Population based forward genetic screen of mutagenized zebrafish identifies loci associated with nicotine preference and human smoking behavior

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ABSTRACT

BACKGROUND: Although there is clear evidence of genetic contributions to susceptibility to nicotine addiction, it has proved difficult to identify causal alleles and pathways from studies in humans. Mutagenesis in model species generates strong phenotypes not present in wildtype populations and can be used to identify biological mechanisms underlying quantifiable behaviours. We tested the hypothesis that a forward genetic screen for nicotine preference in zebrafish can predict loci and biological mechanisms influencing human smoking behaviour.

METHODS: A population-based forward genetic screen of ethylnitrosurea-mutagenized zebrafish was used to identify lines of fish showing altered nicotine preference. Immunohistochemical, behavioral and quantitative PCR analyses were used to characterize mutant larvae. Focussed SNP analysis of the homologous human locus in cohorts from the UK and a Finnish Twin study assessed the predictive validity of the zebrafish data for human smoking behavior.

RESULTS: We show nicotine preference is heritable in fish as in humans and identify loss-of-function mutations in the zebrafish *Slit3* gene as associated with increased nicotine preference. *Slit3* mutant larval fish showed altered sensitivity of habituation to acoustic startle to dopaminergic antagonists and increased *Drd2* and *Drd3* mRNA expression. Dopaminergic neuronal pathfinding was unaffected. Analysis of the SLIT3 locus in two independent human cohorts identified 2 genetic markers that predict level of cigarette consumption and likelihood of cessation.

CONCLUSION: These findings suggest a role for SLIT3 signaling in development of dopaminergic pathways affecting behaviours associated with nicotine dependence and confirm the translational relevance of the zebrafish model in exploring complex human behaviors.

INTRODUCTION

Tobacco smoking is the leading preventable cause of death worldwide and places a heavy social and financial burden on society (1–3). It is well established that aspects of nicotine addiction have a strong genetic component (4–6). However, identifying causal genetic factors and exploring the mechanisms by which they act is challenging in human studies: the field has been characterized by small effect sizes and lack of replication such that there are remarkably few genes and loci that can be confidently linked to nicotine addiction. The strongest evidence for causal effects in humans is for functional variants in *CHRNA5* and *CYP2A6*, affecting amount smoked and nicotine metabolism respectively.

As approaches to identify genetic risk are difficult in humans, research has been facilitated by studies in animal models, with a focus on genomic analysis of inbred and selectively-bred naturally occurring genetic strains (7). This type of study produces quantitative trait loci (QTL) maps of multiple loci, each with a small impact on the phenotype. However, as with human studies, it is inherently difficult to identify relevant genes from QTL maps, as the overall phenotype cannot be predicted by individual genotypes. Mutagenesis studies in animal model systems may overcome these limitations: N-ethyl-N-nitrosourea (ENU) mutagenesis introduces thousands of point mutations into the genome, dramatically increasing variation with the potential to generate much stronger phenotypes than those occurring in a natural population. Although the disruptive mutations generated by ENU mutagenesis are unlikely to occur in a natural population, they may identify pathways the attenuation of which, through 'weaker' variants in orthologous genes, can be reasonably expected to influence the same behavior in humans. Thus, ENU mutagenesis in model species could be used to

distinguish novel, naturally occurring variants influencing human addictive behavior by identifying key genes and pathways affecting conserved behavioural phenotypes.

ENU Mutagenesis screens for addiction-related phenotypes have been undertaken in both invertebrates and vertebrates. They have identified fruit flies (*Drosophila melanogaster*) that vary in their alcohol consumption and response to alcohol (8). Mutagenesis studies for acute effects of drugs of abuse in rodents have had limited success (9,10): they are limited by the small number of offspring in each generation that, given the inherent variability of behavior, makes identification of significant differences difficult. The prolific reproductive nature of zebrafish (up to 300 eggs in a single pairing) overcomes this difficulty but, to date, the predictive validity of behavioral screens for addiction-related phenotypes in fish for human behavior has not been established.

Here, we describe a population-based forward genetic approach to screen families of ENU mutagenized zebrafish for nicotine-induced conditioned place preference (CPP). We identify a novel mutation influencing sensitivity to rewarding effects of nicotine and characterize the mutation using immunohistochemical analysis of neuronal pathways and behavioral responses to acoustic startle, a response known to be modulated by dopaminergic signaling and, in humans, associated with vulnerability to addiction (11–13). We then use focused SNP analysis of human cohorts to assess the predictive validity of findings in fish for human smoking behavior.

MATERIALS AND METHODS

Zebrafish: Fish were housed in a recirculating system (Techniplast, NL) on a 14h:10h light:dark cycle (0830–2230). All fish were age and weight matched for each part of the study and were 3-5 months old at the start of testing. The housing and testing rooms were maintained at ~25–28ºC. Fish were maintained in aquarium-treated water and fed three times daily with live artemia (twice daily) and flake food (once). All procedures were carried out under license in accordance with the Animals (Scientific Procedures) Act, 1986 and under guidance from the local animal welfare and ethical review board at Queen Mary University of London.

Human Cohorts: Human subjects for studies in London were recruited from three clinical groups: patients with chronic obstructive pulmonary disease (COPD) (Cohort 1; n=272); patients with asthma (Cohort 2; n = 293); and residents and carers in sheltered accommodation, with neither condition (Cohort 3; n=298). The methods used for recruitment and definition of phenotypes are reported elsewhere (14–16). The studies were approved by East London and The City Research Ethics Committee 1 (09/H0703/67, 09/H0703/76 and 09/H0703/112) and written informed consent was obtained from all participants.

Details of the Finnish twin cohort are reported elsewhere (17–19). In brief, twin pairs concordant for moderate to heavy smoking were identified from the population-based Finnish Twin Cohort survey responders. The twin pairs and their siblings were invited to a computer-assisted telephone-based structured psychiatric interview (SSAGA) (17), to yield detailed information on smoking behavior and nicotine dependence defined as by Fagerström Test for Nicotine Dependence (FTND) and DSM-IV diagnoses. Human

phenotypes to be investigated in relation to zebrafish nicotine seeking behavior were determined by consensus *a priori*. Detailed definitions of Finnish phenotypes can be found in the supplementary material.

Human genotyping: For the London cohorts, DNA from participants was extracted from whole blood using the salting-out method (20) on the Biomek FX robot (Beckman Coulter), quantified using the Nanodrop spectrophotometer and normalized to 5ng/µl. 10ng DNA was used as template for 2 µl TaqMan assays (Applied Biosystems, Foster City, CA, USA) performed on the ABI 7900HT platform in 384-well format and analyzed with Autocaller software. Pre-developed assays were used to type all SNPs. See supplementary table 3 for primer and reporter sequences. Typing for two SNP (rs6127118 and rs11574010) failed. For the Finnish cohort, DNA was extracted from whole blood and genotyping was performed at the Wellcome Trust Sanger Institute (Hinxton, UK) on the Human670-QuadCustom Illumina BeadChip (Illumina, Inc., San Diego, CA, USA), as previously described (17–19).

