

# **Identification of *Slit3* as a locus affecting nicotine preference in zebrafish and human smoking behaviour.**

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## ABSTRACT

Although there is clear evidence of genetic contributions to smoking behaviour, it has proved difficult to identify causal alleles and pathways from human studies. To facilitate smoking genetics research we examined the ability of a screen of mutagenized zebrafish to predict loci affecting smoking behaviour. We identified *Slit3* as a gene affecting nicotine preference in fish. Focussed SNP analysis of the homologous human locus in cohorts from UK and Finland identified two SNP variants in the *SLIT3* locus that predict level of cigarette consumption and likelihood of cessation. Characterisation of *Slit3* mutant larvae and adult fish revealed altered behavioural sensitivity to amisulpride, a dopaminergic and serotonergic antagonist, and increased *5htr1aa* mRNA expression in mutant larvae. No effect on neuronal pathfinding was detected. These findings reveal a role for SLIT3 signalling in development of pathways affecting responses to nicotine and confirm the translational relevance of zebrafish for exploring complex human behaviours.

# 1 INTRODUCTION

2 Tobacco smoking is the leading preventable cause of death worldwide placing a  
3 heavy social and financial burden on society (1–3). It is well established that aspects  
4 of smoking behaviour have a strong genetic component (4–7). However, identifying  
5 causal genetic factors and exploring the mechanisms by which they act is challenging  
6 in human studies: the field has been characterized by small effect sizes and lack of  
7 replication such that there are remarkably few genes and loci that can be confidently  
8 linked to smoking. The strongest evidence for causal effects is for functional variants  
9 in *CHRNA5* and *CYP2A6*, affecting amount smoked and nicotine metabolism,  
10 respectively. Recent large studies have identified numerous new association loci, but  
11 their significance is yet to be biologically characterised (6,7).

12  
13 As approaches to identify genetic risk are difficult in humans, research has been  
14 facilitated by studies in animal models, with a focus on genomic analysis of inbred  
15 and selectively-bred, naturally occurring genetic strains (8). This type of study  
16 produces quantitative trait loci (QTL) maps of multiple loci, each with a small impact  
17 on the phenotype. However, as with human studies, it is inherently difficult to identify  
18 relevant genes from QTL maps, as the overall phenotype cannot be predicted by  
19 individual genotypes. Mutagenesis studies in animal model systems can overcome  
20 these limitations: e.g. N-ethyl-N-nitrosourea (ENU) mutagenesis introduces thousands  
21 of point mutations into the genome with the potential to generate much stronger  
22 phenotypes than those occurring in a natural population thereby facilitating  
23 identification of causal mutations. Examination of phenotypic variation in ENU  
24 mutagenized model species could be applied to identify novel, naturally occurring

variants influencing human addictive behaviour by identifying key genes and pathways affecting conserved behavioural phenotypes.

Drug-induced reinforcement of behaviour, that reflects the hedonic value of drugs of abuse including nicotine, is highly conserved in both mammalian and non-mammalian species (9–12). Conditioned place preference (CPP), where drug exposure is paired with specific environmental cues, is commonly used as a measure of drug-induced reward or reinforcement (13). ENU Mutagenesis screens for cocaine or amphetamine-induced CPP have been undertaken in zebrafish (8,14), however, despite successful isolation of lines with altered reinforcement responses to these drugs, the causal mutations have not been identified and the predictive validity of these screens for human behaviour has not been established.

Here, we conducted a forward genetic screen of families of ENU-mutagenized zebrafish for nicotine-induced CPP. Zebrafish express the same set of neuronal nicotinic acetylcholine receptors as found in other vertebrates ( $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\alpha 8$ ,  $\beta 2$ ,  $\beta 3$ ,  $\beta 4$ ) (15,16) with similar binding characteristics (17,18). Zebrafish show robust CPP to nicotine (18–21) and nicotinic receptor partial agonists that modulate striatal dopamine release in response to nicotine in mammalian systems also inhibit nicotine-induced CPP in zebrafish (18). Further, on prolonged exposure to nicotine or ethanol, adult zebrafish show conserved adaptive changes in gene expression and develop dependence-related behaviours, such as persistent drug seeking despite adverse stimuli or reinstatement of drug seeking following periods of abstinence (21). These data demonstrate the existence of a conserved nicotine-responsive reward pathway and support the suitability of zebrafish to examine the genetic and molecular mechanisms underlying behavioural responses to nicotine.

