Research Paper

A translational systems biology approach in both animals and humans identifies a functionally related module of accumbal genes involved in the regulation of reward processing and binge drinking in males

David Stacey, PhD*; Anbarasu Lourdusamy, PhD*; Barbara Ruggeri, PhD; Matthieu Maroteaux, PhD; Tianye Jia, PhD; Anna Cattrell, PhD; Charlotte Nymberg, PhD; Tobias Banaschewski, MD, PhD; Sohinee Bhattacharyya, PhD; Hamid Band, MD, PhD; Gareth Barker, PhD; Arun Bokde, PhD; Christian Buchel, MD; Fabiana Carvalho, PhD; Patricia Conrod, PhD; Sylvane Desrivieres, PhD; Alanna Easton, PhD; Mira Fauth-Buehler, PhD; Alberto Fernandez-Medarde, PhD; Herta Flor, PhD; Vincent Frouin, PhD; Jurgen Gallinat, MD; Hugh Garavanh, PhD; Andreas Heinz, MD, PhD; Bernd Ittermann, PhD; Mark Lathrop, PhD; Claire Lawrence, PhD; Eva Loth, PhD; Karl Mann, MD; Jean-Luc Martinot, MD, PhD; Frauke Nees, PhD; Tomas Paus, MD, PhD; Zdenka Pausova, MD; Marcella Rietschel, MD; Andrea Rotter, PhD; Eugenio Santos, PhD; Michael Smolka, MD; Wolfgang Sommer, MD, PhD; Manuel Mameli, PhD; Rainer Spanagel, PhD; Jean-Antoine Girault, MD, PhD; Christian Mueller, PhD; Gunter Schumann, MD; on behalf of the IMAGEN consortium†

Background: The mesolimbic dopamine system, composed primarily of dopaminergic neurons in the ventral tegmental area that project to striatal structures, is considered to be the key mediator of reinforcement-related mechanisms in the brain. Prompted by a genome-wide association meta-analysis implicating the Ras-specific guanine nucleotide-releasing factor 2 (*RASGRF2*) gene in the regulation of alcohol intake in men, we have recently shown that male *Rasgrf2*$^{-/-}$ mice exhibit reduced ethanol intake and preference accompanied by a perturbed mesolimbic dopamine system. We therefore propose that these mice represent a valid model to further elucidate the precise genes and mechanisms regulating mesolimbic dopamine functioning. **Methods:** Transcriptomic data from the nucleus accumbens (NAcc) of male *Rasgrf2*$^{-/-}$ mice and wild-type controls were analyzed by weighted gene coexpression network analysis (WGCNA). We performed follow-up genetic association tests in humans using a sample of male adolescents from the IMAGEN study characterized for binge drinking (n = 905) and ventral striatal activation during an fMRI reward task (n = 608). **Results:** The WGCNA analyses using accumbal transcriptomic data revealed 37 distinct "modules," or functionally related groups of genes. Two of these modules were significantly associated with *Rasgrf2* knockout status: M5 ($p < 0.001$) and M6 ($p < 0.001$). In follow-up translational analyses we found that human orthologues for the M5 module were significantly ($p < 0.01$) enriched with genetic association signals for binge drinking in male adolescents. Furthermore, the most significant locus, originating from the EH-domain containing 4 (*EHD4*) gene ($p < 0.001$), was also significantly associated with altered ventral striatal activity in male adolescents performing an fMRI reward task ($p_{empirical} < 0.001$). **Limitations:** It was not possible to determine the extent to which the M5 module was dysregulated in *Rasgrf2*$^{-/-}$ mice by perturbed mesolimbic dopamine signalling or by the loss of *Rasgrf2* function in the NAcc. **Conclusion:** Taken together, our findings indicate that the accumbal M5 module, initially identified as being dysregulated in male *Rasgrf2*$^{-/-}$ mice, is also relevant for human alcohol-related phenotypes potentially through the modulation of reinforcement mechanisms in the NAcc. We therefore propose that the genes comprising this module represent important candidates for further elucidation within the context of alcohol-related phenotypes.

Correspondence to: D. Stacey, Discipline of Psychiatry, Level 4, Eleanor Harrald Building, Frome Road, University of Adelaide, Adelaide, 5005, Australia; david.stacey@adelaide.edu.au

*These authors contributed equally to this work.

†Consortium members are listed at the end of this article.

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Introduction

Binge drinking is a major public health problem that is particularly prevalent during adolescence.[1] It constitutes an increased risk of substance use in later life as well as mental health problems throughout the lifespan.[2,3] Early onset of excessive drinking is one of the strongest predictors of a lifetime prevalence of alcohol dependence.[4,5] The mesolimbic pathway, which consists primarily of dopaminergic neurons in the ventral tegmental area (VTA) that send projections to the nucleus accumbens (NAcc), is considered to be a central regulator of alcohol-related phenotypes.[6] However, despite the apparent importance of the mesolimbic pathway within the context of alcohol, the specific genes and mechanisms involved are not fully understood.

Large-scale genome-wide association studies (GWAS) have recently implicated several novel genes in the regulation of alcohol-related phenotypes in multiple populations.[7,8] The exploratory nature of GWAS is proving to be instrumental in furthering our understanding of the precise mechanisms in the brain that regulate alcohol-related phenotypes, particularly when combined with extensive follow-up functional work. In a recent GWAS meta-analysis of alcohol consumption (n > 20 000) we identified a novel male-specific signal in the *RASGRF2* gene,[9] and in further support of this, a follow-up candidate-based analysis in a sample of male adolescents revealed a significant association between a *RASGRF2* polymorphism and frequency of binge drinking episodes.[10]

RASGRF2 encodes a Ras-specific guanine nucleotide-releasing factor that is widely expressed across human tissues with particular enrichment in the brain, where expression appears to be neuron-specific.[11] It is a Ca^{2+}/calmodulin-regulated protein responsible for coupling *N*-methyl-D-aspartate receptor (NMDA)–type and calcium-permeable α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)–type glutamate receptors to mitogen activated protein kinase (MAPK) signalling cascades, including the extracellular signal–regulated kinase (ERK) pathway.[12,13] Moreover, via the ERK pathway *Rasgrf2* has been shown to activate the cAMP-response element binding (CREB) protein while also promoting long-term potentiation (LTP) in the mouse hippocampus.[14]

We recently performed behavioural and neurobiological phenotyping of a *Rasgrf2*$^{-/-}$ mouse strain in order to validate and extend upon our GWAS signal implicating *RASGRF2* in alcohol consumption.[9] We found a significant reduction in both ethanol intake and preference in male (no significant differences were found in female mice) *Rasgrf2*$^{-/-}$ mice relative to wild-type controls,[10] which is in accordance with the male-specific GWAS signal.[9] Male *Rasgrf2*$^{-/-}$ mice also exhibited decreased mesolimbic dopamine neuron activity relative to controls, which was accompanied by reduced ethanol-induced dopamine release in the NAcc.[10] This blunted mesolimbic dopamine response to acute ethanol indicates that the mechanisms underlying ethanol reinforcement in the NAcc would either be absent or mitigated, thereby explaining the reduced ethanol intake and preference observed in mutant mice.