Conditioned place preference (CPP): Following habituation and determination of basal preference, fish were conditioned to a predicted threshold concentration of nicotine (Sigma, Catalogue number: N1019. Concentration of 5µM based on previous research (21)) over three consecutive days and assessed for a change in place preference the following day. The CPP procedure was carried out using a balanced assay as described previously (21–23) (see Supplementary Material for details). Of the 30 families included in the screen, four individuals were selected from each to make a total of 120 fish.

Breeding strategy: The zebrafish lines included in the experiment were all generated at the Sanger Institute, as part of the Zebrafish Mutation Project [https://www.sanger.ac.uk/resources/zebrafish/zmp/]. F2 lines, heterozygous for Tuebingen long-fin wildtype were obtained and inbred to generate F3 lines to allow the inclusion of homozygous mutations in the population screen. A total of 120 ENU-mutagenized F3 mutant zebrafish were initially selected for screening. Both male and female age/sex matched individuals were selected from 30 distinct lines (3-4 fish from each line) each containing 10-20 known exonic mutations and approximately 1500 unknown mutations across the entire genome. Following completion of the first round CPP screen the individuals in the upper (H) and lower (L) 5% of the change in preference distribution curve were kept for analysis and further breeding: Fish were bred H:H and L:L for the second round screen. In two cases (AJBQM1,2) four fish from the same family were found to be within the top or bottom 5%. In these cases, only progeny from one member of the family were included in the second generation screen, for which a similar strategy was used.

Rescreening of outlier siblings and independent sa202 Slit3 allele: Once we had determined lines that clustered together in the extremes of the distribution, the siblings of the high (n=10) and low (n=14) responder lines were screened for CPP along with control fish. Larvae from the sa202+/- line were obtained from the Sanger Institute and reared to adulthood then assayed for CPP to 5μM nicotine (n=10) along with sibling controls sa202 +/+ (n=11). All fish were fin-clipped and genotyped using site-specific PCR after CPP.

Genomic DNA extraction and Polymerase Chain Reaction (PCR): Genomic DNA was extracted from fin-clips using QIAGEN DNeasy® Blood and Tissue Kit according to manufacturer's instructions. Samples were eluted into distilled water and stored at -20°C until later use. Site specific PCR used for genotyping and quantitative PCR used for analysis of *Drd2/Drd3* expression levels were conducted as described previously (24,25). Details can be found in Supplementary Material.

Antibody staining: In order to visualize axonal pathways, fluorescent immunohistochemistry was carried out in three day old embryos from wildtype *Slit3*$^{+/+}$, heterozygous mutant *Slit3*$^{sa1569/+}$ and homozygous mutant *Slit3*$^{sa1569/sa1569}$ in-crosses. See supplementary material for detailed method and results.

Startle stimuli response in presence of a D2/D3 receptor antagonist: Five day old larvae, generated from adult *Slit3* wildtype and homozygous mutant (*Slit3*$^{sa1569/sa1569}$) fish as for qPCR (See supplementary material), were individually placed in 24 well plates. In the drug-free condition, each well contained system water and 0.05% of dimethyl sulfoxide (DMSO). In the pharmacological conditions, serial dilutions of the D2/D3 antagonist amisulpride (Tocris, Catalogue number: 71675-86-9) were prepared to give final concentrations of 0.05 mg/L, 0.1 mg/L or 0.5 mg/L amisulpride in 0.05% DMSO. Amisulpride concentrations were chosen based on previous studies in zebrafish (26) and correspond to 50, 100 and 500 times its Ki value for the D2 receptor in mammals (27,28). To ensure that larvae were exposed to the drug for the same amount of time, amisulpride was added 15 minutes before undertaking the experiment. Care was taken regarding the distribution of concentrations and genotypes to ensure that experimental groups were randomly distributed in the plates. Plates were placed in a

custom-made filming tower with a tapping device that applied 10 sound/vibration stimuli with two seconds interval between them. The setup for this device has been described elsewhere (29). Larval movement was recorded using Ethovision XT software (Noldus Information Technology, Wageningen, Netherlands) and data were outputted in one second time-bins.

Statistics: Nicotine preference scores were calculated as proportion of time spent in the vicinity of each stimulus (i.e., for spots, Time(T)spots/ [Tspots + Tstripes]). Population means between generations were compared using independent two-sample t-tests, and effect-sizes ascertained using Cohen's d (30). For the rescreen of outlier siblings and sa202 line, mutant lines were compared with wildtype controls using an independent two-sample t-test.

Response to startle stimuli was defined as the distance travelled (mm) during one second after each tap. Linear mixed models were calculated to assess differences in baseline distance moved, distance moved one second after the first stimulus and distance moved during all the stimuli across groups. To assess habituation to the stimuli, we calculated the number of fish exhibiting a response ≤ 50% of the mean startle response to the first tap stimulus (set to 2.5mm because this was 50% of the mean distance travelled across all groups at tap one, and response to the first stimulus was not significantly different across experimental groups (n.s.)). Differences in the number of fish that moved ≤ 2.5mm across genotype and drug groups were compared using ANOVA. Results of all statistical analyses are reported with respect to a type-1 error rate of α=0.05. Post-hoc tests were conducted using Tukey's HSD.

Relative mRNA expression in qPCR was calculated against reference gene cycle-threshold (Ct) values, and then subjected to one-way ANOVA.

Human association analyses: Association analysis for the London cohorts was performed using PLINK v1.07 (31). SLIT3 SNPs that had been previously associated with disease phenotype and induced an amino acid change were identified and the 20 with the highest linkage disequilibrium score selected for analysis. Of twenty SLIT3 SNPs, one departed from Hardy-Weinberg equilibrium (rs13183458) and was excluded. Linear regression was performed on average number of cigarettes smoked per day, controlling for age, sex and cohort. This analysis was repeated on heavy smokers (≥ 20 cigarettes per day) and light smokers (< 20 cigarettes per day) to investigate whether effects were related to intake level. Smoking cessation (current *vs* ever smokers) was analyzed using logistic regression controlling for age, sex and cohort. All the analyses were performed under the additive genetic model and multiple testing was taken into account using the Benjamini-Hochberg adjustment. Only individuals from European ancestry were included in the analyses.

Association analyses for the Finnish Twin Cohort were performed using GEMMA v0.94 (32) with linear mixed model against the allelic dosages controlling for age and sex. The sample relatedness and population stratification were taken into account by using genetic relatedness matrix as random effect of the model.

RESULTS

To assess heritability of nicotine preference in zebrafish we screened adult F2 and F3 offspring of ENU mutagenized fish (33) for nicotine CPP and bred from extremes of preference (Figure 1A-C). An increasing difference in nicotine preference between offspring of low and high responders in successive generations indicates that the populations are increasingly distinct (Cohen's $d = 0.89$ to $d = 1.64$) (Figure 1D & 1E).