To evaluate the use of a behavioural CPP screen in zebrafish to predict loci affecting human smoking behaviour we initially assessed 1) the ability of varenicline and bupropion, pharmacological agents used to treat human nicotine addiction, to reduce zebrafish nicotine-induced place preference and 2) the heritability of nicotine responses in ENU-mutagenized fish. We then screened 30 families of ENU-mutagenized fish to identify families with increased/decreased CPP for nicotine. For two families with altered CPP response, the phenotype was confirmed following independent replication with a larger number of fish. Exome sequence information was used to generate a list of coding, loss of function candidate mutations affecting the phenotype. One family with a mutation co-segregating with increased nicotine CPP was selected for further study. Firstly, the effect of the identified gene on nicotine-induced CPP was confirmed using an independent line carrying a similar predicted loss of function mutation in the same gene. We then characterized the mutation using gene expression analysis, immunohistochemical analysis of neuronal pathways and behavioural responses to acoustic startle; a response known to be modulated by serotonergic and dopaminergic signalling and, in humans, associated with vulnerability to addiction (22–24). Finally, we used focused SNP analysis of human cohorts to assess the predictive validity of findings in fish for human smoking behaviour.

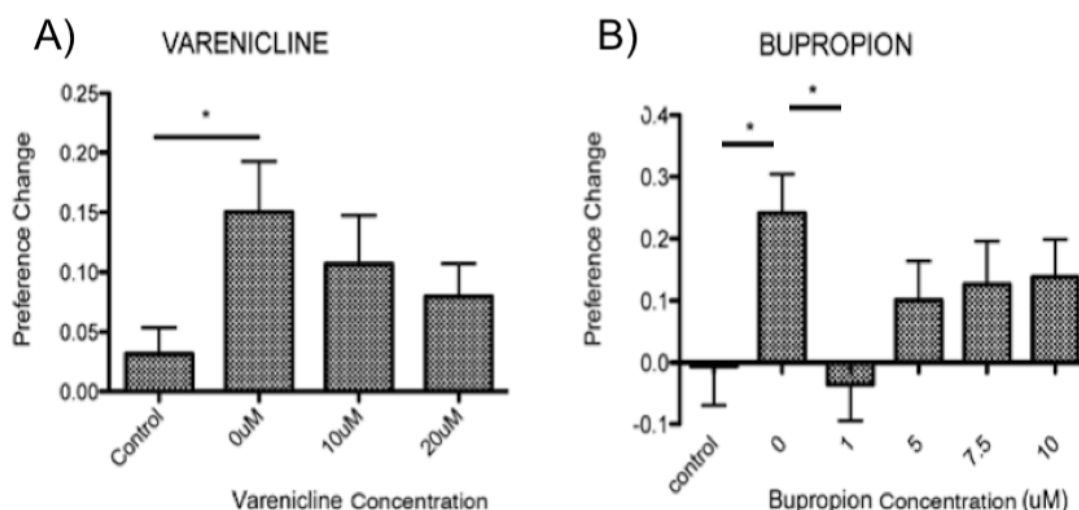
In agreement with previous studies zebrafish showed a robust CPP to nicotine. Nicotine-induced CPP was abolished by varenicline and bupropion and found to be heritable in fish. The screening of ENU mutagenized families identified mutations in the *Slit3* gene influencing sensitivity to rewarding effects of nicotine. *Slit3* mutant larvae and adult fish showed altered behavioural sensitivity to amisulpride and larvae

74 showed increased *5ht1aa* receptor expression. No effect on neuronal pathfinding was  
 75 detected. Analysis of the *SLIT3* locus in two independent human cohorts identified  
 76 two genetic markers that predict level of cigarette consumption and likelihood of  
 77 cessation. This proof of principle study demonstrates that screening of zebrafish is  
 78 able to predict loci affecting complex human behavioural phenotypes and suggests a  
 79 role for SLIT3 signalling in the development of dopaminergic and serotonergic  
 80 pathways affecting behaviours associated with nicotine sensitivity.

