

# A Genomewide Association Study of Nicotine and Alcohol Dependence in Australian and Dutch Populations

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Persistent tobacco use and excessive alcohol consumption are major public health concerns worldwide. Both alcohol and nicotine dependence (AD, ND) are genetically influenced complex disorders that exhibit a high degree of comorbidity. To identify gene variants contributing to one or both of these addictions, we first conducted a pooling-based genomewide association study (GWAS) in an Australian population, using Illumina Infinium 1M arrays. Allele frequency differences were compared between pooled DNA from case and control groups for: (1) AD, 1224 cases and 1162 controls; (2) ND, 1273 cases and 1113 controls; and (3) comorbid AD and ND, 599 cases and 488 controls. Secondly, we carried out a GWAS in independent samples from the Netherlands for AD and for ND. Thirdly, we performed a meta-analysis of the 10,000 most significant AD- and ND-related SNPs from the Australian and Dutch samples. In the Australian GWAS, one SNP achieved genomewide significance ( $p < 5 \times 10^{-8}$ ) for ND (rs964170 in *ARHGAP10* on chromosome 4,  $p = 4.43 \times 10^{-8}$ ) and three others for comorbid AD/ND (rs7530302 near *MARK1* on chromosome 1 ( $p = 1.90 \times 10^{-9}$ ), rs1784300 near *DDX6* on chromosome 11 ( $p = 2.60 \times 10^{-9}$ ) and rs12882384 in *KIAA1409* on chromosome 14 ( $p = 4.86 \times 10^{-8}$ )). None of the SNPs achieved genomewide significance in the Australian/Dutch meta-analysis, but a gene network diagram based on the top-results revealed overrepresentation of genes coding for ion-channels and cell adhesion molecules. Further studies will be required before the detailed causes of comorbidity between AD and ND are understood.

**Keywords:** addiction, alcohol, comorbidity, genetics, smoking

Nicotine and alcohol dependence frequently occur together. Approximately 23% of individuals with nicotine dependence meet past-year criteria for alcohol abuse or dependence, and individuals with alcohol dependence are over 4 times more likely to be nicotine dependent (Grant et al., 2004). Individuals who consume both cigarettes and alcohol are at an increased risk for some forms of cancer (Kuper et al., 2000) and alcohol dependent individuals are actually at greater risk of dying from tobacco-related diseases than dying from an alcohol-related disease (Hurt et al., 1996).

Both nicotine dependence (ND) and alcohol dependence (AD) are highly heritable (Gelernter & Kranzler, 2009; Heath et al., 1997; Knopik et al., 2004; Lessov et al., 2004; Madden et al., 1999; Madden et al., 2004; Vink et al., 2005) and their co-occurrence appears, in part, to be accounted for by common genetic variation (Koopmans et al., 1997; True et al., 1999). A genetic correlation between a history of regular smoking and DSM-III-R alcohol dependence has been observed even after controlling for personality and history of other psychopathology (Madden et al., 2000). Behavioral and genetic studies in both rodents and humans suggest cross-tolerance processes that contribute to the high phenotypic and genetic correlations of these two behaviors (Butt et al., 2003; Butt et al., 2004; Madden & Heath, 2002; Madden et al., 1997; Owens et al., 2003). Substance-specific

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genetic risk factors also play a role in the development of ND and AD (Hettema et al., 1999).

Genomewide association studies (GWAS) provide a means of identifying common polymorphisms contributing to genetic risk without restricting the scope of study to known or suspected candidate genes. Some GWAS results for ND (or related phenotypes) have been reported (Bierut et al., 2007; Caporaso et al., 2009; Drgon et al., 2009; Johnson et al., 2006; Liu et al., 2009; Rosenberger et al., 2005; Saccone et al., 2008; Thorgeirsson et al., 2008; Uhl et al., 2008a; Uhl et al., 2007; Uhl et al., 2008b; Vink et al., 2009). ND-related findings met genomewide significance criteria in only one case (Thorgeirsson et al., 2008) for 7 SNPs on chromosome 15 (mainly represented by rs1051730 in *CHRNA3*). Two previous GWAS have been reported on AD (Johnson et al., 2006; Treutlein et al., 2009); the latter reported genome-wide significant results, after combining samples, for two SNPs located near *PECR*.

Identification of predisposing genetic variants common to ND and AD, may inform and ultimately improve treatments tailored for dependence to these substances, but an appreciation of comorbid ND and AD when designing genetic studies of either disorder has been lacking. The aim of this study was to conduct a GWAS using DNA pools from Australian cases who met DSM-IV criteria for lifetime history of ND, AD or comorbid AD and ND. We tested for replication of the initial findings using GWA data available from Dutch subjects with ND and AD information. We performed a meta-analysis of AD and ND in the Australian sample and in two samples from the Netherlands. This is the first study to our knowledge that has systematically screened genetic variants to identify those that affect a person's liability to developing both AD and ND.

## Methods

### Australian Sample

Participants were twins initially recruited through the Australian Twin Registry, and other members of their families. Individuals were selected from (a) a large-sibship study (BIGSIB) designed to study families with 5 or more offspring sharing both biological parents and unselected for phenotype (Hansell et al., 2008; Saccone et al., 2007); (b) an alcohol extreme discordant and concordant (EDAC) study (Hansell et al., 2008), designed to focus on families extremely discordant or concordant for heavy drinking and alcohol dependence risk; and (c) the Nicotine Addition Genetics (NAG) study, which targeted families based on heavy smoking index cases with one or more full sibling smokers identified in previous interview and questionnaire surveys (Loukola et al., 2008; Pergadia et al., 2009; Saccone et al., 2007). Self-reported ancestry of the participants is predominantly Anglo-Celtic and Northern European (> 90%) with information available on the birthplace and ethnicity of their four grandparents. Eighty percent ( $N = 1906$  unrelated

individuals) of the pooled GWAS sample provided self-report on ancestry and birthplace of all four grandparents. Of these, 95.6% had all European grandparents (with 70% of these Northern European,  $N = 1281$ ), a further 1.7% indicated Australian Aboriginal ancestry and 1.5% reported some Asian or Middle-Eastern ancestry. Three who reported 'Australian' ancestry are thought to have all grandparents born in Australia but with European ancestry. The remaining 1.1% had one or more grandparent from the Americas, Africa or the Pacific region. All individuals were included in the pooling experiment. All participants provided written informed consent under study protocols approved by the Queensland Institute of Medical Research (QIMR) Human Research Ethics Committee. In total, 9669 individuals from 4882 families, comprising 3115 twins, 5665 non-twin siblings, and 889 parents were interviewed.

### Case and Control Definitions of Alcohol and Nicotine Dependence

The focus of this study was discovery of allelic associations for DSM-IV defined nicotine and alcohol dependence in unrelated individuals using a case-control pooling design (Risch & Merikangas, 1996; Sham et al., 2002). Information was collected using a computer-assisted telephone diagnostic interview (CATI). Self-reported symptoms of DSM-III-R and DSM-IV alcohol dependence and quantity and frequency of alcohol consumption were measured using an adaptation of the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) (Bucholz et al., 1994) for telephone administration. The tobacco section for the CATI was derived from the Composite International Diagnostic Interview (CIDI) (Cottler et al., 1991), and incorporated the Fagerstrom Test for Nicotine Dependence (FTND) (Heatherton et al., 1991) and DSM-III-R and DSM-IV assessments of nicotine dependence. For the purposes of this study, nicotine (ND) and alcohol dependent (AD) cases were defined using DSM-IV criteria. ND controls were defined as individuals exposed to smoking (all AD-/ND- subjects tried cigarettes at some point in their lifetime; see Table 1), but had never become dependent. Similarly, all AD controls were exposed to cigarettes and practically all to alcohol (99% of AD-/ND- subjects had a full drink of alcohol at some point in their lifetime) but did not meet criteria for DSM-IV AD (Table 1).

Three case pools were constructed: (i) AD subjects with no history of ND despite a history of cigarette experimentation (AD+/ND-;  $N = 625$ ); (ii) ND subjects with no history of AD (AD-/ND+;  $N = 674$ ), but in almost all cases a history of experimentation with alcohol; and (iii) comorbid AD and ND subjects (AD+/ND+;  $N = 599$ ); an AD-/ND- control pool ( $N = 488$ ) was also created (Table 1). Each pool was constructed separately for males and females to allow for the examination of possible sex-specific effects. No more than one individual per family was used in the pooling experiment.