Male *Rasgrf2*$^{-/-}$ mice therefore represent a convincing model to further explore the precise mechanisms within the mesolimbic dopamine system responsible for mediating the reinforcing effects of alcohol. Considering these mechanisms are coordinated by multiple (as opposed to single) genes, in the present study we applied a systems biology approach to transcriptome-wide mRNA expression data from the NAcc of male *Rasgrf2*$^{-/-}$ mice and wild-type controls to investigate the downstream consequences of an altered mesolimbic dopamine system. We first used these data to construct a weighted gene coexpression network, from which we identified a group (or module) of functionally related genes associated with *Rasgrf2* gene knockout (KO) status. We then validated the relevance of this module for human binge drinking and ventral striatal activation using a sample of adolescent boys from the IMAGEN project.[15]

Methods

Animal experiments

All procedures were carried out according to the UK Animals (Scientific Procedures) Act 1986, and ethics approval was granted by the King's College London research ethics committee. In accordance with our previous article reporting significantly reduced ethanol intake and preference in male *Rasgrf2*$^{-/-}$ mice relative to wild-type controls, but not female mice,[10] we investigated male mice only. All mice were born and weaned from multiple litters in the Santos laboratory at the University of Salamanca, Spain, where the *Rasgrf2*$^{-/-}$ strain is being maintained,[11] and were then shipped to the UK, remaining in quarantine until they cleared a health screen. We used 8- to 10-week-old wild-type and *Rasgrf2*$^{-/-}$ C57BL/6 mice (9 per group), exclusively male, single-housed with free access to food and water.[11] Artificial light was provided daily from 7 am to 7 pm (12 h light/dark cycle), and the room temperature and humidity were kept constant (temperature: 22 + 1° C; humidity: 55 + 5%).

Tissue preparation, RNA extraction and transcriptomic profiling

We dissected the NAcc from animals and extracted total RNA using the RNeasy Micro Kit (QIAGEN). Total RNA was biotinylated and amplified using the Illumina TotalPrep RNA Amplification kit (Applied Biosystems), and transcriptome profiling was performed using the Illumina Mouse WG-6 version 2.0 Expression BeadChip (Illumina) as per the manufacturer's protocol.

Human genetic association studies

In accordance with our animal experiments, we used data exclusively from male participants in our human genetic association studies. All participants were from the IMAGEN study. A detailed description of recruitment and assessment procedures, as well as inclusion and exclusion criteria, has previously been published.[15] In brief, the IMAGEN study is a multisite European research project designed to investigate risk-taking behaviour in teenagers using a longitudinal design beginning at age 14 years with follow-up at ages 16 and 18 years, with the collection of genetic, behavioural, neuropsychological and

neuroimaging data throughout. Participants were collected across 8 IMAGEN assessment centres (London and Nottingham in England; Dublin, Ireland; Mannheim, Berlin, Hamburg and Dresden in Germany; and Paris, France). Local ethics research committees at each site approved the study. On the day of testing, written informed consent was obtained from the parent or guardian and verbal assent was obtained from the adolescent.

Genetic data were available for a total of 907 male adolescents of European origin from the baseline assessment of the IMAGEN sample (mean age 14.42 ± 0.41 [range 12.88–16.04] yr). Binge drinking data were not available for 2 of these participants, leaving a total sample of 905 boys for binge drinking analyses. The binge drinking phenotype was defined as a binary phenotype (i.e., 0 v. 1) using the 2007 European School Survey Project on Alcohol and Other Drugs (ESPAD) questionnaire,[16] where 699 (77.24%) had no previous binge drinking episodes (0) and 206 (22.76%) had 1 or more previous binge drinking episodes (1). Neuroimaging analyses were performed using a subsample of the abovementioned 905 male adolescents consisting of 608 boys (mean age 14.41 ± 0.42 [range 12.73–16.04] yr) for whom fMRI data were also available. Of this subsample of 608 boys, 2 were lacking binge drinking data, 473 (78.05%) had no previous binge drinking episodes and 133 (21.95%) had at least 1 previous episode of binge drinking. These proportions of binge drinkers (1) versus non–binge drinkers (0) are highly comparable to the full sample.

Monetary incentive delay fMRI task

Our adapted version of the monetary incentive delay (MID) task consisted of 66 10-s trials. In each trial, participants were presented with 1 of 3 cue shapes (cue 250 ms) denoting whether a target (white square) would subsequently appear on the left or right side of the screen and whether 0, 2 or 10 points could be won in that particular trial. After a variable delay (4000–4500 ms) of fixation on a white crosshair, participants were instructed to respond by pressing the left or right button as soon as the target appeared. Feedback on whether and how many points were won during the trial was presented for 1450 ms after the response. A tracking algorithm adjusted task difficulty (i.e., target duration varied between 100 ms and 300 ms) such that each participant successfully responded on approximately 66% of trials. For each 5 points won, the participant would receive 1 reward in the form of small chocolate candies. Only successfully trials were included in our analysis.

DNA extraction and genotyping

DNA purification and genotyping were performed at the Centre National de Génotypage in Paris. DNA was extracted from whole blood samples (approximately 10 mL) preserved in BD Vacutainer EDTA tubes (Becton, Dickinson and Company) using a Gentra Puregene Blood Kit (QIAGEN) according to the manufacturer's instructions. Genotype information was collected at 582 982 markers using the Illumina HumanHap610 Genotyping BeadChip (Illumina). Details on quality control of genotype data are provided in Appendix 1, available at jpn.ca.

RNA extraction and gene expression profiling

Total RNA was extracted from whole blood cells using the PAXgene Blood RNA Kit (QIAGEN). Following quality control, labelled complementary RNA (cRNA) was generated using the Illumina TotalPrep RNA Amplification kit (Applied Biosystems). Gene expression profiling was performed using Illumina HumanHT-12 version 4 Expression BeadChips (Illumina). Expression data were normalized using the mloess method.

Statistical and bioinformatics analysis

Gene expression data processing

Raw intensity values were normalized by variance stabilization and quantile normalization using the Lumi Biocondcutor package.[17] To reduce noise, probes not expressed above the background ($p < 0.01$) and low varying probes estimated with coefficient of variation (CV < 0.01) were removed from subsequent analysis. The probe with the highest mean expression value was selected from multiple probes representing the same gene.

Mouse gene coexpression network analysis and module characterization

To identify modules (or groups) of functionally related genes within our mouse transcriptomic data, we applied weighted gene coexpression network analysis (WGCNA) as previously described.[18,19] For further information on these analyses and the methods used to characterize and prioritize modules for follow-up studies, refer to Appendix 1.

Module-based GWAS signal enrichment analysis

We used the efficient mixed-model association eXpedited (EMMAX) software program to run the genome-wide association analysis for binge drinking.[20] Further details are provided in Appendix 1.

Functional MRI data analysis

Functional MRI data were analyzed using SPM8 (www.fil.ion.ucl.ac.uk/spm). Slice-time correction was conducted to adjust for time differences due to multislice imaging acquisition; all volumes were aligned to the first volume, and nonlinear warping was performed to an echo-planar imaging template. Images were then smoothed with a 5 mm full-width at half-maximum Gaussian kernel.