## 81 RESULTS

### 82 Nicotine CPP in zebrafish is inhibited by varenicline and bupropion

83 The hedonic value of drugs of abuse, that gives rise to reinforced behaviour, is  
 84 commonly assessed using either self-administration protocols or CPP. The ability of  
 85 compounds used as therapeutics in humans to prevent rodent nicotine self-  
 86 administration is used to support the translational relevance of nicotine-self  
 87 administration in that model (25–27). As our aim was to use nicotine-CPP to predict  
 88 genes affecting smoking behaviour, we assessed the ability of the nicotine  
 89 therapeutics varenicline and bupropion to inhibit nicotine induced CPP in zebrafish.  
 90 As seen previously (19,21,28), 10 $\mu$ M nicotine induced a robust 15-20% change in  
 91 place preference. Pre-incubation in varenicline or bupropion dose-dependently  
 92 inhibited the nicotine CPP response (Figure 1).



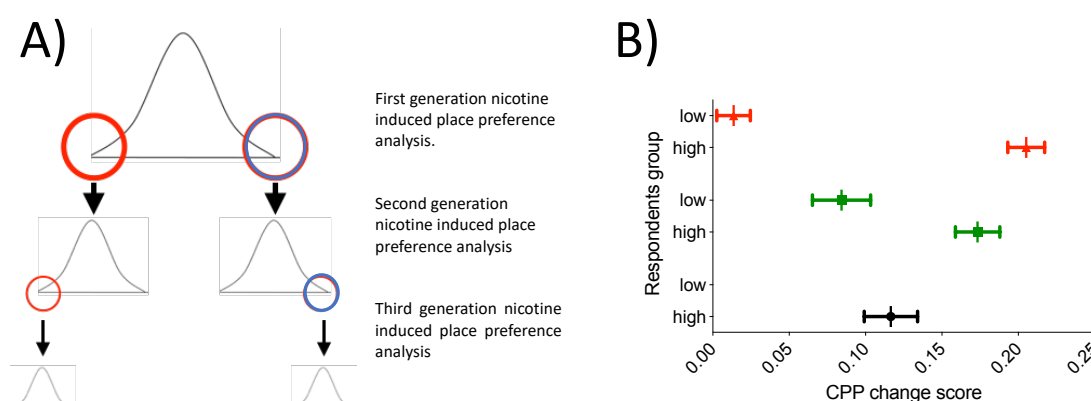
93  
 94 **Figure 1. 10 $\mu$ M nicotine induced place preference in zebrafish is sensitive to**  
 95 **inhibition by therapeutics effective in humans. A) varenicline (nicotine partial**  
 96 **agonist) and B) bupropion (norepinephrine and dopamine reuptake inhibitor with**

nicotine antagonist properties when metabolised). Bars represent mean and error bars represent +SEM. Asterics (\*) represents significance at  $p < 0.05$ .

### **Nicotine CPP is heritable in zebrafish**

We selected a nicotine concentration predicted to induce a minimal detectable CPP in wildtypes (5 $\mu$ M) (19,20), to enable us to detect both increased and decreased response to nicotine in mutants. To ensure that this strategy could detect genetic factors affecting response to nicotine, we assessed the heritability of the CPP response in ENU mutagenized fish using a selective breeding approach over three generations. Figure 2A shows our assessment strategy where fish showing the highest and lowest CPP response are selected for further breeding. In the first generation, the CPP change score phenotype was normally distributed (Shapiro-Wilks  $p = 0.83$ ) and there was a mean CPP change score of 0.11 to the drug paired side. CPP change scores ranged from - 0.4 to 0.6.