Two families of zebrafish AJBQM1 and AJBQM2 clustered at the top and bottom of the nicotine preference distribution respectively, suggesting a dominant genetic effect (Figure 2A). Exome sequencing of F1 fish (33) used to generate AJBQM1 and AJBQM2 identified 26 nonsense and essential splice site mutations. We genotyped fish at these loci and determined the corresponding nicotine preference.

Of the 14 mutations in AJBQM1 (Supplementary Table 1), only *Slit3*$^{sa1569/+}$ (exon 7 splice acceptor site disruption at amino acid position 176), segregated with the response (Figure 2B & Supplementary Table 4). No mutations in AJBQM2 segregated with nicotine preference and this line was not examined further.

To confirm that loss of *Slit3* function was related to nicotine seeking behavior we used a second allele, *Slit3*^{sa202}, with a G>T transversion producing a premature stop codon at position 163. Heterozygous *Slit3*^{sa202} fish showed enhanced nicotine conditioned place preference (P=0.03) compared to wildtype siblings (Figure 2C & 2D). Both *Slit3*^{sa1569} and *Slit3*^{sa202} mutations affect splicing in the region before the second leucine rich repeat (LRR) domain in the encoded protein (Figure 2F), which is essential for interaction with ROBO receptor proteins (34).

SLIT3 is a member of a family of proteins with established axon guidance properties

and previously suggested to be involved in dopaminergic pathfinding (35). Therefore we performed immunostaining of the axonal projections in three-day-old zebrafish larvae. No differences between *Slit3*^{sa1569} mutant fish and wildtype fish were observed in the axonal tracts labelled by anti-acetylated tubulin antibody (Supplementary Figure 1B) nor in the catecholaminergic tracts labelled by anti-tyrosine hydroxylase antibody (Supplementary Figure 1A).

As subtle effects on dopamine circuit formation may not have been detected by our antibody staining, we examined dopamine signaling in wildtype and mutant fish using habituation of locomotor response to acoustic startle, a response known to be sensitive to dopaminergic antagonists such as the D2/D3 antagonist amisulpride (36). For all groups tested the tap startle induced a robust increase in distance travelled one second after stimulus. The mean distance travelled in response to each tap decreased with tap number. There were no significant differences in locomotion in the 15 sec before tap startle, in magnitude of the response to the first tap stimulus nor in total distance moved across all tap stimuli across any of the experimental groups (Supplementary Figure 2).

In line with the habituation response paradigm (37), for all wildtype experimental groups a higher number of fish showed a 50% reduction in distance travelled as the number of stimuli increased (Effect of stimuli number on the number of fish reaching 50% habituation [$F(8,24)=3.262$, $p=0.0117$]) (Figure 3A). In wildtype fish, amisulpride caused a dose-dependent increase in the number of fish showing a 50% reduction in response to each stimulus (Effect of amisulpride dose on the number of fish reaching 50% habituation [$F(3, 24)=12.150$, $p<0.001$]) (Figure 3A).

In absence of amisulpride, *Slit3*^{sa1569} mutants showed increased habituation compared to wildtype fish; a greater number of fish showed 50% reduction in startle response

across all stimuli (Figure 3B, effect of genotype in drug free conditions [F(2,18)=23.83, p<0.001]). In contrast to wildtype fish, *Slit3*^{sa1569} mutants showed a U-shaped response to amisulpride: a reduction of habituation at low doses and increase at high doses. The presence of a *Slit3*^{sa1569} genotype by amisulpride dose interaction was confirmed by ANOVA [F(6, 99)=8.076, p < 0.001] (Figure 3B).

As mutant fish showed altered sensitivity to the D2/D3 antagonist, we examined *Drd2* and *Drd3* receptor expression using quantitative PCR. Expression of both *Drd2a* and *Drd3* was significantly upregulated in *Slit3*^{sa1569} heterozygous and homozygous mutant larvae compared to controls. Effect of genotype was confirmed for *Drd2a*: [F(2,12)=5.418, P=0.0211] and *Drd3* [F(2,12)=4.434, p=0.0362] by ANOVA (Figure 2E).

We next examined associations between 19 single nucleotide polymorphisms (SNPs) in the human *SLIT3* gene and smoking behavior in the London cohorts. Two SNPs, rs12654448 and rs17734503 in high linkage disequilibrium (Figure 4) were associated with level of cigarette consumption (p=0.00125 and p=0.00227). We repeated the analysis on heavy smokers: rs12654448 (p=0.0003397) and rs17734503 (p=0.0008575) were again associated with cigarette consumption together with rs11742567 (p=0.004715), (Table 1A & 1B). The SNP rs11742567 was associated with cigarette consumption in light smokers (<20 cigarettes per day, p=0.003909)) (Table 1C) and with quitting (Table 1D). No other SLIT3 polymorphisms were associated with smoking initiation, persistent smoking or cessation (Supplementary Tables 5 & 6).

We then investigated associations with more detailed smoking phenotypes in the Finnish twins (Table 2). Associations were observed between rs17734503 and DSM-IV nicotine dependence symptoms (p=0.0322) and age at onset of weekly smoking

(p=0.00116) and between rs12654448 and age at onset of weekly smoking (p=0.00105). Associations were seen elsewhere between *SLIT3* markers and FTND, cigarettes smoked each day, sensation felt after smoking first cigarette and time to first cigarette in the morning. In keeping with the London studies the minor allele was associated with a lower degree of dependence and decreased cigarette consumption.

DISCUSSION

The aim of this study was to use forward genetic screening in zebrafish to identify loci affecting human smoking behavior. We identified two loss of function mutations in the zebrafish *Slit3* gene that were associated with increased nicotine place preference. We established the relevance in humans by identifying markers in *SLIT3* where the presence of the minor allele was associated with fewer cigarettes smoked each day and with smoking cessation. Studies in a separate twin cohort showed that these same alleles were associated with DSM-IV nicotine dependence symptoms and age at onset of weekly smoking. Taken together these findings suggest that the alleles are linked in humans with a disruption of SLIT3 function which may affect the propensity to develop tobacco dependence.

We used a population-based pre-screen of ENU-mutagenized zebrafish lines followed by sibling re-screen to identify lines of fish showing altered nicotine preference. This proof of principle study indicates the relevance of zebrafish for human studies and emphasizes the advantage of using a population-based pre-screen to increase efficiency. The classic three generation forward genetic screen examines phenotypes in groups of 20 or more individuals from each family (38). Logistical considerations make it difficult to apply such an approach to adult behavioral screens. Use of a population-based pre-screening approach increases efficiency of the screen by initially screening a small number of individuals from a large number of F3 families and only selecting those families that occur at the extremes of the distribution for further analysis. Although in the study described here we were able to confirm phenotypes using a relatively small population of siblings, re-screening of a larger number would increase the power of the analysis and allow more subtle phenotypes to be identified.