**Table 1**  
Characteristics of the Australian Case and Controls Pools.

Pool	AD+/ND-	AD-/ND+	AD+/ND+	AD-/ND-
Pool description	AD Cases	ND Cases	Comorbid Cases	Controls
No. of subjects	625	674	599	488
Gender				
Male	395 (63%)	304 (45%)	320 (53%)	225 (46%)
Female	230 (37%)	370 (55%)	279 (47%)	263 (54%)
Age (years)				
Range	21–73	25–78	25–73	30–79
M ± SD <sup>a</sup>	41.0 ± 8.0	45.5 ± 9.9	41.3 ± 8.1	46.7 ± 10.1
Smoker status				
Ever smokers <sup>b</sup>	100%	100%	100%	100%
Regular smokers <sup>c</sup>	51%	100%	100%	36%
MaxCigs <sup>d</sup>				
Female (M ± SD)	10.0 ± 13.6	33.4 ± 13.1	36.9 ± 16.2	6.3 ± 10.7
Male (M ± SD)	13.1 ± 16.5	42.3 ± 17.8	43.8 ± 16.2	9.8 ± 16.0
DSM-IV ND symptom count				
0–1 symptoms	67%	0%	0%	82%
≥4 symptoms	3%	69%	79%	1%
Alcohol use status				
Ever drinkers <sup>e</sup>	100%	99.9%	100%	99%
Regular drinkers <sup>f</sup>	100%	95%	100%	91%
MaxDrinks <sup>g</sup>				
Female (M ± SD)	14.5 ± 8.1	12.1 ± 10.1	19.0 ± 11.9	8.3 ± 6.1
Male (M ± SD)	29.9 ± 14.8	24.4 ± 12.3	33.6 ± 14.8	18.4 ± 11.9
DSM-IV AD symptom count				
0–1 symptom	0%	60%	0%	76%
≥4 symptoms	49%	2%	62%	1%

Note: AD+, alcohol dependent; AD-, not alcohol dependent; ND+, nicotine dependent; ND-, not nicotine dependent. <sup>a</sup>Mean ± standard deviation. <sup>b</sup>Individuals who have at least tried cigarette smoking ('have you ever tried cigarette smoking, even a puff?'). <sup>c</sup>Individuals who have smoked 100 or more cigarettes in their lifetime. <sup>d</sup>The largest number of cigarettes an individual had ever smoked in a 24-hour period. <sup>e</sup>Individuals who have at least tried alcohol ('have you ever had a drink of alcohol?'). <sup>f</sup>Individuals who have consumed alcohol at least once a month for six months or more. <sup>g</sup>The largest number of alcoholic beverages an individual had ever consumed in a 24-hour period.

### DNA Pool Construction

Association analysis using pooled samples is based on the addition of equal amounts of DNA from each relevant individual to either Case or Control pools, followed by quantitative measurement of the signals from each allele at each typed SNP, as a measure of the case or control allele frequency (Macgregor et al., 2008). Genomic DNA was extracted from peripheral venous blood samples. DNA concentrations in each sample were measured using PicoGreen (Molecular Probes) for the quantitation of double-stranded DNA in solution on a Fluoroskan Ascent CF plate reader (LabSystems, Chicago, IL). DNA samples were initially diluted to 70 ng/μL using 1X TE before another quantification measurement using the PicoGreen method. Each sample was then diluted to 45 ng/μL, re-quantified using Pico Green and diluted to a final concentration of 30 ng/μL. Pools were constructed by combining equal volumes (10 μL) of each DNA sample. Variance introduced by different pool sizes was taken into account during data analysis. All pipetting steps requiring volumes greater than 1 μL were performed on an Eppendorf EpMotion 5070 or EpMotion 5075 robot (Eppendorf AG, Hamburg, Germany).

### Genotyping of Australian Sample

Array-based genotyping was performed on an Illumina BeadStation platform using Infinium 1M (singleton) v1 (1.05 million markers) arrays and Infinium 1M (duo) v3 (1.2 million markers) arrays (Illumina Inc. California, USA). The BeadStation software was set to output bead level intensity data (i.e., raw green/red beadcores). Each of the 8 pools was genotyped in triplicate twice; once on a BeadStation at QIMR (1M v1 arrays) and once on a Beadstation at deCODE Genetics (deCODE Genetics Inc., Reykjavik, Iceland) (1M v3 arrays). In each case, each pool was hybridized to three Illumina arrays. Thus, 6 replicates are available for each of the 8 pools.

### Analysis of Pooled Genotype Data

A small number of SNPs had negative values for one or more beadcores. For some of these SNPs, the vast majority of beadcores had valid positive values with only a small number of slightly negative values. To allow such SNPs to be included in the analysis (hence maximizing genomic coverage), a small amount (+50) was added to negative beadcores. Each SNP was then assessed to see if >90% of SNPs had positive scores. If

so, the small number (<10%) of negative scores were assumed to represent small positive values (+1). SNPs which still had one or more negative scores after this process were not analyzed further. All SNPs designed to detect copy number variants (i.e., invariants) were discarded. SNPs were removed in the initial quality control based on their frequency in HapMap CEU reference samples (The International HapMap Consortium, 2003). SNPs with CEU minor allele frequency <1% were dropped from further analysis (this reduced the number of 1M v1 SNPs of interest to ~800k SNPs and the number of 1M v3 SNPs of interest to ~920k SNPs). Removing SNPs which are likely to be monomorphic in our Caucasian samples is desirable as the subsequent normalization and analysis steps are designed primarily for SNPs which are polymorphic. Furthermore, SNPs with very low (or zero) minor allele frequencies are unlikely to show significant results in genetic association studies.

The beadscores required normalization/calibration because green beadscores were generally larger than red beadscores. The Illumina 1M v1 arrays had 20 stripes per array, each with ~50000 SNPs. The Illumina 1M v3 arrays had 6 stripes per array, each with ~200000 SNPs. Note that these 'stripes' were previously denoted 'strands' but this is now changed to avoid confusion with top/bottom strand. Within each stripe, approximately half of the SNPs were from the Illumina 'top' strand (A/C and A/G SNPs) and half were from the Illumina 'bottom' strand (T/C and T/G SNPs). Normalization was done within stripe by rescaling the red beadscore to make the mean value of the pooling allele frequency (PAF) = 0.5 (over all SNPs on that stripe); PAF was computed as the corrected red intensity divided by the total (corrected red plus green) intensity.

Due to an array reagent issue on the QIMR run arrays which affected 'top' strand SNPs more than 'bottom' strand SNPs, the degree of normalization (amount by which red beadscores were rescaled) on the arrays varied by strand. While results are broadly similar on both strands, the array reagent issue meant that the results from 'bottom' strand SNPs showed lower levels of pooling error. We hence focused on the set of 'bottom' strand SNPs in QIMR run arrays. In the deCODE run arrays (where there were no array reagent issues), both strands performed similarly.

Each SNP had approximately 15 PAF estimates per array. For each pool, a small number of SNPs had fewer than 20 PAF estimates available and these SNPs were dropped. After normalization, SNPs were removed from the analysis if they had a  $-\log_{10}(p)$  value > 6 for the quality control score described in Macgregor et al. (2008). SNPs from the sex chromosomes were also dropped. Overall, on the deCODE run 1M v3 arrays, ~918000 SNPs passed quality control (the vast majority of omitted SNPs were dropped due to their having <1% frequency in the CEU HapMap samples). For the QIMR run 1M v1

arrays, ~375k SNPs from the 'bottom' strand were included in the analysis with the deCODE generated data (i.e., ~40% of SNPs had data from 6 arrays per pool, with the remainder typed on 3 arrays per pool).