An increasing difference in nicotine preference between offspring of fish from the upper vs lower extremes of the distribution (Shift of Cohen's  $d = 0.89$  in Second generation CPP to  $d = 1.64$  in Third generation CPP) indicates that nicotine CPP behaviour is heritable in zebrafish (Figure 2B), and that our CPP strategy is able to identify heritable differences in both extremes of the distribution. Phenotypes in the second and third generation screen are presumably stronger as a result of selecting for multiple co-segregating mutations in each generation.

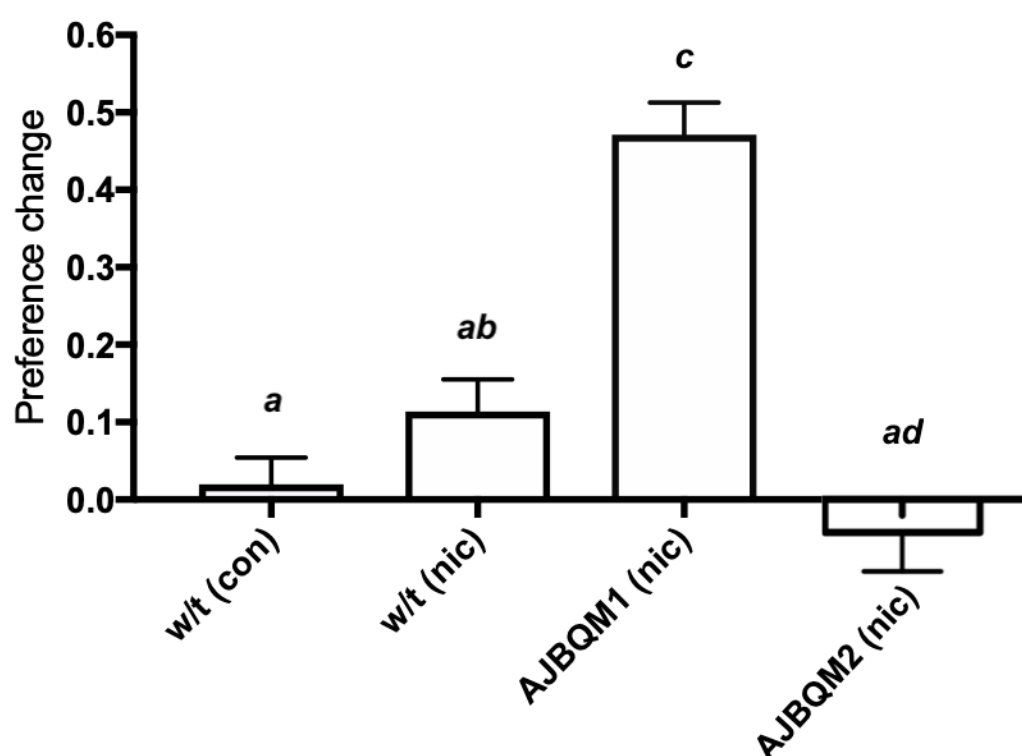


**Figure 2. A) Breeding and selection to assess heritability of nicotine-induced place preference in ENU-mutagenized zebrafish.** To test whether nicotine preference is heritable, fish in the upper and lower 10% of the change in preference distribution curve were inbred and screened for CPP (Second generation CPP assay). A similar approach was used for the third generation CPP assay. **B) CPP for nicotine is heritable.** Mean preference change is increasingly distinct for the second and third generation of CPP assay. Plot represents mean and  $\pm$ SEM. First generation (corresponding to the F<sub>3</sub> families used for the screen) (n=120): mean=0.11; SD=0.17. Second generation: Offspring of fish from *upper* 10% of the first generation screen (n=92): mean=0.17; SD=0.14. Offspring of fish from *lower* 10% of the first generation screen (n=64): mean=0.08; SD=0.15. Third generation. Offspring of fish from *upper* 10% of the second generation screen (n=69): mean=0.21; SD=0.10. Offspring of fish from *lower* 10% of the second generation screen (n=67): mean=0.01; SD=0.09.