At the first level of analysis, changes in the blood oxygen level–dependent (BOLD) response for each participant were assessed using linear combinations at the individual level; for each experimental condition, each trial (i.e., reward anticipation high gain, reward feedback) was convolved with the hemodynamic response function to form regressors that accounted for potential noise variance associated with the processing of reward anticipation. Estimated movement parameters were added to the design matrix in the form of 18 additional columns (3 translations, 3 rotations, 3 quadratic and 3 cubic translations, and each 3 translations with a shift of ± 1 repetition time). Single-participant contrast images were normalized to Montreal Neurological Institute (MNI) space. These normalized and smoothed images were then taken to a second-level random effects analysis. The left and right ventral striatal (VS)

regions of interest (ROI) were extracted using the MarsBaR toolbox (http://marsbar.sourceforge.net) from the anticipation high gain versus no gain contrast as well as from the feedback high gain versus no gain contrast. The extracted ROIs were based on the study by O'Doherty and colleagues[21] (MNI coordinates: $x, y, z = \pm 15, -9, 9$; radius = 9 mm).

Establishment of linkage disequilibrium structures

We used Haploview 4.2 software to define the haplotype blocks of the *EHD4* gene.[22] We selected the haplotype block 3 for further analyses, where the candidate single nucleotide polymorphism (SNP) rs1648821 is located and the minimum pairwise linkage disequilibrium (LD), measured as D', is higher than 0.99. We used Plink software to generate the haplotype phases for each individual and performed the association analyses.[23]

Kernel-based association measure

We used the kernel generalized variance[24] to quantify the dependence between the *EHD4* SNPs and both binge drinking behaviour and fMRI BOLD response in the IMAGEN sample. Statistical inference was based on a permutation procedure where both a parametric approximation of the *p* value and an empirical *p* value were calculated; we report the latter in this article. For more details on the kernel-based association measure, refer to Appendix 1 and the study by Bach and Jordan.[25]

Results

Construction of a gene coexpression network

We began by constructing a coexpression network based on NAcc transcriptomic data derived from 9 male *Rasgrf2*$^{-/-}$ mice and 9 male wild-type controls (total $n = 18$) by applying WGCNA.[18] This analysis revealed 37 groups, or modules, of genes that exhibited highly significant coexpression patterns from a total of 12 043 unique genes (Appendix 1, Fig. S1). The number of genes in a module varied widely from 39 to 3706.

Identification and characterization of coexpression modules associated with KO status

To determine whether any of the identified coexpression modules were associated with KO status (binomial trait), we correlated each module with KO status using Pearson *r* with module eigengenes (i.e., the first principal component of the standardized gene expression profile of each module). After Bonferroni correction for 37 modules, we found that 2 modules, modules 5 (M5; $p_{\text{uncorrected}} < 0.001$, $p_{\text{corrected}} < 0.001$) and 6 (M6; $p_{\text{uncorrected}} < 0.001$, $p_{\text{corrected}} = 0.025$), were significantly correlated with KO status (Fig. 1A). In order to provide validation, we also correlated these 2 modules with *Rasgrf2* mRNA expression levels (quantitative trait). We found a significant correlation with the M5 module (Fig. 1B), which comprises 367 highly interconnected genes, but not with the M6 module (Fig. 1C). Specifically, the M5 module eigengene in *Rasgrf2*$^{-/-}$ mice was reduced compared with wild-type controls, indicating that the majority of the genes in this module were downregulated in *Rasgrf2*$^{-/-}$ mice (Appendix 1, Fig. S2). We therefore took the M5 module forward for further characterization.

At the individual gene level, more than 96% of genes in M5 were differentially expressed significantly between *Rasgrf2*$^{-/-}$ mice and wild-type controls, with positive correlations evident between numerous M5 genes and *Rasgrf2* expression levels. The top 40 genes (ranked by module membership) and their correlation with *Rasgrf2* expression levels ($r > 0.50$), differentially expressed genes significantly between *Rasgrf2*$^{-/-}$ mice and wild-type controls ($p_{\text{FDR}} < 0.05$), and the number of connections (> 50) with other genes in the module are presented in Table 1. A complete list of genes comprising the M5 module can be found in Appendix 1, Table S1.

To begin to characterize the M5 module, we first performed gene ontology (GO) term enrichment analysis. This revealed significant enrichment of M5 genes for protein phosphorylation and kinase activity, transferase and nuclease activities and (cytoskeletal) protein binding in both the cytoplasm and nucleus (Appendix 1, Table S2). To determine whether the M5 module functions in a cell-specific manner, we tested whether M5 gene expression significantly overlaps with markers of the 4 major central nervous system (CNS) cell classes (astrocytes, microglia, neurons and oligodendrocytes). We found significant enrichment with neuronal marker genes ($p_{\text{corr}} = 0.018$; Appendix 1, Table S3), suggesting that M5 module functioning is neuron-specific.

The M5 module is enriched with alcohol-related genes in mice and humans

Because the M5 module was correlated with both KO status (binomial trait) and *Rasgrf2* mRNA expression levels (quantitative trait), we took this module forward to determine its relevance for alcohol-related phenotypes. We first set out to determine to what extent M5 module genes overlap with previously identified quantitative trait loci (QTL) for alcohol preference in mice.[26] We identified a total of 36 M5 genes located within these QTL, including *Adamtsl2, Atm, Cryab, Neo1, Pgm2* and *Rasl12* (Appendix 1, Table S4), indicating that the M5 module does contain genes already implicated in the regulation of alcohol preference.

We then shifted our focus to human alcohol-related phenotypes by investigating whether human orthologues to M5 module genes are enriched with genetic association signals for binge drinking in a sample of 905 14-year-old adolescent males from the IMAGEN project.[15] We identified 250 human orthologues for M5 module genes, 216 of which contained SNPs covered by the Illumina HumanHap610 Genotyping BeadChip (see Table 1 for orthologues of the top 40 M5 genes). This set of 216 orthologues was significantly enriched with genetic association signals for binge drinking GWAS data, even after correction for multiple testing ($p = 0.01$, $p_{\text{FDR}} = 0.02$; Fig. 2A).

In order to filter this list of orthologues to identify the most prominent candidates for involvement in alcohol-related phenotypes, we revisited our mouse coexpression data and preferentially selected genes that exhibited a high module membership (MM > 0.5) and a highly significant correlation

with *Rasgrf2* mRNA expression ($p < 0.01$). Thus, of the initial set of 216 human genes, we highlighted 28 candidates for alcohol-related phenotypes based on the apparent prominence of their orthologues in the M5 module (Table 2).

EHD4 *is associated with binge drinking and VS activation during a reward task*

The SNP showing the most highly significant association with binge drinking was rs1648821 ($p < 0.001$), which is located in intron 3 of the EH-domain containing 4 (*EHD4*) gene (Fig. 2B and Appendix 1, Table S5). In order to further validate our overall findings, we decided to take *EHD4* forward for candidate-based analyses. First, we extended upon the single SNP association between rs1648821 and binge drinking by performing a haplotype analysis in the same sample of male adolescents.[15] We selected haplotype block 3, which consists of rs1648821 and 5 other SNPs (Appendix 1, Fig. S3 and Table S5), to test for a kernel-based association with binge drinking behaviour. This yielded a highly significant result ($p_{empirical} < 0.001$; all empirical p values presented in this study are based on 1000 permutations).