### Statistical Analysis of Pool Genotype Data

Given our design, we ran stratified analyses with our Australian sample, i.e., testing for effects of ND after controlling for AD (AD+ND+ versus AD+ND- and AD-ND+ versus AD-ND-) and testing for the effects of AD (AD+ND+ versus AD-ND+ and AD+ND- versus AD-ND-), in addition to testing for comorbid effects (AD+ND+ versus AD-ND-). For each pair of pools (e.g., AD+/ND+ versus AD+/ND- QIMR arrays, AD+/ND+ versus AD+/ND- deCODE arrays, AD-/ND+ versus AD-/ND- QIMR arrays, etc.) run separately, a linear model based approach was used to test for allelic association in which the response variable is the set of PAF estimates for each SNP. The predictor variable is case/control status. The linear model was used to estimate the pooling error across all SNPs. A test statistic which corrects for the pooling error was used to rank SNPs, with  $p$  values based on a Chi-Square distribution. The test statistic is:

$$T_{2\_X} = T_{\text{simple}} * (V / (V + 2 * \text{vare\_pool\_2} + \text{varSNPspecific}))$$

$T_{\text{simple}}$  is  $d^2/V$ , where  $d$  is the mean case pool PAF minus the mean control pool PAF and  $V$  is binomial sampling variance.  $\text{vare\_pool\_2}$  and  $\text{varSNPspecific}$  are the two components of the pooling error — see appendix 2 of Macgregor et al. (2006) for full details. Since this method is based upon contrasting case and control pools, the effect of unequal amplification of alleles is minimal as such effects cancel out.

Results from the different array sets were analyzed separately to allow for the possibility of different levels of pooling error in each experiment. The results for each SNP were then combined by calculating a composite version of  $T_{2\_X}$ , which weighted the result by the inverse of the estimated variance for that SNP — the estimated variance comprised the binomial sampling variance, together with the terms  $2\text{vare\_pool\_2}$  and  $\text{varSNPspecific}$ . In cases where a particular SNP passed quality control for a subset of pools, only the relevant pools contributed to the test statistic. That is, failing quality control for some pools did not necessarily remove that SNP completely from the analysis (although the sample size for that SNP would be reduced).

### Dutch Samples

To confirm the findings from the pool-based GWAS, we sought to replicate our findings *in silico* in two cohorts: the Netherlands Study of Depression and Anxiety (NESDA; Penninx et al., 2008) and the Netherlands Twin Registry (NTR; Boomsma et al., 2006). The NESDA and NTR studies were approved by the Central Ethics Committee on Research Involving Human Subjects of the VU University

Medical Center Amsterdam. Subjects were selected for a GWAS for major depressive disorder (Boomsma et al., 2008; Sullivan et al., 2009) and the sample included 1738 depressed cases and 1802 controls. Details on the recruitment, sample composition, screening procedure and phenotype assessment are described in detail elsewhere (Boomsma et al., 2008). The number of AD and ND cases and controls is summarized in Table 2.

#### Case and Control Definitions of Alcohol and Nicotine Dependence

In both the NESDA and NTR samples, ND cases ( $N = 611$ ) were defined as scoring 4 or more on the Fagerstrom Test for Nicotine Dependence (FTND) (Heatherton et al., 1991) whereas ND controls ( $N = 835$ ) had FTND scores  $<4$ . For NTR subjects longitudinal FTND data (3 timepoints: 2000, 2002 and 2004) were available in smokers and ex-smokers whereas for NESDA subjects FTND data were available in current smokers only. For the ex-smokers from NESDA ( $N = 523$ ) it was not possible to determine their FTND score for the period they smoked so they were excluded from analyses. For both samples the 'never smokers' ( $N = 1208$ ) were excluded because their liability to ND is unknown. Alcohol dependence was assessed by the DSM-IV based lifetime CIDI interview (version 2.1) in the NESDA sample ( $N = 1760$ ), with 318 AD cases and 1242 controls identified. The 200 participants identified as alcohol abusers were excluded. In the NTR sample ( $N = 1777$ ), participants were defined as being alcohol dependent if they ever reported a score of 2 or higher ( $N = 237$ ) on the CAGE (Ewing, 1984) questionnaire and classified as a control if they *never* scored 2 or higher ( $N = 1526$ ). Subjects with missing data on the CAGE were excluded ( $N = 14$ ).

#### Genotyping of Dutch Samples

Genomewide association genotyping for 600,000 SNPs was conducted by Perlegen Sciences (Mountain View, CA, USA). After a series of quality control processes (Sullivan et al., 2009), individual genotype data for 427,037 autosomal SNPs were available. This set of SNPs was used as the basis for an imputation procedure

whereby ~2.5M HapMap (The International HapMap Consortium., 2003) SNPs were imputed. Imputation was carried out in IMPUTE (Marchini et al., 2007) using the HAPMAP Phase II CEU data (Build 35) available on the IMPUTE website (<http://www.stats.ox.ac.uk/~marchini/software/gwas/impute.html#>). The threshold (for genotype uncertainty) to include SNPs in the analyses was 0.70. Genome wide association analyses were carried out in PLINK (Purcell et al., 2007) (option --assoc and --model, no covariates were included).

#### Meta-Analysis of the Australian and Dutch Data

Meta-analysis was performed by extending the analysis of the different array sets run on the pools. First the difference between case and control allele frequencies was computed for each SNP in the Dutch data set. The results for each SNP were then combined by calculating a composite version of  $T_{2\_X}$  which weighted the pooling (Australian data) and individual genotyping (Dutch data) frequency difference values by the inverse of their variance. For the pool data, the variance comprised the binomial sampling variance, together with the terms  $\text{vare}_{\text{pool}_2}$  and  $\text{varSNP}_{\text{specific}}$ . For the individual genotyping data, the variance comprised just the binomial sampling variance. In a limited number of cases, the allele frequency estimates from pooling were different from those obtained from individual genotyping; this was due largely to the potential for unequal amplification of alleles in the pooling analysis. To minimize the effects of this on the meta-analysis results, SNPs with minor allele frequency less than 5% (as measured in the Dutch samples) were filtered out in the final analysis. Our study had very limited power to detect variants with small minor allele frequencies so this is unlikely to have adversely affected results.

#### Connectivity Diagram

SNPs with a  $p$  value  $< .001$  in the meta-analyses of AD ( $N = 515$  SNPs) and ND ( $N = 386$ ) were selected. Of these, 261 AD and 213 ND SNPs were located in or close to ( $<10,000$  bp) 200 and 165 genes respectively. In total, 169 AD and 143 ND genes/gene-products were found in the IPA database (Ingenuity Systems, release IPA6.0). The Ingenuity database contains information

**Table 2**

Number of Dutch Alcohol and Nicotine Dependent Cases and Controls by Sex

Sample	Female	Male	Total
AD cases (NESDA)	178	140	318
AD controls (NESDA)	929	313	1242
Total GWAS alcohol (NESDA)	1107	453	1560
AD cases (NTR)	103	134	237
AD controls (NTR)	1015	511	1526
Total GWAS alcohol (NTR)	1118	645	1763
ND cases (NESDA/NTR)	368	243	611
ND controls NESDA/NTR)	555	280	835
Total GWAS nicotine (NESDA/NTR)	923	523	1446

about structure, biological function and subcellular localization of proteins as well as information about protein-protein interactions. The available information was summarized in a connectivity diagram and the gene-products were manually reorganized by grouping the proteins with related structure or function.

**Results**

**Genome-Wide Association in the Australian Sample**

The lowest *p* values for ND, AD and comorbid AD/ND respectively were  $4.43 \times 10^{-8}$  (rs964170 in *ARHGAP10* on chromosome 4),  $3.91 \times 10^{-7}$  (rs12761801 in *CTBP2* on chromosome 10) and  $1.91 \times 10^{-9}$  (rs7530302 located in a region upstream of *MARK1* on chromosome 1), with two additional SNPs for comorbid AD/ND (rs1784300 near *DDX6* on chromosome 11 and rs12882384 in *KIAA1409* on chromosome 14) giving *p* values less than  $5 \times 10^{-8}$ . Tables 3–5 list detailed results for the 30 SNPs with lowest *p* values

from the analysis of the ND, AD and comorbid AD/ND DNA pools in the Australian sample.

