymerase (Qiagen) in a total volume of 12 μL containing 0.25 μM both forward and reverse primer and 24 ng genomic DNA. Thermal cycler conditions consisted of an initial step of 95 °C for 15 min followed by 35 cycles of 95 °C for 40 s, 35 cycles of 55 °C for 30 s, and 35 cycles of 72 °C for 40 s, with a final step of 72 °C for 10 min. Primers used were forward: 5′-AAACTCAAAACCCACTCCTGAA-3′ and reverse: 5′-CAGTATACATAAACA-TTGGAAAAGAGG-3′. Amplified samples were incubated with 1 U shrimp alkaline phosphatase (USB) and 2 U exonuclease I (New England Biolabs) for 1 h at 37 °C followed by 15 min at 85 °C. Single-base extension was performed using the SNaPshot Multiplex Kit (Applied Biosystems) in a total volume of 10 μL containing 2 μL clean PCR product, 1.25 μL SNaPshot master mix, 0.1 μM extension primer, and ddH$_2$O. Thermal cycler conditions consisted of an initial step of 95 °C for 2 min followed by 30 cycles of 95 °C for 10 s, 30 cycles of 50 °C for 5 s, and 30 cycles of 60 °C for 10 s. Extension primer used was 5′-ACATA-AACATTGGAAAAGAGGGAAA-3′. After single-base extension, reaction products were incubated with 1 U shrimp alkaline phosphatase for 1 h at 37 °C followed by 15 min at 85 °C. Then, 2 μL SNaPshot product was added to 8 μL HiDi formamide and loaded onto a 3130xl Genetic Analyzer (Applied Biosystems). Data were collected by the Run 3130xl Data Collection (v3.0) software (Applied Biosystems), and genotypes were ascertained using Gene-Marker (v1.71) software (Softgenetics). Genotyping yielded 6 minor homozygotes (AA), 33 heterozygotes (AT), and 57 major homozygotes (TT).

Quantitative PCR. Samples were amplified using an ABI Prism 7900HT sequence detection system (Applied Biosystems) in a final volume of 20 μL containing 2× power SYBR Green (Applied Biosystems), 4 μL diluted cDNA, and 0.07 μM each primer. The following thermal cycler conditions were used: 95 °C for 15 min followed by 95 °C for 30 s and 59 °C for 30 s for 40 cycles each; then, the PCR was evaluated by dissociation curve analysis. Primers used were AUTS2 forward: 5′-CGAGAAAATGACCGCAATCT-3′ and AUTS2 reverse: 5′-ACTGTCCCTGCA-GCTGTTCT-3′; GAPDH (housekeeping gene) forward: 5′-CATGAGAAGTAT-GACAACAGCCT-3′ and GAPDH reverse: 5′-AGTCCTTCCACGATACCAAAGT-3′.

Statistical analysis. The relative gene expression levels of AUTS2 to GAPDH (ΔCt) were transformed using normal quantile transformation. Neither cause of death nor ancestry affected AUTS2 gene expression levels ($P = 0.770$

and $P = 0.739$, respectively). We used a generalized linear model to assess the effect of allele on AUTS2 mRNA levels, with sex, age, postmortem interval, and RNA integrity number included as covariates.

Drosophila Studies. Drosophila strains were obtained from the following sources: KG00195 (strain 13059) and elav-GAL4^{c155}, Bloomington Drosophila Stock Center at Indiana University; NP0941 (DGRC 103–825), Drosophila Genetic Resource Center, Kyoto Institute of Technology; RNAi to dAUTS2 (CG9056/tay), Vienna Drosophila RNAi Center (31). All strains were outcrossed for five generations to the WT strain 2202U (32) before behavioral assays.

Measurement of alcohol tolerance. Sedation assays were performed (33). Each sample consisted of ~30 male flies aged 2–4 d posteclosion. Flies were exposed continuously in perforated tubes to a mixture of ethanol vapor and humidified air at a relative ratio (E/A) of 90 U/60 U (Fig. 1 D and E) or 100 U/50 U (Fig. 1 F and G).

Ethanol absorption assay. Flies were exposed to ethanol vapor as follows: 15 min at 100 U/ 50 U E/A for the tay-RNAi experiment (Fig. 1H) and 10 min at 90 U/60 U E/A for the mutants (Fig. 1I); after exposure, flies were snap-frozen and processed to determine internal ethanol concentration in fly extracts using a kit (229-29; Genzyme).

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