We have previously shown that *RASGRF2* appears to exert its effect on alcohol-related phenotypes by altering mesolimbic dopamine function, resulting in a mitigation of the rewarding effects of alcohol.[10] Thus, to determine whether *EHD4* might exert its effect on binge drinking through similar mechanisms, we examined whether there was an association between haplotype block 3 in *EHD4* and activation of the VS, which contains the NAcc, during a reward task (i.e., the MID task). Our sample consisted of 608 14-year-old boys from the IMAGEN baseline assessment with fMRI data, which was a subsample the study's 905 participants.[15] Whole brain contrast maps during reward anticipation showed a widespread positive BOLD

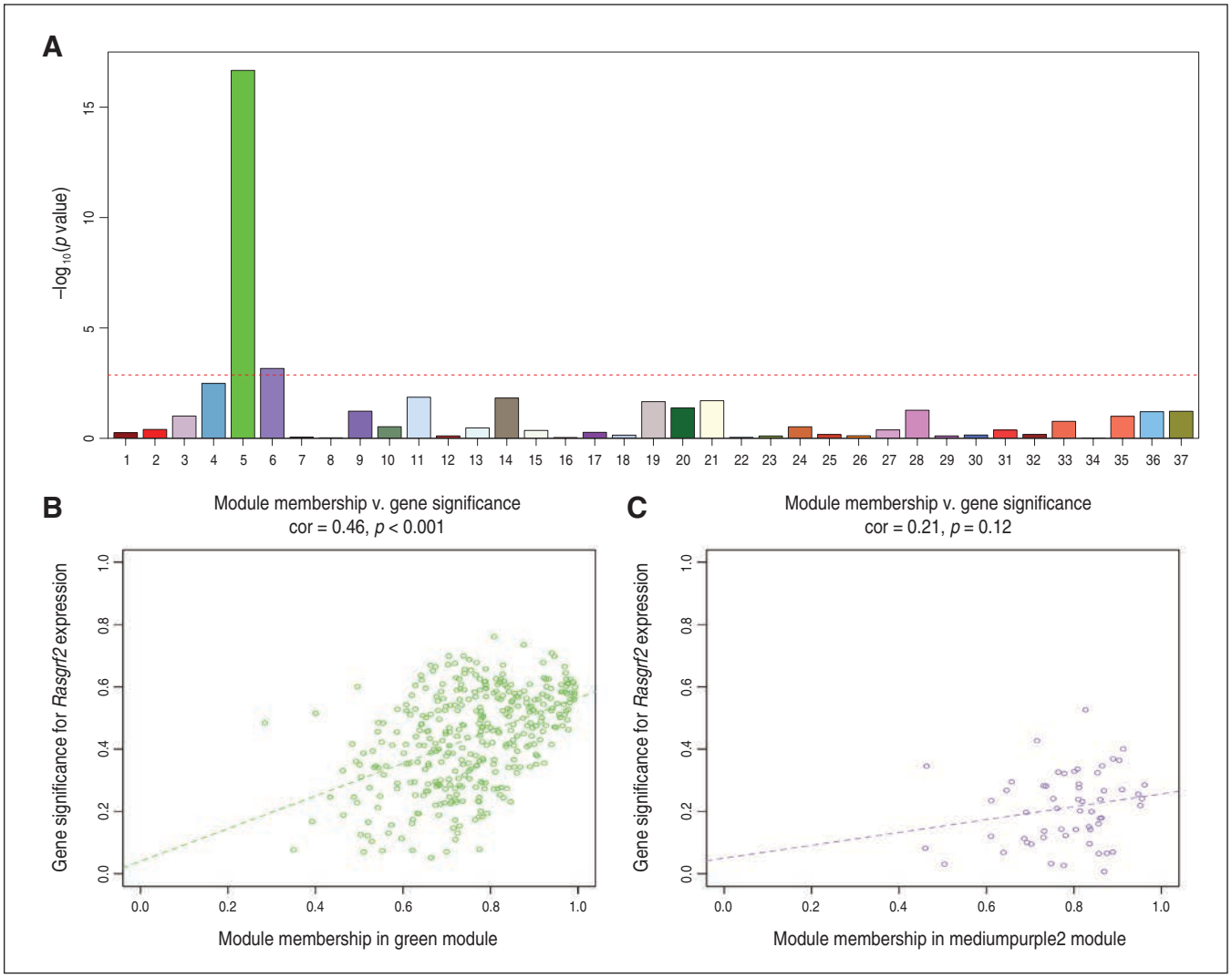

Fig. 1: Identifying gene coexpression modules associated with knockout (KO) status. (**A**) Association of each module's eigengene with the KO status. The Y axis indicates the $-\log_{10}$ of the association p value. The dotted red line represents the significance level at $p < 0.001$ after Bonferroni correction. (**B, C**) Correlations between gene significance (i.e., correlation with *Rasgrf2* gene expression levels) and module membership for (**B**) M5 and (**C**) M6 modules. Colour-coding is equivalent to module names (green and medium purple).

response, with highest activation in subcortical regions, including the VS, extending into the insula, prefrontal cortex and the parietal cortex (Fig. 3A). Reward feedback induced significant positive BOLD responses predominantly in the cingulate gyrus, the prefrontal cortex and the parietal cortex (Fig. 3B). In the anticipation phase, we found a significant association between haplotype block 3 in *EHD4* and the right ($p_{empirical} < 0.001$) but not the left VS ($p_{empirical} = 0.41$; Appendix 1, Table S6). There was no significant association between haplotype block 3 and VS activity upon reward feedback (data not shown).

Evidence of a cis*-acting haplotypic effect on* EHD4 *mRNA expression*

Considering rs1648821 and the other 5 SNPs constituting haplotype block 3 are all intronic and do not tag ($r^2 > 0.8$) any coding SNPs, we set out to test whether this haplotype influenced *EHD4* mRNA expression levels. *EHD4* expression data from peripheral blood were available from a subsample of adolescent boys ($n = 190$) from the IMAGEN project. By applying a kernel-based association test, we found an association between

Table 1: Top 40 M5 module genes ranked by module membership. Differential gene expression between wild-type and *Rasgrf2*$^{-/-}$ mice and module membership statistics, as well as human orthologues, are also included.