The SNP rs964170 within *ARHGAP10*, which ranked number one for ND, also ranked number 11 for the comorbid analyses. The SNP rs2189816 within *GRM3*, which ranked number two for ND ( $p = 5.5 \times 10^{-7}$ ), also ranked number 29 for the comorbid analyses. The SNP rs7530302 upstream of *MARK1* which ranked first for comorbid AD/ND also showed suggestive association with ND ( $p = 6.87 \times 10^{-6}$ ) and nominal association with AD ( $p = 3.70 \times 10^{-4}$ ).

**Genome-Wide Association in Dutch Samples**

The top results of the Australian sample (rs964170 for ND and rs12761801 for AD) were not replicated in the Dutch samples. In the Dutch analyses, the lowest *p* values were  $4.3 \times 10^{-6}$  for ND (rs11631180 in *TBC1D2B* on chromosome 15),  $2.3 \times 10^{-7}$  for AD in NESDA sample (rs16882302 in *BACH2* on chromosome 6) and  $1.3 \times 10^{-6}$  for AD in NTR sample (rs16953659 on chromosome 16; see Tables S1-S3 in

**Table 3**

Thirty Most Significant Association Results From the Analysis of Nicotine Dependence in the Australian DNA-Pools

Chr	Location (bp)	SNP	Gene	SNP Type	P value
4	148915406	rs964170	<i>ARHGAP10</i>	Intron	$4.43 \times 10^{-8}$
7	86179382	rs2189816	<i>GRM3</i>	Intron	$5.48 \times 10^{-7}$
8	121997550	rs10505382			$9.10 \times 10^{-7}$
2	62611059	rs1997247			$9.70 \times 10^{-7}$
2	157381074	rs11683474			$1.51 \times 10^{-6}$
5	9981862	rs1035961			$1.80 \times 10^{-6}$
20	41771289	rs285171	<i>MYBL2</i>	Intron	$2.76 \times 10^{-6}$
1	224621971	rs3219123	<i>PARP1</i>	Intron	$6.23 \times 10^{-6}$
20	3778462	rs6084496	<i>VISA</i>	Intron	$6.79 \times 10^{-6}$
1	218731575	rs7530302			$6.87 \times 10^{-6}$
6	132210055	rs7768555	<i>ENPP1</i>	Intron	$8.24 \times 10^{-6}$
4	62201416	rs6551640	<i>LPHN3</i>	Intron	$9.59 \times 10^{-6}$
10	74909983	rs11000671	<i>PPP3CB</i>	Intron	$1.02 \times 10^{-5}$
10	115584715	rs10567	<i>DCLRE1A</i>	3' UTR	$1.12 \times 10^{-5}$
8	6603162	rs1046449	<i>AGPAT5</i>	3' UTR	$1.14 \times 10^{-5}$
10	18677238	rs12240995	<i>CACNB2</i>	Intron	$1.33 \times 10^{-5}$
7	141412174	rs4276595	<i>MGAM</i>	Intron	$1.58 \times 10^{-5}$
11	7466142	rs12805648	<i>OLFML1</i>	Coding	$1.79 \times 10^{-5}$
17	52767460	rs9890811	<i>MSI2</i>	Intron	$1.83 \times 10^{-5}$
1	219781759	rs11118748			$1.84 \times 10^{-5}$
7	153598213	rs10274025	<i>NULL</i>	Intron	$1.85 \times 10^{-5}$
1	244772762	rs3120701	<i>TFB2M</i>	Intron	$1.86 \times 10^{-5}$
14	93055200	rs12882384	<i>KIAA1409</i>	Intron	$1.94 \times 10^{-5}$
13	34976427	rs7332116			$1.98 \times 10^{-5}$
18	19916814	rs2305024	<i>C18orf17</i>	Intron	$2.05 \times 10^{-5}$
2	62925712	rs1877026	<i>EHBP1</i>	Intron	$2.06 \times 10^{-5}$
22	33497583	rs137270			$2.11 \times 10^{-5}$
16	74809643	rs2866619			$2.38 \times 10^{-5}$
14	77004340	rs3825694	<i>AHSA1</i>	Intron	$2.42 \times 10^{-5}$
9	367066	rs12348944	<i>DOCK8</i>	Coding	$2.80 \times 10^{-5}$

**Table 4**

Thirty Most Significant Association Results From the Analysis of Alcohol Dependence in the Australian DNA-Pools

Chr	Location (bp)	SNP	Gene	SNP Type	P value
10	126762971	rs12761801	<i>CTBP2</i>	Intron	$3.91 \times 10^{-7}$
12	92595143	rs9668896			$5.62 \times 10^{-7}$
11	118184839	rs1784300			$6.28 \times 10^{-7}$
12	51470992	rs12312467	<i>KRT3</i>	Intron	$1.30 \times 10^{-6}$
10	94192885	rs2798253			$1.93 \times 10^{-6}$
18	67894444	rs17085505			$2.18 \times 10^{-6}$
15	27805919	rs7179270	<i>TJP1</i>	Coding	$2.35 \times 10^{-6}$
18	26365184	rs8085261			$3.15 \times 10^{-6}$
5	2544585	rs462437			$3.43 \times 10^{-6}$
6	52171060	rs2154225			$3.90 \times 10^{-6}$
9	4160457	rs7851070			$7.02 \times 10^{-6}$
2	113537285	rs2472188	<i>IL1F5</i>	3' UTR	$8.72 \times 10^{-6}$
9	10851806	rs2890930			$9.23 \times 10^{-6}$
9	1990684	rs10512034			$1.02 \times 10^{-5}$
15	27788620	rs17683205	<i>TJP1</i>	Intron	$1.07 \times 10^{-5}$
4	54438003	rs2102010			$1.09 \times 10^{-5}$
21	42351204	rs17114247			$1.13 \times 10^{-5}$
4	185302289	rs11132226	<i>ENPP6</i>	Intron	$1.16 \times 10^{-5}$
9	107269811	rs2149592	<i>FSD1L</i>	Intron	$1.17 \times 10^{-5}$
17	59931967	rs16947824	<i>DDX5</i>	Intron	$1.43 \times 10^{-5}$
10	100789822	rs2487891	<i>HPSE2</i>	Intron	$1.48 \times 10^{-5}$
10	122831260	rs10886848			$1.77 \times 10^{-5}$
7	141339277	rs6947481			$1.88 \times 10^{-5}$
8	1588552	rs17064176	<i>DLGAP2</i>	Intron	$2.01 \times 10^{-5}$
12	31686631	rs11051507			$2.07 \times 10^{-5}$
8	103136803	rs2155235			$2.10 \times 10^{-5}$
15	27766632	rs10519663			$2.25 \times 10^{-5}$
2	199387355	rs10189905			$2.29 \times 10^{-5}$
14	28523792	rs12587874			$2.30 \times 10^{-5}$
9	132541165	rs11244096	<i>PRDM12</i>	Intron	$2.38 \times 10^{-5}$

the Appendix A). The results for ND show an association for rs2718771 and rs11771941 ( $p$  values  $4.87 \times 10^{-5}$  and  $4.99 \times 10^{-5}$ ) which are both located in *CNTNAP2* on chromosome 7. Interestingly, we also found a SNP (rs851712) in *CNTNAP2* associated with AD in the NTR sample ( $p = 2.61 \times 10^{-6}$ ).

#### Meta-Analysis and Connectivity Diagram

Tables 6 and 7 list detailed results for the 30 most significant SNPs from the meta-analysis of AD and ND, respectively. None of the SNPs reached genome-wide significance in the meta-analysis. To explore whether the marginally significant results contain SNPs from genes that form biological meaningful networks we constructed a connectivity diagram based on information in the Ingenuity database. The gene-products ( $N = 308$ ) were manually grouped based on the related structure or function of their encoded proteins. Figure 1 shows a group of 15 ion-channels, of which 6 contained one or more SNPs with a  $p$  value smaller than 0.0001 in the meta-analyses, including the potassium

voltage-gated channels *KCNMA1* on chromosome 10 ( $p = 8.91 \times 10^{-5}$ , rs592676) and *KCNC2* on chromosome 12 ( $p = 2.08 \times 10^{-5}$ , rs17803126) for ND. The Figure also shows a group of cell adhesion molecules. The most significant results are found for *PCDH15* on chromosome 15 ( $p = 1.58 \times 10^{-5}$ , rs1935468) and *GPC5* ( $p = 5.26 \times 10^{-5}$ , rs716623) on chromosome 13, both associated with AD. Other interesting results in this group are *DSCAM* on chromosome 21 ( $p = 4.99 \times 10^{-4}$ , rs2837524) and *CNTNAP2* ( $p = 1.11 \times 10^{-4}$ , rs2707592), which are both associated with ND. *CNTNAP2* is also ranked second for AD in the NTR sample ( $p = 2.61 \times 10^{-6}$ , rs851712).