We identified the line showing increased nicotine preference as carrying a loss of function mutation in the zebrafish *Slit3* gene and confirmed the phenotype in an independent line. SLIT molecules bind to ROBO receptors through a highly conserved leucine-rich repeat (LRR) domain (39). In the AJBQM1 line the gene breaking mutation causes a truncation at amino acid 176 and in the sa202 line at amino acid 163. These are immediately adjacent to the LRR2 domain responsible for SLIT3's functional interaction with ROBO proteins (39) and would therefore be predicted to lead to formation of non-functional proteins. Initially identified as a family of axon guidance molecules, SLIT proteins are known to be expressed in a range of tissues and, by regulating cell polarity, to play major roles in many developmental process including cell migration, proliferation, adhesion, neuronal topographic map formation and dendritic spine remodeling (40). *In vitro* SLIT proteins bind promiscuously to ROBO receptors suggesting that the proteins may cooperate *in vivo* in areas in which they overlap. However, their restricted spatial distributions, particularly of SLIT3 in the CNS (41) suggest the individual proteins play subtly different roles in vivo.

Despite its neuronal expression, the most prominent phenotype seen in *Slit3* deficient mice is postnatal diaphragmatic hernia (42,43) with no obvious neuronal or axon pathfinding defects having been reported. Similarly, we did not detect any major differences in axon pathfinding in *Slit3* mutant zebrafish larvae. As suggested previously (44) it may be that overlap of expression with other SLIT molecules compensates for loss of SLIT3 in the brain preventing gross neuronal pathfinding defects. However, subtle differences in circuit formation and/or axon branching may have escaped our analysis.

In support of this suggestion, *Slit3* mutants showed increased habituation to acoustic startle and altered sensitivity of the startle response to the D2/D3 antagonist amisulpride. As there were no differences in startle magnitude or mean distance travelled during the test period across groups, differences in habituation response cannot be due to differences in health or reactivity of the mutants and presumably reflect subtle differences in underlying neuronal circuits and/or their regulation. Acoustic startle has previously been shown to be sensitive to modulation of dopaminergic signaling in all species studied (36,45,46). Data from dopamine receptor loss of function mice indicate that the dopaminergic system regulates both sensitization and habituation of the startle response in an 'antithetical manner' (45); dopamine D1 receptors inhibit sensitization and augment habituation whereas D2-like receptors augment sensitization and inhibit habituation. Although results with dopamine agonists and antagonists in rodents are less clear cut (45), our finding that the selective dopamine D2/3 antagonist amisulpride increased habituation to acoustic startle in wildtype fish is consistent with this model, and is in agreement with the effect of amisulpride on habituation in humans (36)

In contrast to results in wildtype fish, *Slit3* mutants showed decreased habituation in the presence of low dose amisulpride. This finding, together with the observation that *Slit3* mutants have increased *Drd2a* and *Drd3* receptor expression indicate disruption of dopaminergic signalling pathways that may underlie both the differences in habituation response and the increased sensitivity to nicotine in our place preference assay. Although it is possible that an anxiolytic effect of nicotine contributed to the increased nicotine induced place preference, preliminary assessment of anxiety-like responses (tank diving) in *Slit3* mutants (n.s) argue against this. The disrupted dopaminergic signalling and increased receptor expression supports a role of *Slit3* in

the regulation of nicotine reward processes. Further analysis is required in order to tease out the exact processes affected.

Whilst we confirmed the translational effects of *Slit3* gene variants in a human study and the association was validated in another independent cohort, there are limitations to our findings: larger studies would be necessary to obtain greater precision on estimates of the effect size. Further studies are also required to determine the effects of genetic variation in *SLIT3* on anatomical pathways in the human brain and their functioning with view to identifying people who are at high risk of developing dependence. This could be achieved by using imaging techniques to study brain activation in response to smoking related cues in smokers who have the *SLIT3* polymorphisms linked to smoking (particularly rs12654448).

To our knowledge, this is the first report of a novel human functional polymorphism, identified using a forward genetic screen of adult zebrafish to uncover loci affecting a complex human behavioral trait. Taken together, these results provide evidence for a role for *SLIT3* in regulating smoking behavior in humans and establish adult zebrafish as an animal model for exploration of addictive behaviors. Further work analyzing the cellular processes affected as a result of the *Slit3* mutation may provide useful targets when designing tailored treatments to aid smoking cessation.

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Disclosures

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Figures and Legends

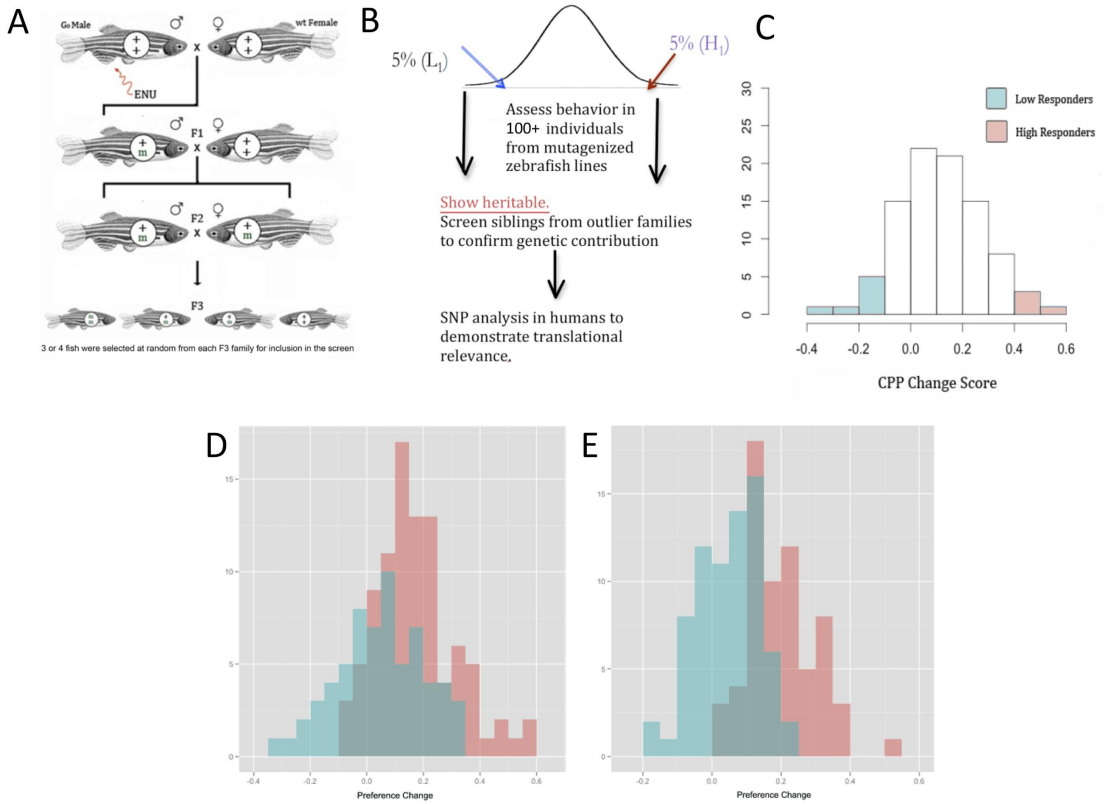

Figure 1: Establishing heritability of nicotine preference in zebrafish. A: Zebrafish breeding scheme. Zebrafish lines were obtained from the Zebrafish Mutation Project. Fish were selected from 30 F2 lines each containing 8-15 nonsense or essential splice site mutations and about 4000 additional exonic and intronic point mutations. F2 fish were incrossed to create F3 families. Three or four randomly selected F3 fish from each family were assessed for nicotine preference. **B: Schematic of study design.** Fish from mutagenized lines were screened for nicotine preference. **C: First generation nicotine preference screen (n = 100).** Change in preference normally distributed (Shapiro-Wilks P = 0.83), mean increase of 0.11 preference to the drug paired side. Blue and red bars indicate low and high responders selected for breeding. **D: Second generation screen (n=184).** Nicotine preference differed from first generation (low responders 0.08, $P = 0.001$, high responders 0.17, $P = 0.0002$). **F:**

Third generation screen (n=187). Mean preference change 0.21 for high responders, 0.01 for low responders.