		Differential expression			Module membership			
Rank	Mouse gene symbol	logFC	*t*	*p* value	MM	*p* value	kWithin	Human gene symbol
1	*Glrx1*	3	38.27	< 0.001	0.99	< 0.001	76.61	—
2	*LOC666403*	4.48	59.71	< 0.001	0.99	< 0.001	76.23	—
3	*1200015F23Rik*	2.15	42.63	< 0.001	0.99	< 0.001	75.97	—
4	*Tmem87a*	–2.38	–42.17	< 0.001	–0.99	< 0.001	75.47	*TMEM87A*
5	*Lrrc57*	2.44	34.6	< 0.001	0.99	< 0.001	75.23	*LRRC57*
6	*Ccndbp1*	1.43	24.13	< 0.001	0.99	< 0.001	73.6	*CCNDBP1*
7	*9930021D14Rik*	1.31	22.86	< 0.001	0.99	< 0.001	72.98	—
8	*Slc25a18*	2.25	23.78	< 0.001	0.98	< 0.001	72.8	*SLC25A18*
9	*2810410P22Rik*	1.84	19.75	< 0.001	0.98	< 0.001	71.23	—
10	*Pla2g4b*	0.43	11.35	< 0.001	0.98	< 0.001	70.12	*PLA2G4B*
11	*Gfer*	0.66	14.77	< 0.001	0.98	< 0.001	68.74	*GFER*
12	*Cops8*	–2.91	–21.1	< 0.001	–0.98	< 0.001	67.93	*COPS8*
13	*Adamts9*	0.85	15.38	< 0.001	0.98	< 0.001	67.84	*ADAMTS9*
14	*Eme2*	–1.74	–20.75	< 0.001	–0.98	< 0.001	68	*EME2*
15	*C920004C08Rik*	–0.98	–16.06	< 0.001	–0.98	< 0.001	66.72	—
16	*Slc39a9*	0.65	12.83	< 0.001	0.97	< 0.001	66.17	*SLC39A9*
17	*Arrdc3*	2.07	16.82	< 0.001	0.97	< 0.001	68.47	*ARRDC3*
18	*2810423A18Rik*	1.51	17.65	< 0.001	0.97	< 0.001	68.02	—
19	*Mad*	1.21	17.31	< 0.001	0.97	< 0.001	68.05	—
20	*A330021E22Rik*	1.28	16.43	< 0.001	0.97	< 0.001	64.42	*C7orf63*
21	*Arl5a*	1.7	16.24	< 0.001	0.97	< 0.001	66.31	*ARL5A*
22	*LOC229810*	–1.64	–18.56	< 0.001	–0.97	< 0.001	68.29	—
23	*Zap3-pending*	0.58	13.07	< 0.001	0.97	< 0.001	65.62	—
24	*Clstn1*	–1.51	–18.54	< 0.001	–0.97	< 0.001	66.34	*CLSTN1*
26	*Vrk1*	0.85	–13.51	< 0.001	0.96	< 0.001	62.83	*VRK1*
27	*2310002B06Rik*	–0.6	–12.66	< 0.001	–0.96	< 0.001	61.92	—
28	*Polr3f*	–0.38	–9.54	< 0.001	–0.96	< 0.001	61.86	*POLR3F*
29	*Gpr19*	0.81	11.99	< 0.001	0.96	< 0.001	62.33	*GPR19*
30	*Mid1*	1.61	17.27	< 0.001	0.96	< 0.001	62.82	*MID1*
31	*A630064P09Rik*	–0.46	–10.17	< 0.001	–0.96	< 0.001	59.67	—
32	*Ccl27*	–1.79	–15.52	< 0.001	–0.96	< 0.001	60.68	—
33	*8430432M10Rik*	0.67	11.92	< 0.001	0.95	< 0.001	59.82	—
34	*Ndufb10*	1.09	15.91	< 0.001	0.95	< 0.001	59.86	*NDUFB10*
35	*Ube2i*	–1.04	–12.22	< 0.001	–0.95	< 0.001	56.05	*UBE2I*
36	*Tubgcp4*	0.62	10.28	< 0.001	0.95	< 0.001	55.84	*TUBGCP4*
37	*Bub1b*	0.36	8.56	< 0.001	0.94	< 0.001	53.49	*BUB1B*
38	*Ehd4*	0.67	9.79	< 0.001	0.94	< 0.001	54.66	*EHD4*
39	*Rps2*	–0.51	–10.47	< 0.001	–0.94	< 0.001	53.67	*RPS2*
40	*Arhgap12*	1.12	11.84	< 0.001	0.94	< 0.001	53.33	*ARHGAP12*

logFC = fold change on log2 scale between wild-type and *Rasgrf2*$^{-/-}$; MM = module membership; kWithin = number of connections between a gene and other genes in a module.