#### Discussion

We have used a GWAS strategy in a search for genes involved in AD, ND and comorbid AD/ND in samples from Australia and the Netherlands. Within the Australian data set, there were four genome-wide significant results ( $p < 5 \times 10^{-8}$ ): a SNP in *ARHGAP10* for ND; and SNPs in *KIAA1409* and near *MARK1*

**Table 5**

Thirty Most Significant Association Results From the Analysis of Comorbid Alcohol and Nicotine Dependence in the Australian DNA-Pools

Chr	Location (bp)	SNP	Gene	SNP Type	P value
1	218731575	rs7530302			$1.90 \times 10^{-9}$
11	118184839	rs1784300			$2.60 \times 10^{-9}$
14	93055200	rs12882384	<i>KIAA1409</i>	Intron	$4.86 \times 10^{-8}$
1	218668246	rs7512221			$7.44 \times 10^{-7}$
10	126762971	rs12761801	<i>CTBP2</i>	Intron	$7.89 \times 10^{-7}$
18	3166377	rs10153321	<i>MYOM1</i>	Intron	$8.73 \times 10^{-7}$
9	124552591	rs10985760	<i>OR1L6</i>	Coding	$8.79 \times 10^{-7}$
2	45435823	rs10167668			$1.12 \times 10^{-6}$
18	54567449	rs2319974	<i>MALT1</i>	3' UTR	$1.17 \times 10^{-6}$
21	42351204	rs17114247			$1.31 \times 10^{-6}$
4	148915406	rs964170	<i>ARHGAP10</i>	Intron	$1.49 \times 10^{-6}$
2	157381074	rs11683474			$2.00 \times 10^{-6}$
21	22071121	rs8131512			$2.10 \times 10^{-6}$
12	92595143	rs9668896			$2.79 \times 10^{-6}$
4	185302289	rs11132226	<i>ENPP6</i>	Intron	$3.08 \times 10^{-6}$
15	67031734	rs4365239			$3.26 \times 10^{-6}$
8	8267771	rs2921010	<i>PRAGMIN</i>	Intron	$3.35 \times 10^{-6}$
1	235038621	rs7526063	<i>MTR</i>	Intron	$4.42 \times 10^{-6}$
1	218692709	rs7553380			$5.29 \times 10^{-6}$
9	32855569	rs6476383			$5.68 \times 10^{-6}$
7	8767093	rs994247			$5.68 \times 10^{-6}$
1	224322577	rs6664668	<i>H3F3A</i>	Intron	$6.96 \times 10^{-6}$
17	59931967	rs16947824	<i>DDX5</i>	Intron	$7.93 \times 10^{-6}$
3	162707232	rs7644500			$9.51 \times 10^{-6}$
7	6118208	rs12667392	<i>USP42</i>	Intron	$9.77 \times 10^{-6}$
16	82112614	rs4074375	<i>CDH13</i>	Intron	$1.14 \times 10^{-5}$
9	10851806	rs2890930			$1.17 \times 10^{-5}$
6	94996990	rs9360055			$1.26 \times 10^{-5}$
7	86179382	rs2189816	<i>GRM3</i>	Intron	$1.29 \times 10^{-5}$
10	49120681	rs169439	<i>FRMPD2</i>	Intron	$1.30 \times 10^{-5}$

and *DDX6* for the comorbid analyses. There were no significant associations for AD alone.

The protein coded by *ARHGAP10* is a member of the Rho GTPase activating protein (Rho-GAP) family which are negative regulators of Rho-GTPase signaling pathways related to actin cytoskeleton dynamics, cell proliferation, and differentiation (Basseres et al., 2002). The fact that this gene is highly expressed in muscle and brain further supports the hypothesis that *ARHGAP10* is important for cell differentiation and might be implicated in neuronal plasticity (Basseres et al., 2002). *KIAA1409* has not been extensively studied, but is believed to form part of a cation channel activated by neuropeptides substance P or neurotensin. *MARK1* is a member of the MARK family of protein kinases and plays a key role in phosphorylation of microtubules involved in dendritic growth. Variants have been associated with autism spectrum disorders (ASDs) and over-expression of *MARK1* in the prefrontal cortex of post-mortem brain tissue of patients with ASDs has been reported

(Maussion et al., 2008). *DDX6* lies at 121 cM on chromosome 11; this locus may harbor genes associated with smoking-related behavior (Li et al., 2008). We have previously found suggestive linkage for DSM-IV defined nicotine withdrawal at 123 cM (LOD = 1.68; Pergadia et al., 2009), near this SNP. Others have reported linkage (LOD = 1.97) for DSM-IV ND at 109 cM (Gelernter et al., 2007).

Another interesting result is *GRM3* (metabotropic glutamate 3 receptor gene) on chromosome 7. In animal models of ND, chronic nicotine exposure was associated with increased activity of mGlu3 receptors (Kenny et al., 2003) and administration of an mGlu2/3 agonist was associated with decreased nicotine consumption, and nicotine self-administration was associated with a downregulation of mGlu2/3 receptors (Liechti et al., 2007). In humans, eight studies have found at least nominal associations with SNPs within *GRM3* and schizophrenia (Chen et al., 2005; Egan et al., 2004; Fallin et al., 2005; Fujii et al., 2003; Marti et al., 2002; Mossner et al., 2008;



**Table 6**

Thirty Most Significant Association Results From the Meta-Analysis of Nicotine Dependence in the Australian and Dutch Samples

Chr	Location (bp)	SNP	Gene	SNP Type	P value
2	1529316	rs6705087			$4.80 \times 10^{-6}$
11	7466142	rs12805648	<i>OLFML1</i>	Coding	$1.35 \times 10^{-5}$
12	120811195	rs2230681	<i>PSMD9</i>	Coding	$1.36 \times 10^{-5}$
5	39288779	rs7700754			$1.89 \times 10^{-5}$
2	1524117	rs4927632	<i>TPO</i>	Intron	$2.04 \times 10^{-5}$
12	73863133	rs17803126	<i>KCNC2</i>	Intron	$2.08 \times 10^{-5}$
22	37544720	rs7288826	<i>NPTXR</i>	3' UTR	$2.13 \times 10^{-5}$
7	36044340	rs10215376			$2.53 \times 10^{-5}$
4	10929509	rs17383790			$2.58 \times 10^{-5}$
6	124483985	rs594664	<i>NKAIN2</i>	Intron	$2.63 \times 10^{-5}$
2	102845067	rs12052617			$2.92 \times 10^{-5}$
1	223741626	rs3795443	<i>ENAH</i>	3' UTR	$4.56 \times 10^{-5}$
11	73830888	rs12800641			$4.60 \times 10^{-5}$
2	102927558	rs11691730			$4.65 \times 10^{-5}$
5	122949002	rs1579036	<i>CSNK1G3</i>	Intron	$4.88 \times 10^{-5}$
2	1498071	rs17732233	<i>TPO</i>	Intron	$4.97 \times 10^{-5}$
8	24918626	rs2950347			$5.28 \times 10^{-5}$
7	31940499	rs11770536	<i>PDE1C</i>	Intron	$5.45 \times 10^{-5}$
5	131755579	rs274551	<i>SLC22A5</i>	Intron	$5.50 \times 10^{-5}$
3	172663379	rs12634193			$6.05 \times 10^{-5}$
5	121345819	rs4496732	<i>SRFBP1</i>	Intron	$6.35 \times 10^{-5}$
4	10742243	rs4697674			$6.85 \times 10^{-5}$
12	24184569	rs11047279	<i>SOX5</i>	Intron	$7.00 \times 10^{-5}$
19	55030265	rs752522	<i>MED25</i>	Intron	$7.84 \times 10^{-5}$
3	45058932	rs10514712			$8.07 \times 10^{-5}$
7	21413414	rs12700284			$8.44 \times 10^{-5}$
8	87674247	rs1992405	<i>CNGB3</i>	Intron	$8.47 \times 10^{-5}$
4	110789544	rs9996730	<i>CCDC109B</i>	Intron	$8.82 \times 10^{-5}$
10	78723963	rs592676	<i>KCNMA1</i>	Intron	$8.91 \times 10^{-5}$
6	33848360	rs2296744	<i>LEMD2</i>	3' UTR	$8.99 \times 10^{-5}$

Norton et al., 2005; Tochigi et al., 2006), and it is known that rates of smoking are very high (> 90%) in patients with schizophrenia (de Leon et al., 2002).