### Identification of Slit3 mutations affecting nicotine place preference in zebrafish

To identify candidate mutations affecting nicotine preference in fish, we focussed on families where all individuals included in the screen clustered at one or other extreme of the distribution curve. Two families (called AJBQM1 and AJBQM2 after the

researcher who conducted the screen), which clustered at the top (AJBQM1) and bottom (AJBQM2) of the nicotine preference distribution, were selected for further study. We first assessed nicotine CPP in the remaining siblings not initially included in the screen. As shown in Figure 3, the phenotypes were conserved when remaining siblings were assessed. Exome sequencing of fish (29) used to generate AJBQM1 and AJBQM2 identified 25 nonsense and essential splice site mutations. We genotyped fish at these 25 loci and determined the co-segregation with nicotine preference.

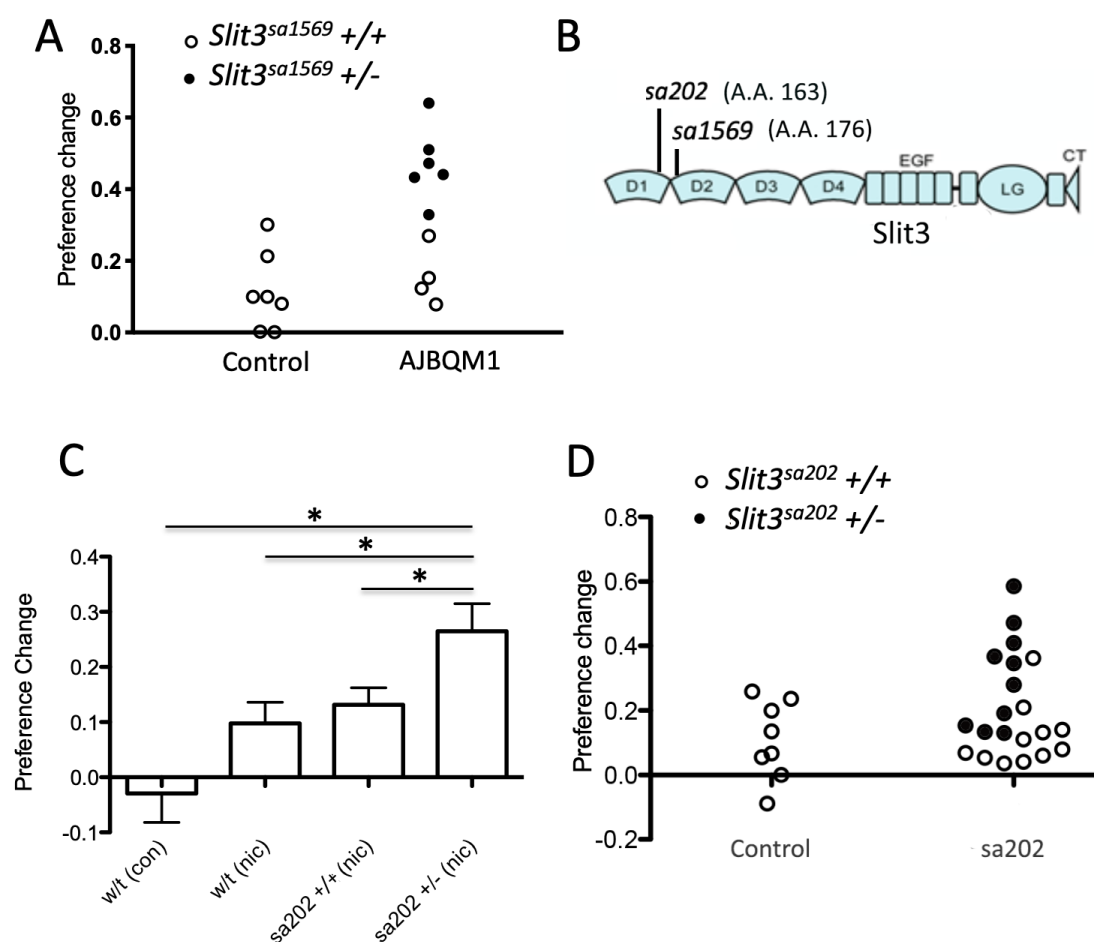


**Figure 3. Identification of *Slit3* mutations affecting nicotine place preference in zebrafish. AJBQM1 and AJBQM2 families show increased and decreased nicotine place preference, respectively. AJBQM1 and AJBQM2 siblings, not included in the screen (n=10 for AJBQM1; n=14 for AJBQM2), AJBQM1 significantly differed from TLF wildtype (w/t) saline control (n=17) and wildtype nicotine exposed fish (n=7). AJBQM2 differed from wildtype nicotine exposed fish**

but not wildtype saline controls. Different superscript letters indicate significant difference ( $p < 0.05$ ). Bars indicate Mean  $\pm$  SEM.

Of the 25 coding, predicted loss of function mutations in AJBQM1 and AJBQM2 (Listed in Supplementary Table 1), only *Slit3*<sup>sa1569/+</sup> (exon 7 splice acceptor site disruption at amino acid position 176), segregated with nicotine preference (Figure 4A & Supplementary Table 5A). None of the coding, predicted loss of function mutations in AJBQM2 segregated with nicotine preference and this line was not examined further (Supplementary Table 5B).

To confirm that loss of *Slit3* function was related to nicotine seeking behaviour we used a second, independent allele, *Slit3*<sup>sa202</sup>, with a G>T transversion producing a premature stop codon at amino acid position 163. Although not as marked as in AJBQM1 mutants (hereafter called *Slit3*<sup>sa1569</sup>), heterozygous *Slit3*<sup>sa202</sup> fish showed enhanced nicotine CPP ( $p = 0.03$ ) compared to wildtype siblings (Figure 4C & 4D). Both *Slit3*<sup>sa1569</sup> and *Slit3*<sup>sa202</sup> mutations affect splicing in the region before the second leucine rich repeat (LRR) domain in the encoded protein (Figure 4B), which is essential for interaction with ROBO receptor proteins (30).