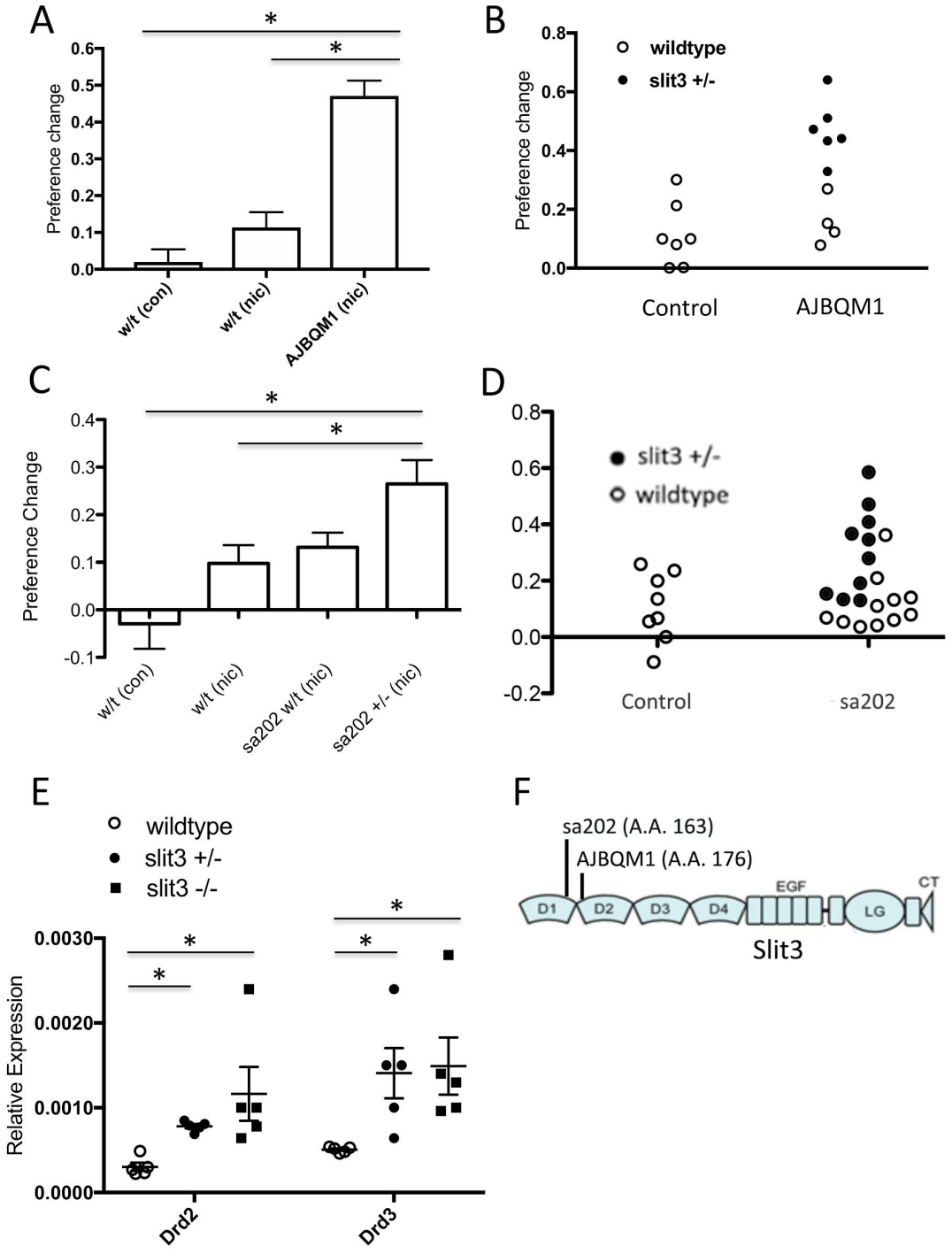

Figure 2: Nicotine preference in AJBQM1 and *slit3*^{sa202} lines. A: Conservation of phenotype in AJBQM1. AJBQM1 fish clustered to the extreme right of preference distribution, indicating a dominant mutation affecting nicotine preference. This phenotype was conserved ($p<0.05$ compared to wildtype). **B: Segregation of *slit3*^{sa1569} mutation with nicotine seeking.** Heterozygosity for *slit3*^{sa1569} segregates with

increased nicotine seeking behavior. **C: Nicotine preference of *slit3*^{sa202} line.** *slit3*$^{sa202/+}$ fish show increased nicotine preference compared to wildtype siblings (p = 0.03) or wildtype controls (p = 0.001). **D: Segregation of *slit3*^{sa202} allele with nicotine seeking**. **E: qPCR analysis of *Drd2 and Drd3* mRNA in wildtype and *slit3*^{sa1569} heterozygous and homozygous mutants (5 day) larvae (n=15, 5 per group).** There is upregulation of *Drd2* (p=0.0211) *and Drd3* (p=0.0362) at 5 days. **F: Position of ENU-induced mutations in zebrafish Slit3 protein.** *slit3*^{sa1569} (A>G transition) disrupts a splice site in intron 7 affecting translation at amino acid 176. *slit3*^{sa202} (G>T transversion) introduces a stop codon at amino acid 163. Both mutations truncate the protein before the leucine rich repeat domain 2 (LRR D2), which interacts with membrane bound ROBO during SLIT-ROBO signaling.