These associations in the Australian sample did not replicate in the Dutch samples. Furthermore, a meta-analysis on the Australian and Dutch samples, for AD and ND did not yield SNPs with genomewide significance. Pathway analysis has been proposed as a strategy to deal with highly polygenic traits in which effect sizes of single SNPs may be too low to be detected even in large studies (Wang et al., 2007). A number of open-access or proprietary systems are available to assess whether groups of genes in pathways are over-represented among the top genes in the GWA results. A feature of this approach is that prior knowledge in correctly assigning genes to particular pathways is crucial. Moreover, genes may have diverse functions which may not be reflected in pathway assignment. Vink et al. (2009) used a more liberal approach in which replicated genes were grouped by their biological functions, cellular locations, and possible interactions of their encoded

proteins. The gene networks were visualized in a connectivity diagram. Using the same strategy, the top meta-analyses SNPs located in or close to genes were summarized in a connectivity diagram (Figure 1). Inspection of those top-ranked SNPs, in the light of potential gene networks involved in AD or ND, yielded a number of interesting findings. One group consisted of ion-channels, including several potassium voltage-gated ion channels, calcium ion-channels and several other types of ion-channels. *KCNMA1* encodes a potassium large conductance calcium-activated channel. It has been postulated as a protein targeted by Lobeline which is a smoking cessation aid (Hu & Agarwal, 2009). In addition, chronic smoking has been shown to down-regulate *KCNMA1* protein synthesis and mRNA expression in bronchial and bronchiolar smooth muscles in rats (Ye et al., 2004). *KCNMA1* also affects the level of response to alcohol in humans (Schuckit et al., 2005) and *C. elegans* (Davies et al., 2003). Because *KCNMA1* has been implicated in nicotine- and alcohol-related disorders in other studies (Davies et al., 2003; Hu & Agarwal, 2009;

**Table 7**

Thirty Most Significant Association Results From the Meta-Analysis of Alcohol Dependence in the Australian and Dutch Samples

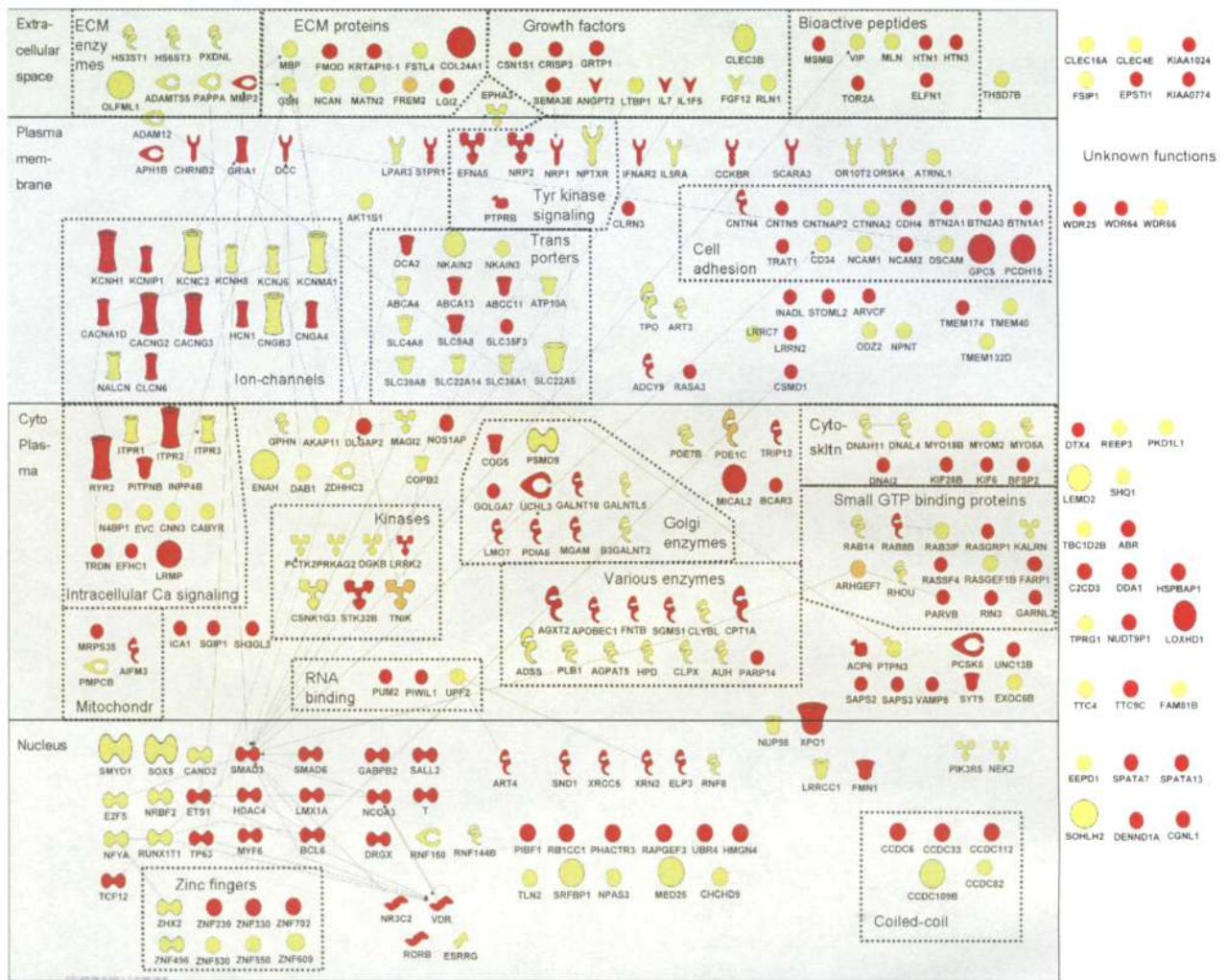
Chr	Location (bp)	SNP	Gene	SNP Type	P value
7	82905406	rs2247219	<i>SEMA3E</i>	Intron	$6.23 \times 10^{-6}$
11	105736354	rs563816			$7.17 \times 10^{-6}$
1	29907191	rs187954			$8.65 \times 10^{-6}$
10	55753731	rs1935468	<i>PCDH15</i>	Intron	$1.58 \times 10^{-5}$
1	11760686	rs10864540	<i>C1orf167</i>	Intron	$1.72 \times 10^{-5}$
11	68298116	rs11228346	<i>CPT1A</i>	Intron	$1.97 \times 10^{-5}$
11	105738304	rs618453			$2.11 \times 10^{-5}$
2	206348076	rs3755233	<i>NRP2</i>	Intron	$2.13 \times 10^{-5}$
13	37920191	rs7328507			$2.43 \times 10^{-5}$
6	63922941	rs6454050			$2.57 \times 10^{-5}$
8	54493239	rs16919143			$2.59 \times 10^{-5}$
7	30843167	rs10229281	<i>FLJ22374</i>	Intron	$2.85 \times 10^{-5}$
22	35340813	rs6000351	<i>CACNG2</i>	Intron	$2.95 \times 10^{-5}$
13	96586447	rs2282424			$2.98 \times 10^{-5}$
13	75069332	rs4885322	<i>UCHL3</i>	Intron	$3.16 \times 10^{-5}$
8	54529044	rs10429416			$3.17 \times 10^{-5}$
5	106936959	rs6881619	<i>EFNA5</i>	Intron	$3.24 \times 10^{-5}$
4	5132896	rs6811343	<i>STK32B</i>	Intron	$3.43 \times 10^{-5}$
5	35081502	rs40200	<i>AGXT2</i>	Intron	$3.65 \times 10^{-5}$
3	179187762	rs6443523			$3.66 \times 10^{-5}$
1	101497539	rs7532171			$3.81 \times 10^{-5}$
2	116787683	rs10496515			$3.92 \times 10^{-5}$
9	77787045	rs4416887	<i>PCSK5</i>	Intron	$3.96 \times 10^{-5}$
6	83427171	rs376096			$4.04 \times 10^{-5}$
17	2380774	rs11655295			$4.19 \times 10^{-5}$
4	66266501	rs4629506			$4.28 \times 10^{-5}$
1	235489612	rs10495392	<i>RYR2</i>	Intron	$4.33 \times 10^{-5}$
12	25135160	rs1979522	<i>LRMP</i>	Intron	$4.38 \times 10^{-5}$
11	12177958	rs10831759	<i>MICAL2</i>	Intron	$4.48 \times 10^{-5}$
12	40033695	rs10785246			$4.67 \times 10^{-5}$