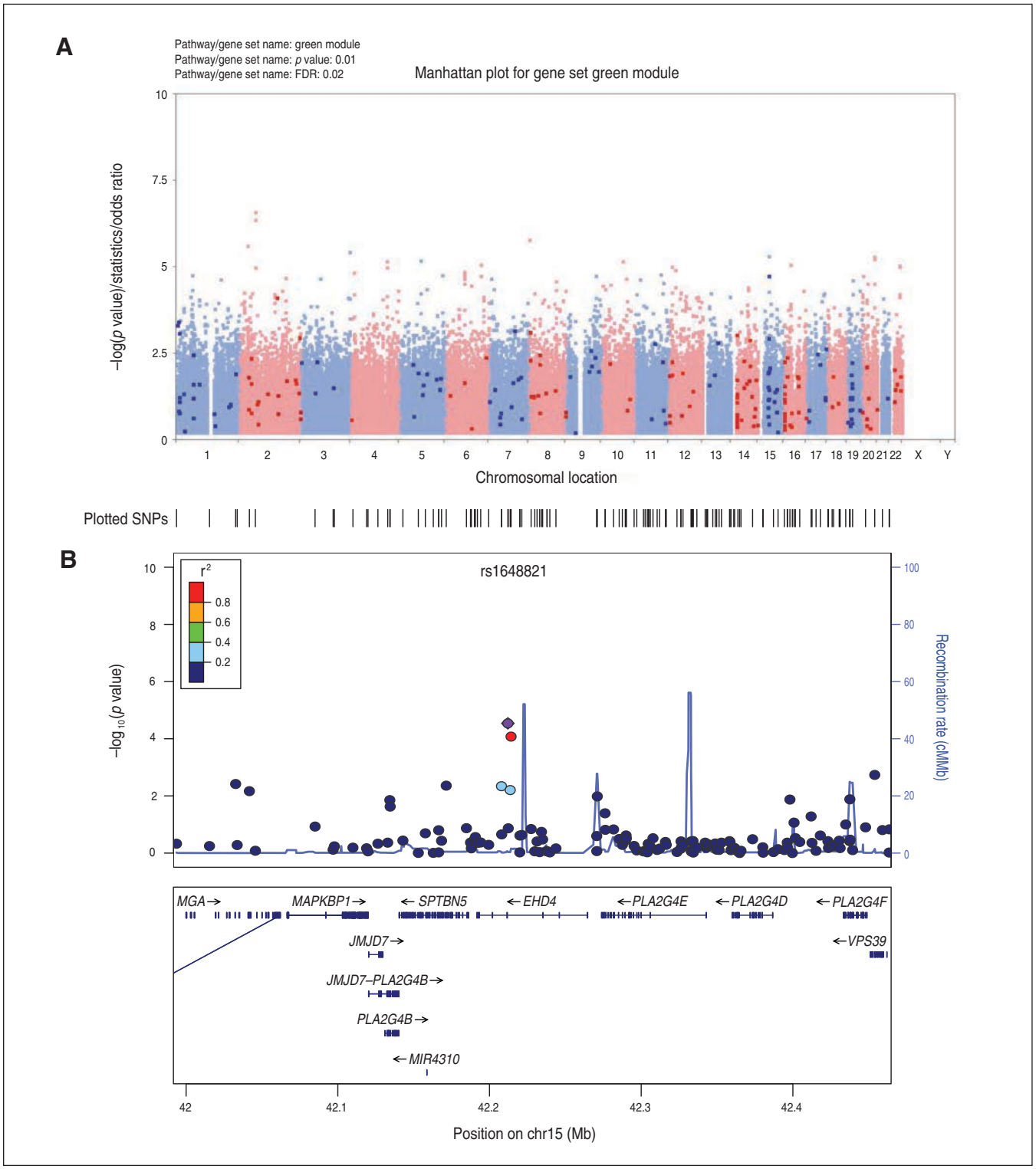

Fig. 2: Manhattan and regional association plot for the M5 (green) module associations with male adolescent binge drinking from the IMAGEN study sample. (**A**) Genome-wide association study signals for binge drinking in male adolescents. The single-nucleotide polymorphism (SNP) with the lowest –log(*p*) located within 100 kb either upstream or downstream was used to present each gene. In total 372 813 variants were used and 16 449 genes were mapped for enrichment analysis. (**B**) Regional association of the *EHD4* gene locus for binge drinking in male adolescents. Association *p* values (–log10 scale, Y axis) of variants are shown according to their chromosomal positions (X axis). Blue lines indicate the genetic recombination rate (cM/Mb).

haplotype block 3 and *EHD4* mRNA expression ($p_{empirical}$ < 0.001), indicating this haplotype exerts a *cis*-acting effect.

Discussion

Previous characterization of male *Rasgrf2*$^{-/-}$ mice revealed significant reductions in ethanol intake and preference relative to wild-type controls as well as altered mesolimbic dopamine neuron activity and dopamine release in the NAcc.[10] In the present study we therefore applied a systems biology approach to elucidate the downstream consequences of altered mesolimbic dopamine signalling in the NAcc of this same mouse model. We constructed a coexpression network using transcriptome-wide data derived from the NAcc of male *Rasgr2*$^{-/-}$ mice and wild-type controls, which revealed 37 distinct modules of functionally related genes. One of these modules, M5 green, was significantly associated with both *Rasgrf2* KO status (binomial trait) and *Rasgrf2* mRNA expression levels (quantitative trait), thereby highlighting this module as a putative central regulator of mesolimbic dopamine signalling and reinforcement mechanisms in the NAcc.

Initial characterization of this module by GO term enrichment analysis indicated that this module plays a role in the regulation of protein phosphorylation and kinase activity, which is in keeping with the known function of *Rasgrf2* as a guanine nucleotide-releasing factor and activator of MAP kinase signalling pathways.[11,24,27] We also found significant coexpression of our M5 module genes with neuronal marker genes indicating that the functioning of this module is largely neuron-specific. Again, this is in accordance with previous research showing that *Rasgrf2* expression in the CNS is localized to neurons only, not glia.[11,24] This apparent neuron-specific expression of M5 module genes is potentially interesting considering the relatively homogeneous neuronal make-up of the NAcc, whereby approximately 90% of these neurons belong to the class of medium-sized spiny neurons (MSNs).[6] They are known to play a critical role in the regulation of alcohol reinforcement by functioning as integrators of dopaminergic and glutamatergic signals,[28] so a neuron-specific role for the M5 module in the NAcc is highly suggestive of an important role for this module in alcohol reinforcement mechanisms. Further research to determine whether M5 module

Table 2: Summary of mouse genes in the M5 module with high module membership and gene significance (correlated with *Rasgrf2* expression levels) and their corresponding genetic association with binge drinking in humans.

	Mouse gene expression					Human genetic association			
	Wild-type v. *Rasgrf2*$^{-/-}$			*Rasgrf2* expression		Binge drinking			
Mouse gene	MM	FC	*p* value	*r*	*p* value	Human gene	*n*	SNP*	*p* value
Ehd4	0.94	0.67	< 0.001	0.71	< 0.001	*EHD4*	30	rs1648821	< 0.001
Klc1	0.77	0.78	< 0.001	0.64	0.004	*KLC1*	10	rs861550	0.009
Slc5a5	0.74	1.23	< 0.001	0.66	0.003	*SLC5A5*	6	rs7508025	0.011
Capn3	0.66	0.17	0.006	0.67	0.002	*CAPN3*	9	rs4924675	0.017
Dcp1b	0.83	0.58	< 0.001	0.61	0.008	*DCP1B*	16	rs972657	0.022
Snapc3	0.82	0.38	< 0.001	0.62	0.006	*SNAPC3*	10	rs6474915	0.023
Emp2	0.79	0.44	< 0.001	0.59	0.009	*EMP2*	30	rs6498070	0.024
Lypd6b	0.79	0.39	< 0.001	0.60	0.008	*LYPD6B*	49	rs10490383	0.026
Ccl17	0.63	0.35	0.004	0.60	0.008	*CCL17*	8	rs4784805	0.026
Snapc1	0.81	0.42	< 0.001	0.63	0.005	*SNAPC1*	8	rs7143626	0.036
Adamts9	0.98	0.85	< 0.001	0.61	0.007	*ADAMTS9*	67	rs6793277	0.037
Gpr176	0.73	0.32	< 0.001	0.63	0.005	*GPR176*	26	rs17646847	0.045
Map4k5	0.83	0.23	< 0.001	0.64	0.004	*MAP4K5*	20	rs12881869	0.049
Neo1	0.93	0.61	< 0.001	0.63	0.005	*NEO1*	30	rs4132535	0.06
Pgpep1	0.85	0.45	< 0.001	0.61	0.008	*PGPEP1*	11	rs1978423	0.09
Pbx4	0.67	0.24	0.003	0.65	0.003	*PBX4*	7	rs12610185	0.11
Rnd3	0.92	0.89	< 0.001	0.63	0.005	*RND3*	12	rs1528429	0.12
Zfp609	0.73	0.14	0.005	0.61	0.008	*ZNF609*	10	rs547818	0.12
Polr3k	0.81	0.28	< 0.001	0.76	< 0.001	*POLR3K*	5	rs216600	0.13
A330021E22Rik	0.97	1.28	< 0.001	0.60	0.008	*C7orf63*	15	rs11563435	0.17
Tmco5	0.75	0.18	0.002	0.69	0.002	*TMCO5A*	5	rs1604528	0.19
2410076I21Rik	0.79	0.26	< 0.001	0.62	0.006	*C15orf60*	13	rs7176074	0.24
Lrrc57	0.99	2.44	< 0.001	0.61	0.007	*LRRC57*	1	rs10851410	0.50
Tubgcp4	0.95	0.62	< 0.001	0.70	0.001	*TUBGCP4*	3	rs2412780	0.53
Itgb3bp	0.88	0.61	< 0.001	0.74	< 0.001	*ITGB3BP*	9	rs3790867	0.54
Dguok	0.83	0.38	< 0.001	0.62	0.006	*DGUOK*	9	rs13411881	0.56
Xkr8	0.72	0.35	< 0.001	0.63	0.005	*XKR8*	6	rs4908382	0.74
Arl5a	0.97	1.70	< 0.001	0.64	0.004	*ARL5A*	2	rs10182533	0.78