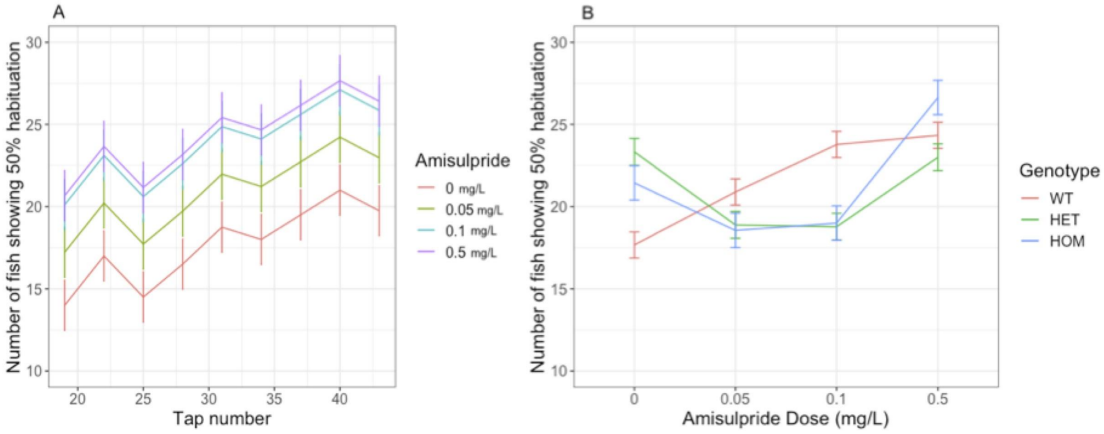

Figure 3. Habituation response in the presence and absence of amisulpride. A: Habituation response in wildtype zebrafish: The number of fish showing 50% habituation increases with stimulus number [$F(8, 24) = 3.262$, $p = 0.0117$] and with concentration of amisulpride [$F(3, 24) = 12.150$, $p < 0.001$]. **B**: Average number of fish that reached 50% reduction in response across *Slit3* ^{sa1569} genotype and amisulpride dose experimental groups. For each dose and genotype group, we calculated the number of fish reaching 50% reduction for each startle stimulus. The figure illustrates the mean number of fish across the nine stimuli +/- SE. The effect of amisulpride on habituation varies by genotype: *Slit3*^{sa1569} mutants show a U-shaped response to amisulpride, in contrast with wildtype fish (Genotype by amisulpride dose interaction: [$F(6, 99)=8.076$, $p < 0.001$]). * ($p<0.05$). n=45 individuals per genotype and dose group.

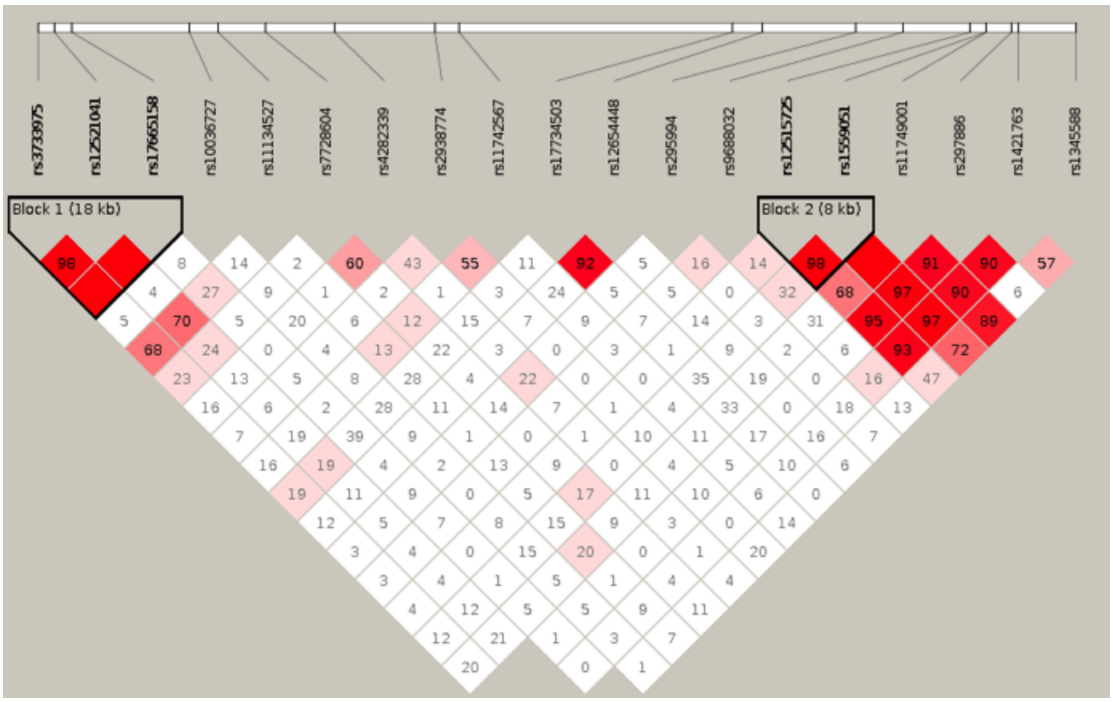

Figure 4: Linkage disequilibrium (LD) plot of *SLIT3* SNPs in human smoking association analysis. Numbers within each square indicate D' values (white: D' < 1, LOD < 2; blue: D' = 1, LOD < 2; pink: D' < 1, LOD ≥ 2; and bright red: D' = 1, LOD ≥ 2.)

Tables and Legends

Table 1A. Associations of *SLIT3* SNPs with level of tobacco consumption for the London study groups. Regression coefficients, confidence intervals and p-values from linear regression of cigarettes smoked per day (CPD) on minor allele count for smokers from COPD, asthma and general cohorts, adjusted for age, sex and cohort. β coefficient represents effect of each additional minor allele. Benjamini-Hochberg cut-off at q-value 0.1 = 0.01053. **Table 1B. Associations of *SLIT3* SNPs with tobacco consumption in a subset of heavy smokers (≥20 cigs/day).** Adjusted for age, sex and cohort. (q-value 0.1 = 0.01579). **Table 1C. Associations of *SLIT3* SNPs in a subset of light smokers (<20 cigs/day).** Adjusted for age, sex and cohort. (q-value 0.1 = 0.00526). **Table 1D. Association analysis of *SLIT3* SNPs with smoking cessation.** Logistic regression of current smokers *vs* ever smokers controlling for age, sex and cohort. Odds ratio >1 indicates minor allele increases odds of persistent smoking relative to major allele. SE: standard error, L95: lower limit of 95% confidence interval, U95: upper limit. For all panels, associations in bold remained significant after adjustment for multiple comparisons using a Benjamin-Hochberg procedure to control false discovery rate at 10%.

1A

SNP	P VALUE	β	SE	95% CI
rs12654448	**0.0013**	**-4.241**	**1.307**	**(-6.803, -1.680)**
rs17734503	**0.0023**	**-3.987**	**1.299**	**(-6.534, -1.441)**
rs11749001	0.0594	1.972	1.044	(-0.074, 4.018)
rs17665158	0.1308	1.338	0.884	(-0.394, 3.070)
rs11742567	0.1351	-1.166	0.779	(-2.691, 0.361)
rs2938774	0.1403	1.101	0.745	(-0.359, 2.562)
rs11134527	0.2175	1.014	0.822	(-0.596, 2.625)
rs1345588	0.2401	-1.268	1.078	(-3.380, 0.845)
rs1421763	0.2724	-0.982	0.894	(-2.735, 0.770)
rs12515725	0.5915	-0.406	0.756	(-1.888, 1.076)

rs297886	0.6198	0.442	0.8903	(-1.303, 2.187)
rs10036727	0.6285	-0.388	0.8018	(-1.960, 1.183)
rs4282339	0.6690	-0.434	1.013	(-2.419, 1.552)
rs7728604	0.7007	0.286	0.7444	(-1.173, 1.745)
rs295994	0.7135	0.283	0.7702	(-1.227, 1.793)
rs12521041	0.9036	-0.105	0.8652	(-1.801, 1.591)
rs3733975	0.9087	-0.099	0.86	(-1.784, 1.587)
rs9688032	0.9478	-0.050	0.7658	(-1.551, 1.451)
rs1559051	0.9611	-0.040	0.8186	(-1.644, 1.564)