Schuckit et al., 2005; Ye et al., 2004) and several potassium voltage-gated ion channels were present in our top-30 results for ND it is likely that these ion channels play a role in the liability for ND and AD. A second group is formed by the genes coding for cell adhesion molecules. Several of these genes were also detected in previous GWAS to addiction phenotypes; *PCDH15* (Uhl et al., 2008b), *DSCAM* (Liu et al., 2006; Uhl et al., 2008a), *CNTN5* (Bice et al., 2009), *CNTN4* (Bice et al., 2009). *CNTNAP2* is a member of the neuroligin family which act as cell adhesion molecules and receptors in synaptic signaling. Several neuroligins are associated with addictive behaviors (e.g., *NRXN3* (Hishimoto et al., 2007; Lachman et al., 2007; Novak et al., 2009)), and *CNTNAP2* is previously associated with autism (Alarcon et al., 2008; Arking et al., 2008), schizophrenia (Friedman et al., 2008) and openness to experience (Terracciano et al., 2008).

Compared to the results of a GWAS for smoking initiation and current smoking in the Dutch NESDA/NTR sample and 3 replication samples (Uhl et al.,

2008a), some gene groups overlapped with the results of the present study (Figure 1), including the cell adhesion proteins *DSCAM* and *CNTN4*, transporters *ABCA13* and *ATP10A*, and cytoskeleton proteins *MYOM2* and *DNAH11*. In contrast, the study of Vink et al. (2009) showed a large group of glutamate signaling proteins which was not found in the present study (only *GRIA1*) whereas the group of genes involved in intracellular calcium signaling identified in the present study was not found by Vink et al. The overlapping groups (and genes) may reflect mechanisms involved in addictive behavior in general, while the specific groups may reflect mechanisms specific for a phenotype, for example glutamate genes for smoking initiation and intracellular calcium signaling for nicotine dependence.

Our approach to discovery of genes affecting AD and ND has both strengths and limitations. The main strength is that we have taken a joint approach to alcohol and nicotine dependence, which are known to have a partially overlapping genetic basis. This approach should allow the identification of common



**Figure 1** Connectivity diagram of the proteins encoded by the top genes (SNP with  $p$  value  $< .001$  in or close to gene) from the meta-analyses for AD and ND.

Note: SNPs with a  $p$  value  $< .001$  in the meta-analyses of AD ( $N = 515$  SNPs) and ND ( $N = 386$ ) were selected. Of these, 261 AD and 213 ND SNPs were located in or within 10,000 bp of 200 and 165 genes, respectively. Those gene names were entered in the IPA database (Ingenuity Systems, release IPA 6.0) and 169 AD and 143 ND genes/gene-products were found in the database (gene names that were not mapped included loci named LOCxxx). We applied a network-based approach that grouped these genes by the biological functions, cellular locations, and possible interactions of their encoded proteins. The proteins are represented as nodes and are displayed in various shapes that represent the functional class of the gene product (see symbol legend). Nodes were manually reorganized with the 'pathway designer' and the location of the nodes is based on the subcellular location of the gene products: extracellular, plasma membrane, cytoplasm, nucleus or unknown localization. Small symbols indicate genes with lowest SNP  $0.001 < p$  value  $< .001$ , large symbols indicate genes with  $p$  value  $< .0001$ . Depicted in red are the genes for AD, in yellow are those for ND and in orange those that are associated with both AD and ND. The resulting connectivity diagram showed several noteworthy groups of genes belonging to the same functional class (see dotted boxes), of which some are described in more detail in the text.

genes and mechanisms for AD and ND. A main limitation is that, for the Australian sample, a DNA-pooling approach was used. Although DNA-pooling is theoretically sound (Macgregor et al., 2006; Macgregor et al., 2008) and can produce important results at a low genotyping cost (Brown et al., 2008; Melquist et al., 2007; Papassotiropoulos et al., 2006; Spinola et al., 2007; Steer et al., 2007), it was necessary to discount data from SNPs with low minor allele frequency and poor signal-to-noise ratios which might have been captured in an individual-genotyping design. A second limitation is the comparatively small sample size, although we estimate that power would be adequate to detect variants accounting for about 1-2% of variation in liability to AD or ND. Furthermore, we attempted to replicate

the Australian results in another population with data available for both alcohol- and nicotine- dependence-related phenotypes. The lack of reproducible results on SNP level reinforces the view that genetic risk of AD or ND arises from multiple polymorphisms of individually small effect. Other published genomewide association studies of AD or ND have similarly found a paucity of large effects (e.g., Bierut et al., 2007; Treutlein et al., 2009). A third limitation of the current meta-analysis was the difference in dependence definitions in the three samples. Nicotine dependence was assessed using DSM-IV criteria for lifetime ND in the Australian sample and for FTND criteria in both Dutch samples. Alcohol dependence was defined as DSM-IV lifetime AD in the Australian and NESDA samples whereas the broader

CAGE criteria were used in the NTR sample. A fourth limitation may be that specific genetic effects on AD may be harder to detect as it is much more likely to be comorbid with ND than vice versa. For instance in our general Australian population sample in ever smokers 32% of ND cases have a history of AD, whereas 56% of our AD cases have a history of ND. Also the small number of comorbid cases available within the Dutch samples precluded the comorbid AD+ND+ vs AD-ND-analysis in those samples. A final feature, which could be considered either a strength or a limitation, is recruitment of subjects from the general population rather than a clinical source. The severity of dependence may well be less among a population-based sample, but most alcohol-related problems occur in the large number of people who are only moderately affected; and smoking is also a problem affecting the general community. It should also be noted that this is a study of alcohol and nicotine dependency versus non-dependency. As such, we did not use a group of hypercontrols, but rather allowed AD and ND controls to represent the general population. This is similar to many other GWA studies in which controls are not explicitly sampled from the low tail of the risk distribution. For example, most controls in studies of cardiovascular disease are presumably carrying some atheromatous plaques but haven't (yet) had the cardiovascular event which leads to the case diagnosis.

In conclusion, we have identified a number of gene networks (ion-channels, cell adhesion molecules) and genes (*ARHGAP10*, *KIAA1409*, *GRM3*) that may play a role in AD, ND or comorbid AD/ND although confirmation in a larger GWAS consortium is clearly needed.