FC = fold change on log2 scale between wild-type and *Rasgrf2*$^{-/-}$ mice; MM = module membership; SNP = single-nucleotide polymorphism.
*SNP with the lowest *p* value (best SNP) for the gene.

genes influence the activity of the DARPP-32 pathway, a major regulator of synaptic plasticity in response to alcohol in MSNs, may prove informative in the elucidation of this role.[28]

Additional characterization of the M5 module revealed an enrichment of genes residing within previously identified QTL for alcohol preference in a recombinant mouse strain.[26] Furthermore, we also found an enrichment of GWAS signals for binge drinking originating from M5 orthologous human genes in a human sample consisting of adolescent boys, indicating that this module, initially identified in mice, is indeed translatable to humans. After filtering these human orthologues to identify those genes representing central regulators of the M5 module (i.e., high module membership and a highly significant correlation with *Rasgrf2* mRNA expression), we highlighted 28 genes that represent highly convincing candidates for a role in the regulation of reinforcement mechanisms in MSNs.

A literature search revealed that several of these 28 candidates have previously been implicated in alcohol- and addiction-related phenotypes, thereby providing proof of principle. For example, *Snapc3* was identified as a candidate involved in ethanol consumption and withdrawal in a recent QTL study using a recombinant panel of BxD mice.[29] *Snapc3* encodes a component of the small nuclear RNA (snRNA) activating complex required for the transcription of both RNA polymerase II and III snRNA genes.[30] Interestingly, our list of 28 genes also contains *Snapc1*, which encodes another component of this complex potentially highlighting snRNA as an important class of molecules in the regulation of reinforcement. In addition, *Rnd3*, which encodes the GTPase RhoE, has previously been implicated in an astroglial inflammatory response to ethanol administration,[31] whereas another gene, *Klc1*, has been implicated in morphine-induced conditioned place preference in mice.[32] Interestingly, *Klc1* encodes kinesin chain light 1, a component of kinesin motor proteins responsible for intracellular molecule trafficking,[33] which in the context of reinforcement may play a role in the transport of proteins regulating synaptic plasticity.

Our list of 28 genes also includes numerous novel candidates that warrant further validation and characterization. Indeed, the most highly significant association with binge drinking from this list originated from the *EHD4* gene, which encodes the ets-homology (EH) domain containing 4 protein,[34] whereby the minor T allele of rs1648821 was associated with a higher frequency of binge drinking. Extended characterization of this single SNP association in our sample of adolescent boys revealed a 6-marker haplotype featuring rs1648821, which not only appears to influence peripheral mRNA expression levels of

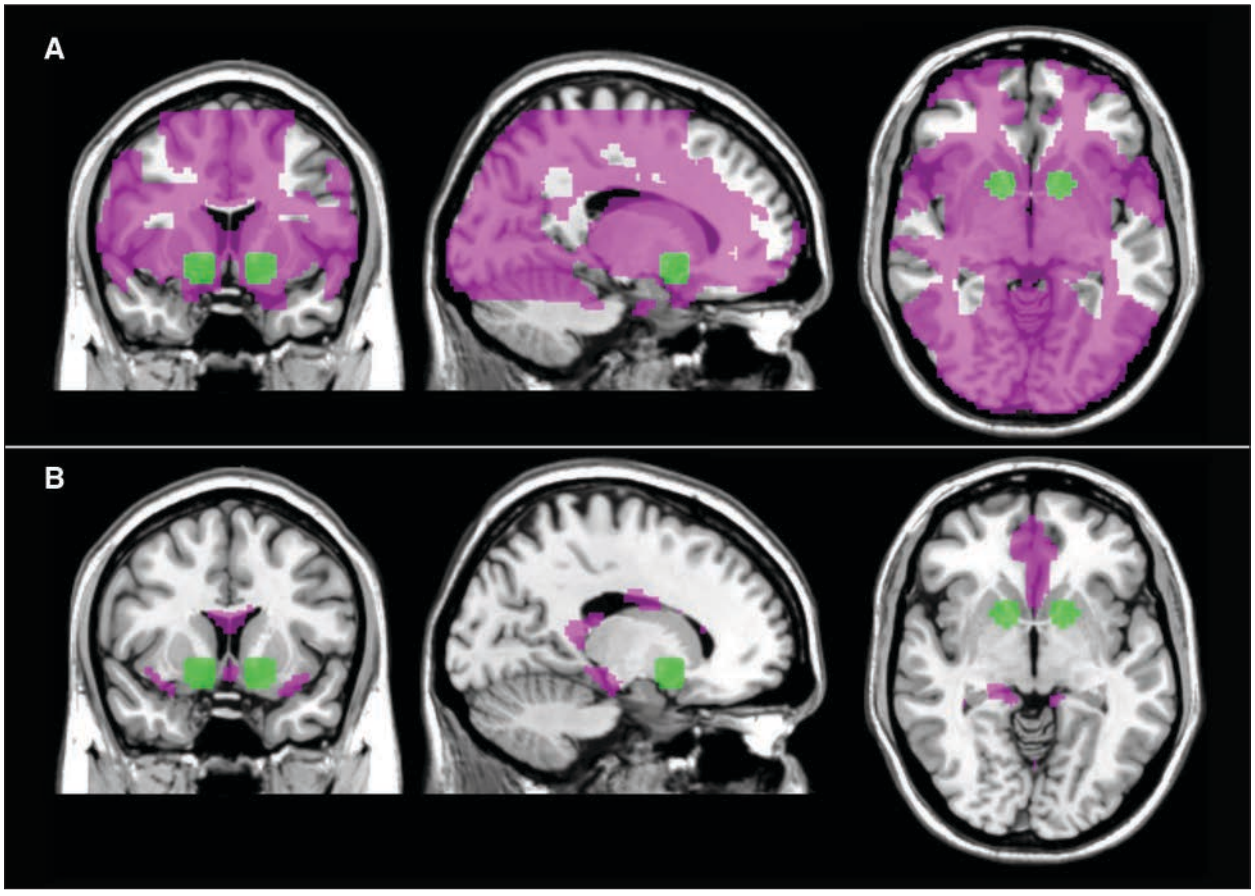

Fig. 3: Whole brain analysis of reward anticipation and reward feedback in male adolescents from the IMAGEN study sample. Positive blood oxygen level–dependent response during (**A**) reward anticipation and (**B**) reward feedback (family-wise error–corrected $p < 0.05$). The locations of the left and right ventral striatal regions of interest (Montreal Neurological Institute coordinates: x, y, z = ±15, 9, –9; radius = 9 mm) are depicted in green and purple, respectively.

EHD4, but also exhibits a highly significant association with binge drinking. In addition, this haplotype was also associated with altered ventral striatal activity during the reward anticipation phase of the MID task, indicating that *EHD4* may influence binge drinking by regulating reward and reinforcement mechanisms in the NAcc. This is concordant with the earlier proposed idea that M5 module genes identified in mice may influence alcohol-related phenotypes by altering reinforcement mechanisms in MSNs of the NAcc.