1B

SNP	P VALUE	β	SE	95% CI
rs12654448	**0.0003**	**-4.830**	**1.334**	**(-7.444, -2.216)**
rs17734503	**0.0009**	**-4.458**	**1.325**	**(-7.055, -1.861)**
rs11742567	**0.0047**	**-2.346**	**0.825**	**(-3.962, -0.730)**
rs17665158	0.2363	1.114	0.939	(-0.727, 2.955)
rs1345588	0.2527	-1.334	1.164	(-3.616, 0.948)
rs7728604	0.3206	0.827	0.832	(-0.803, 2.457)
rs11134527	0.3268	-0.867	0.883	(-2.599, 0.864)
rs10036727	0.4483	-0.653	0.860	(-2.337, 1.032)
rs1559051	0.4576	0.656	0.882	(-1.073, 2.384)
rs12515725	0.4875	-0.565	0.813	(-2.159, 1.029)
rs2938774	0.5281	0.496	0.786	(-1.044, 2.036)
rs295994	0.6426	0.378	0.813	(-1.215, 1.971)
rs9688032	0.7698	0.246	0.839	(-1.398, 1.890)
rs11749001	0.8730	0.177	1.103	(-1.985, 2.338)
rs4282339	0.9422	-0.080	1.103	(-2.241, 2.081)
rs297886	0.9613	-0.048	0.986	(-1.979, 1.884)
rs1421763	0.9775	-0.027	0.959	(-1.908, 1.853)
rs3733975	0.9817	0.022	0.934	(-1.809, 1.852)
rs12521041	0.9956	-0.005	0.942	(-1.851, 1.841)

1C

SNP	P VALUE	β	SE	95% CI
rs11742567	**0.0039**	**1.888**	**0.644**	**(0.6258 ,3.151)**
rs2938774	0.0153	-1.655	0.674	(-2.976, -0.333)
rs17665158	0.0341	1.620	0.758	(0.1354, 3.106)
rs3733975	0.0585	-1.354	0.710	(-2.746, 0.038)
rs12521041	0.0585	-1.354	0.710	(-2.746, 0.038)
rs9688032	0.0797	-1.076	0.610	(-2.272, 0.119)
rs1421763	0.1620	-1.074	0.764	(-2.571, 0.424)
rs11749001	0.2057	1.200	0.944	(-0.651, 3.051)
rs4282339	0.2380	-1.006	0.849	(-2.670, 0.658)
rs295994	0.2381	-0.796	0.672	(-2.114, 0.521)
rs11134527	0.2608	0.795	0.705	(-0.586, 2.176)
rs12515725	0.2781	-0.688	0.631	(-1.925, 0.550)
rs12654448	0.4095	-1.034	1.251	(-3.486, 1.417)
rs17734503	0.4095	-1.034	1.251	(-3.486, 1.417)
rs297886	0.4890	0.488	0.704	(-0.891, 1.867)
rs1559051	0.5072	0.455	0.685	(-0.880, 1.797)
rs7728604	0.6540	0.262	0.583	(-0.880, 1.404)

rs1345588	0.8691	-0.150	0.907	(-1.927, 1.627)
rs10036727	0.9403	-0.051	0.686	(-1.396, 1.293)

1D

SNP	OR	SE	L95	U95	P value
rs11742567	**1.586**	**0.1629**	**1.153**	**2.183**	**0.0046**
rs11134527	0.665	0.1646	0.482	0.918	0.0132
rs12521041	1.554	0.1783	1.096	2.205	0.0134
rs3733975	1.488	0.1759	1.054	2.101	0.0238
rs2938774	0.753	0.1484	0.563	1.007	0.0558
rs17665158	0.723	0.1724	0.516	1.013	0.0597
rs17734503	1.616	0.2754	0.942	2.773	0.0813
rs12654448	1.625	0.2790	0.941	2.808	0.0818
rs1345588	1.417	0.2216	0.918	2.189	0.1155
rs295994	0.799	0.1544	0.591	1.082	0.1465
rs297886	1.108	0.1769	0.784	1.568	0.5606
rs1559051	0.919	0.1630	0.668	1.265	0.6058
rs1421763	0.917	0.1759	0.649	1.294	0.6216
rs7728604	0.935	0.1493	0.698	1.253	0.6533
rs9688032	1.066	0.1555	0.786	1.446	0.6804
rs10036727	0.947	0.1596	0.693	1.295	0.7344
rs11749001	0.953	0.2055	0.637	1.426	0.8165
rs12515725	1.028	0.1508	0.765	1.381	0.8547
rs4282339	0.984	0.2027	0.661	1.464	0.9362

Table 2: Associations between detailed nicotine dependence phenotypes and SLIT3 genotype in a Finnish twin cohort. Associations of *SLIT3* SNPs with DSM-IV nicotine dependence symptoms (Table 2A), Fagerstrom scores (Table 2B), cigarettes smoked each day (CPD) (Table 2C), and sensation felt after smoking first cigarette and time to first cigarette in the morning (Table 2D). The three SNPs that were linked to smoking behavior in the London cohorts are shown in bold.

2A

	DSM-IV ND diagnosis			DSM-IV ND symptoms		
SNP	**β**	**SE**	**P value**	**β**	**SE**	**P value**
rs12654448	-0.0343	0.0262	0.190975	-0.1839	0.0964	0.056728
rs17734503	-0.0354	0.0259	0.171821	-0.2044	0.0954	**0.032199**
rs11742567	0.0006	0.0163	0.972620	-0.0359	0.0601	0.550860
rs17665158	0.0117	0.0190	0.538639	0.1536	0.0696	**0.027544**
rs1345588	-0.0031	0.0222	0.889847	-0.0389	0.0817	0.634184
rs7728604	-0.0049	0.0162	0.761485	-0.0442	0.0597	0.459743
rs11134527	0.0296	0.0171	0.084576	0.0927	0.0630	0.141369
rs10036727	0.0067	0.0165	0.684060	0.0207	0.0605	0.732266
rs1559051	0.0249	0.0193	0.198647	0.0492	0.0703	0.484353
rs12515725	0.0072	0.0159	0.650200	0.0277	0.0584	0.635739
rs2938774	0.0042	0.0173	0.807170	0.0096	0.0642	0.881054
rs295994	-0.0140	0.0171	0.410864	-0.0144	0.0622	0.816397
rs9688032	-0.0174	0.0173	0.312990	-0.0347	0.0636	0.585081
rs11749001	0.0178	0.0235	0.448278	-0.0062	0.0865	0.942516
rs4282339	0.0118	0.0201	0.557544	0.0526	0.0739	0.476216
rs297886	-0.0216	0.0171	0.207835	-0.0450	0.0634	0.478517
rs1421763	0.0079	0.0187	0.671624	0.0178	0.0687	0.795522
rs3733975	-0.0130	0.0167	0.436903	-0.0798	0.0613	0.192755
rs12521041	-0.0098	0.0167	0.559173	-0.0669	0.0613	0.275274