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## APPENDIX A

**Table S1**

Thirty Most Significant Association Results From the Analysis of Nicotine Dependence in the Dutch Sample

Chr	Location (bp)	SNP	Gene	SNP Type	P value
15	76161636	rs11631180	<i>TBC1D2B</i>	Promoter	$4.264 \times 10^{-6}$
20	38475285	rs3092155			$4.371 \times 10^{-6}$
7	154997865	rs6459707			$8.485 \times 10^{-6}$
7	154996933	rs6971850			$1.145 \times 10^{-5}$
5	141168499	rs17097654			$1.269 \times 10^{-5}$
5	39249453	rs696748	<i>FYB</i>	Intron	$1.531 \times 10^{-5}$
10	28774361	rs7085106			$1.695 \times 10^{-5}$
15	76136405	rs12148365	<i>TBC1D2B</i>	Intron	$2.060 \times 10^{-5}$
16	51113243	rs8046979			$2.300 \times 10^{-5}$
18	7509001	rs576192			$3.056 \times 10^{-5}$
6	106644483	rs6923608	<i>PRDM1</i>	Intron	$3.204 \times 10^{-5}$
18	7478970	rs567338			$3.700 \times 10^{-5}$
11	2743637	rs231872	<i>KCNQ1</i>	Intron	$3.720 \times 10^{-5}$
15	96426216	rs12148124			$3.759 \times 10^{-5}$
3	188453990	rs3105782	<i>MASP1</i>	Intron	$3.850 \times 10^{-5}$
15	76131977	rs4886554	<i>TBC1D2B</i>	Intron	$3.869 \times 10^{-5}$
18	4016587	rs1372631			$4.131 \times 10^{-5}$
5	158920307	rs17729321			$4.171 \times 10^{-5}$
18	7481478	rs533682			$4.237 \times 10^{-5}$
12	104628953	rs4590982			$4.240 \times 10^{-5}$
5	160228811	rs12188645			$4.421 \times 10^{-5}$
11	2707296	rs2283214	<i>KCNQ1</i>	Intron	$4.527 \times 10^{-5}$
20	1558939	rs3746722	<i>SIRPB2</i>	Coding exon	$4.571 \times 10^{-5}$
12	12037208	rs12582693			$4.596 \times 10^{-5}$
7	147310468	rs11771941	<i>CNTNAP2</i>	Intron	$4.874 \times 10^{-5}$
7	147294425	rs2178771	<i>CNTNAP2</i>	Intron	$4.990 \times 10^{-5}$
12	74699761	rs7297654	<i>PHLDA1</i>	Downstream	$5.087 \times 10^{-5}$
11	2707861	rs231910	<i>KCNQ1</i>	Intron	$5.102 \times 10^{-5}$
20	1560819	rs1535883	<i>SIRPB2</i>	Intron	$5.386 \times 10^{-5}$
8	87695579	rs2362267	<i>CNGB3</i>	Intron	$5.752 \times 10^{-5}$

**Table S2**

Thirty Most Significant Association Results From the Analysis of Alcohol Dependence in the Dutch NESDA Sample

Chr	Location (bp)	SNP	Gene	SNP Type	P value
6	90731172	rs16882302	<i>BACH2</i>	Intron	$2.278 \times 10^{-7}$
22	46049871	rs11090754			$2.161 \times 10^{-6}$
21	42006728	rs13051745	<i>C21orf129</i>	Intron (boundary)	$4.778 \times 10^{-6}$
13	83474030	rs9546562			$6.829 \times 10^{-6}$
10	43383818	rs2393938	<i>ZNF239</i>	5' UTR	$7.126 \times 10^{-6}$
10	43371825	rs10793442	<i>ZNF239</i>	3' UTR	$7.640 \times 10^{-6}$
10	43370721	rs7893169	<i>ZNF239</i>	Downstream	$7.640 \times 10^{-6}$
10	43374973	rs7894102	<i>ZNF239</i>	Intron	$7.640 \times 10^{-6}$
10	51231805	rs17178655	<i>MSMB</i>	Intron	$7.784 \times 10^{-6}$
13	83489863	rs9546566			$1.023 \times 10^{-5}$
22	19654308	rs178260	<i>AIFL</i>	Intron	$1.365 \times 10^{-5}$
14	68270943	rs8019389			$1.956 \times 10^{-5}$
1	218174540	rs12129498	<i>SLC30A10</i>	Promoter	$2.706 \times 10^{-5}$
11	16791086	rs216495	<i>PLEKHA7</i>	Intron	$2.880 \times 10^{-5}$
19	41939312	rs826969	<i>MGC62100</i>	Intron	$3.146 \times 10^{-5}$
4	109150488	rs4956145	<i>HADHSC</i>	Coding exon	$4.110 \times 10^{-5}$
7	4162514	rs619672	<i>SDK1</i>	Intron	$4.593 \times 10^{-5}$
3	110120120	rs2715689	<i>GUCA1C</i>	Intron	$4.905 \times 10^{-5}$
13	29542418	rs9314995			$5.051 \times 10^{-5}$
5	118701596	rs17164572			$5.286 \times 10^{-5}$
5	118703145	rs3813301			$5.408 \times 10^{-5}$
5	118695541	rs7704398			$5.408 \times 10^{-5}$
6	83427171	rs376096			$5.557 \times 10^{-5}$
1	94889745	rs947386			$5.675 \times 10^{-5}$
6	46050909	rs3777599	<i>CLIC5</i>	Intron	$5.699 \times 10^{-5}$
14	40625514	rs1954456			$5.729 \times 10^{-5}$
14	40627078	rs1959909			$5.729 \times 10^{-5}$
6	46055426	rs4714899	<i>CLIC5</i>	Intron	$6.538 \times 10^{-5}$
14	26880311	rs1456796			$6.861 \times 10^{-5}$
20	48368185	rs928072	<i>LOC284751</i>	Downstream	$6.939 \times 10^{-5}$

**Table S3**

Thirty Most Significant Association Results From the Analysis of Alcohol Dependence in the Dutch NTR Sample

Chr	Location (bp)	SNP	Gene	SNP Type	P value
16	53144933	rs16953659			$1.328 \times 10^{-6}$
7	147159664	rs851712	<i>CNTNAP2</i>	Intron	$2.607 \times 10^{-6}$
11	47933458	rs7936879			$3.425 \times 10^{-6}$
19	55443610	rs11670669	<i>MYH14</i>	Intron	$4.237 \times 10^{-6}$
5	170302206	rs7701805	<i>RANBP17</i>	Intron	$4.945 \times 10^{-6}$
11	48128941	rs17198978	<i>PTPRJ</i>	Intron	$5.455 \times 10^{-6}$
3	82540820	rs17019771			$7.083 \times 10^{-6}$
18	29032084	rs17667691	<i>C18orf34</i>	Intron	$7.690 \times 10^{-6}$
13	105448745	rs4483718			$7.733 \times 10^{-6}$
5	146655326	rs13170855	<i>STK32A</i>	Intron	$8.271 \times 10^{-6}$
6	135121310	rs7749709			$1.156 \times 10^{-5}$
17	34317874	rs82977	<i>LASP1</i>	Intron	$1.190 \times 10^{-5}$
14	76788374	rs2287386	<i>TMEM63C</i>	Intron	$1.233 \times 10^{-5}$
21	23991310	rs2828434			$1.325 \times 10^{-5}$
11	73777540	rs658126	<i>PGM2L1</i>	Intron	$1.509 \times 10^{-5}$
4	166365270	rs12513327	<i>KLHL2</i>	Intron	$1.645 \times 10^{-5}$
5	54586456	rs12523234	<i>DHX29</i>	Downstream	$1.728 \times 10^{-5}$
5	54594729	rs897669	<i>DHX29</i>	Intron	$1.795 \times 10^{-5}$
11	48473182	rs7944287	<i>OR4A47</i>	Downstream	$1.864 \times 10^{-5}$
11	46923384	rs2903517	<i>C11orf49</i>	Intron	$2.174 \times 10^{-5}$
3	72626886	rs10511022			$2.240 \times 10^{-5}$
7	137927174	rs7799136	<i>LOC136306</i>	Downstream	$2.427 \times 10^{-5}$
4	189484767	rs11930915			$2.514 \times 10^{-5}$
5	54735640	rs12518892	<i>SKIV2L2</i>	Intron	$2.533 \times 10^{-5}$
5	54647490	rs13154710	<i>DHX29</i>	Promoter	$2.533 \times 10^{-5}$
3	143743252	rs7636909	<i>ATR</i>	Intron	$2.686 \times 10^{-5}$
2	211427130	rs13002544			$3.016 \times 10^{-5}$
5	54524581	rs12109297			$3.533 \times 10^{-5}$
2	86961722	rs6709175			$3.617 \times 10^{-5}$
7	47249414	rs6463401			$3.673 \times 10^{-5}$