The primary function of *EHD4* is in the regulation of endocytosis, a process by which a small portion of plasma membrane, including receptors or receptor-ligand complexes, becomes internalized into the cell and packaged into early endosomes for either degradation or recycling.[35] A well-characterized member of the EH domain-containing family of genes, *EHD1*, has been shown to regulate the recycling of AMPA receptor subunits to the plasma membrane during NMDA receptor–dependent LTP,[35,36] a process known to be vital in the promotion of synaptic plasticity underlying alcohol reinforcement in the NAcc.[37] Considering the high levels of homology between *EHD1* and *EHD4*, as well as the tendency of their encoded proteins to form functional heterodimers,[34,38] *EHD4* may be involved in alcohol reinforcement mechanisms through the regulation of synaptic plasticity.

Limitations

The major potential limitation of the present study relates to the difficulty in determining the extent to which the M5 module was dysregulated in *Rasgrf2*$^{-/-}$ mice by perturbed mesolimbic dopamine signalling or by the loss of *Rasgrf2* function in the NAcc. However, our follow-up validation studies investigating genetic associations between M5 module orthologues and binge drinking in male adolescents support a role for this module in the regulation of alcohol-related phenotypes, which suggests that the M5 module dysregulation observed in mutant mice was most likely a result of altered mesolimbic dopamine functioning. Furthermore, it should be noted that the findings from our coexpression network analyses in mice are merely correlational, so the cause–effect relationship between the M5 module and alcohol-related phenotypes cannot be determined on the basis of these data alone. However, given that this module is significantly enriched with GWAS signals for binge drinking episodes in a sample of adolescent males, this implies the M5 module — or at least certain components of it — may regulate binge drinking in humans.

Conclusion

By means of a translational systems approach using gene expression data from the NAcc of *Rasgrf2*$^{-/-}$ mice, we have identified a module of coexpressed genes whose human orthologues are significantly enriched with GWAS signals for frequency of binge drinking in adolescent boys. We propose that these genes represent novel candidates for a role in the regulation of binge drinking and other alcohol-related phenotypes, and we have highlighted *EHD4* as a particularly prominent candidate. Overall, our findings support both the validity and necessity of functional characterization of GWAS signals using animal models, as well as the translational benefits of applying systems biology approaches to these models as a means to generate additional candidate genes in humans. Considering that binge drinking and excessive alcohol use during adolescence are major contributors toward the development of alcohol abuse in later life, *EHD4* and its associated M5 module genes may have predictive value with the potential to inform future preventative measures for alcohol problems in later life.

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Affiliations: From the Discipline of Psychiatry, University of Adelaide, Australia (Stacey); the Institute of Psychiatry, King's College, London, UK (Lourdusamy, Ruggeri, Maroteaux, Jia, Cattrell, Nymberg, Barker, Carvalho, Conrod, Desrivières, Easton, Loth, Müller, Schumann); the MRC Social, Genetic and Developmental Psychiatry (SGDP) Centre, London, UK (Lourdusamy, Ruggeri, Jia, Nymberg, Carvalho, Desrivières, Easton, Loth, Müller, Schumann); the Institut du Fer à Moulin, UMR-S 839, INSERM and Université Pierre et Marie Curie, Paris, France (Maroteaux, Mameli, Girault); the Department of Child and Adolescent Psychiatry, Central Institute of Mental Health, Faculty of Clinical Medicine Mannheim, University of Heidelberg, Germany (Banaschewski); the University of Nebraska Medical Center, Omaha, USA (Bhattacharyya, Band); the Institute of Neuroscience, Trinity College Dublin, Dublin, Ireland (Bokde, Garavan); the Institute for Systemic Neuroscience, UMC Hamburg Eppendorf, Hamburg, Germany (Büchel); the Department of Psychiatry, Université de Montreal, CHU Ste Justine Hospital, Montreal, Canada (Conrod); the Department of Addiction Medicine, Central Institute of Mental Health, Faculty of Clinical Medicine Mannheim, University of Heidelberg, Germany (Fauth-Buehler, Mann); the CIC-IBMCC, University of Salamanca – CSIC, Spain (Fernandez-Medarde, Santos); Department of Neuropsychiatry and Cognitive Psychology, Central Institute of Mental Health, Faculty of Clinical Medicine Mannheim, University of Heidelberg, Germany (Flor, Nees); the CEA, DSV, I2BM, Neurospin Bât 145 Gif-sur-Yvette, France (Frouin); the Department of Psychiatry and Psychotherapy, Campus Charité Mitte, Charité – Universitätsmedizin Berlin, Germany (Gallinat); the Departments of Psychiatry and Psychology, University of Vermont, Burlington, USA (Garavan); the Physikalisch-Technische Bundesanstalt (PTB), Braunschweig and Berlin, Germany (Ittermann); the McGill University and Genome Quebec Innovation Centre, Montreal, Canada (Lathrop); the School of Psychology, University of Nottingham, UK (Lawrence, Paus); the Institut National de la Santé et de la Recherche Médicale, INSERM Unit 1000 "Neuromaging & Psychiatry", University Paris Sud, Orsay, and University Paris Descartes, Paris, France (Martinot); the Rotman Research Institute, University of Toronto, Toronto, Canada (Paus); the Montreal Neurological Institute, McGill University, Montreal, Canada (Paus); the Hospital for Sick Children, University of Toronto, Toronto, Canada (Pausova); the Department of Genetic Epidemiology, Central Institute of Mental Health, Faculty of Clinical Medicine Mannheim, University of Heidelberg, Germany (Rietschel); the Department of Psychiatry and Psychotherapy, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany (Rotter, Müller); the Department of Psychiatry and Psychotherapy, Technische Universität Dresden, Germany (Smolka); the Neuroimaging Center, Department of Psychology, Technische Universität Dresden, Germany

(Smolka); and the Deptartment of Psychopharmacology, Central Institute of Mental Health, Faculty of Clinical Medicine Mannheim, University of Heidelberg, Germany (Sommer, Spanagel).

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Contributors: D. Stacey, G. Barker, C. Buchel, A. Fernández-Medarde, J. Gallinat, H. Garavan, A. Heinz, B. Ittermann, K. Mann, T. Paus, Z. Pausova, E. Santos and G. Schumann designed the study. D. Stacey, B. Ruggeri, M. Maroteaux, A. Cattrell, C. Nymberg, T. Banaschewski, S. Bhattacharyya, H. Band, A. Bokoe, F. Carvalho, S. Desrivières, A. Easton, M. Fauth-Bühler, H. Flor, J. Gallinat, H. Garavan, A. Heinz, B. Ittermann, G. Lathrop, C. Lawrence, E. Loth, J.-L. Martinot, F. Nees, T. Paus, M. Rietschel, A. Rotter-Neubert, M. Smolka, C. Mueller and G. Schumann acquired the data, which D. Stacey, A. Lourdusamy, T. Jia, C. Nymberg, P. Conrod, V. Frouin, M. Rietschel, W. Sommer, M. Mameli, R. Spanagel, J.-A. Girault and G. Schumann analyzed. D. Stacey, A. Lourdusamy, T. Jia, P. Conrod, K. Mann, T. Paus and G. Schumann wrote the article, which all authors reviewed and approved for publication.

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