2B

	FTND (≥4)			FTND score		
SNP	**β**	**SE**	**P value**	**β**	**SE**	**P value**
rs12654448	0.0526	0.0287	0.066509	0.0750	0.1365	0.582860
rs17734503	0.0474	0.0283	0.094383	0.0443	0.1350	0.743052
rs11742567	0.0134	0.0179	0.453840	0.0449	0.0851	0.597682
rs17665158	0.0178	0.0207	0.391096	0.0935	0.0988	0.344157
rs1345588	0.0578	0.0242	**0.017080**	0.1901	0.1157	0.100729
rs7728604	0.0004	0.0177	0.980706	-0.0261	0.0846	0.757849
rs11134527	0.0324	0.0187	0.083498	0.1376	0.0891	0.122807
rs10036727	0.0022	0.0180	0.903865	0.0460	0.0857	0.591583

rs1559051	-0.0433	0.0208	**0.037736**	-0.1011	0.0995	0.309836
rs12515725	0.0586	0.0172	**0.000696**	0.2482	0.0824	**0.002637**
rs2938774	-0.0157	0.0191	0.411630	-0.0173	0.0907	0.848978
rs295994	-0.0160	0.0184	0.385420	-0.0985	0.0879	0.262584
rs9688032	0.0234	0.0189	0.216144	0.0626	0.0900	0.486900
rs11749001	0.0224	0.0257	0.383552	0.0067	0.1222	0.956097
rs4282339	-0.0077	0.0219	0.724058	0.1623	0.1045	0.120641
rs297886	-0.0256	0.0188	0.173314	-0.1354	0.0897	0.131469
rs1421763	0.0641	0.0203	**0.001641**	0.2582	0.0971	**0.007892**
rs3733975	-0.0384	0.0181	**0.034371**	-0.2083	0.0866	**0.016295**
rs12521041	-0.0365	0.0182	**0.044962**	-0.1905	0.0868	**0.028295**

2C

	CPD			max CPD		
SNP	**β**	**SE**	**P value**	**β**	**SE**	**P value**
rs12654448	-0.3509	0.5669	0.536029	-1.0602	0.7743	0.171106
rs17734503	-0.4790	0.5608	0.393086	-1.2329	0.7657	0.107544
rs11742567	0.0179	0.3532	0.959588	-0.4621	0.4823	0.338159
rs17665158	0.8135	0.4096	**0.047191**	1.5424	0.5587	**0.005828**
rs1345588	0.2940	0.4805	0.540660	0.3968	0.6562	0.545420
rs7728604	-0.0772	0.3511	0.825888	0.0691	0.4795	0.885363
rs11134527	0.1831	0.3705	0.621187	0.7441	0.5057	0.141392
rs10036727	0.1482	0.3557	0.676970	0.4246	0.4858	0.382161
rs1559051	-0.4816	0.4130	0.243693	-0.4779	0.5641	0.397066
rs12515725	0.5491	0.3429	0.109480	0.7708	0.4684	0.100032
rs2938774	-0.2796	0.3770	0.458350	0.1945	0.5149	0.705670
rs295994	-0.2793	0.3651	0.444276	-0.2585	0.4988	0.604451
rs9688032	0.2452	0.3738	0.511921	0.4211	0.5107	0.409766
rs11749001	-0.0301	0.5078	0.952789	0.0574	0.6939	0.934054
rs4282339	0.4086	0.4340	0.346592	0.3083	0.5930	0.603197
rs297886	-0.1375	0.3727	0.712273	-0.4782	0.5091	0.347622
rs1421763	0.5585	0.4037	0.166723	0.6702	0.5515	0.224417
rs3733975	-0.7784	0.3597	**0.030606**	-1.0555	0.4911	**0.031758**
rs12521041	-0.7312	0.3602	**0.042534**	-0.8864	0.4920	0.071805

2D

	Age of onset of weekly smoking			First time sensation			FTND time to first cigarette (TTF)		
SNP	**β**	**SE**	**P value**	**β**	**SE**	**p-value**	**β**	**SE**	**P value**
rs12654448	0.7826	0.2384	**0.001051**	-0.0861	0.1423	0.545206	0.0047	0.0802	0.953291
rs17734503	0.7689	0.2362	**0.001156**	-0.1039	0.1406	0.460344	0.0188	0.0795	0.812682
rs11742567	0.0965	0.1493	0.518066	-0.1003	0.0884	0.256427	-0.0216	0.0500	0.665265
rs17665158	0.0562	0.1732	0.745385	0.2476	0.1027	**0.016030**	-0.0884	0.0580	0.127870
rs1345588	0.0989	0.2031	0.626431	-0.0618	0.1204	0.607606	-0.1303	0.0680	0.055678
rs7728604	-0.0261	0.1486	0.860643	-0.0290	0.0875	0.740682	-0.0193	0.0497	0.697800
rs11134527	-0.2347	0.1563	0.133517	0.1142	0.0930	0.219650	-0.1089	0.0523	**0.037681**
rs10036727	-0.0456	0.1507	0.762197	0.0289	0.0896	0.747110	-0.0639	0.0504	0.205061
rs1559051	0.1437	0.1750	0.411533	-0.0289	0.1045	0.782381	0.0731	0.0586	0.212174

rs12515725	-0.1629	0.1452	0.261920	-0.0368	0.0865	0.670165	-0.1385	0.0485	**0.004340**
rs2938774	-0.0575	0.1598	0.718862	0.0430	0.0933	0.645221	-0.0136	0.0533	0.797909
rs295994	0.1543	0.1548	0.318928	0.0625	0.0926	0.499881	0.0527	0.0517	0.307869
rs9688032	-0.1142	0.1584	0.471283	-0.2227	0.0937	**0.017550**	-0.0517	0.0531	0.329867
rs11749001	-0.1994	0.2147	0.353064	0.1497	0.1274	0.240255	0.0075	0.0718	0.916823
rs4282339	0.0952	0.1836	0.603958	-0.0394	0.1084	0.716204	-0.0524	0.0614	0.394221
rs297886	0.1262	0.1575	0.423104	0.0519	0.0928	0.576255	0.0632	0.0527	0.230861
rs1421763	-0.1481	0.1706	0.385475	-0.0799	0.1018	0.432497	-0.1269	0.0571	**0.026442**
rs3733975	0.0902	0.1521	0.553335	-0.2932	0.0896	**0.001085**	0.1373	0.0509	**0.007035**
rs12521041	0.0522	0.1523	0.731943	-0.3129	0.0897	**0.000499**	0.1257	0.0510	**0.013730**