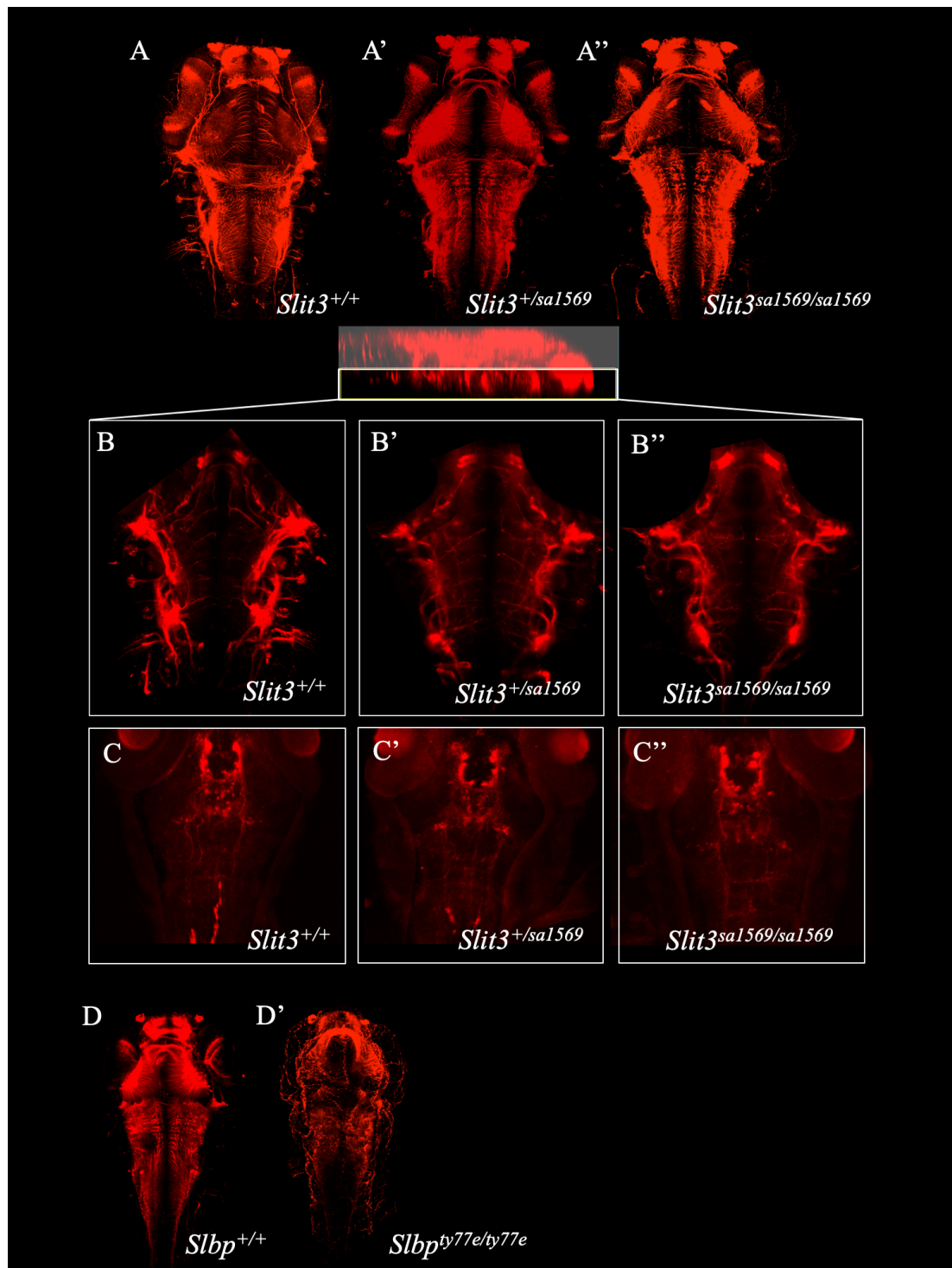


**Figure 4. A. Segregation of *Slit3<sup>sa1569</sup>* mutation with nicotine seeking.** CPP change scores for individual un-mutagenized TLF wildtype fish (n=7) and AJBQM1 fish (n=10). Following CPP analysis, fish were genotyped for 25 loss of function mutations contained within the family. Black dots indicate *Slit3<sup>sa1569/+</sup>* heterozygous mutant fish. White dots indicate *Slit3<sup>sa1569+/+</sup>* fish. Heterozygosity for *Slit3<sup>sa1569</sup>* segregates with increased nicotine seeking behaviour. **B. Position of ENU-induced mutations in zebrafish *Slit3* protein.** *Slit3<sup>sa1569</sup>* (A>G transition) disrupts a splice site in intron 7 affecting translation at amino acid 176. *Slit3<sup>sa202</sup>* (G>T transversion) introduces a stop codon at amino acid 163. Both mutations truncate the protein before the leucine rich repeat domain 2 (D2), which interacts with membrane bound ROBO during SLIT-ROBO signalling. **C: Nicotine preference of *Slit3<sup>sa202</sup>* line.** *Slit3<sup>sa202/+</sup>*

fish (n=18) show increased nicotine preference compared to wildtype TLF controls (n=8) (p = 0.001) and wildtype siblings *Slit3*<sup>+/+</sup> (n=14) (p<0.05). Bars indicate mean +SEM. **D: Segregation of *Slit3*<sup>sa202</sup> allele with nicotine seeking.** CPP change scores for individual un-mutagenised TLF wildtype parent strain fish (n=8) and *Slit3*<sup>sa202</sup> fish (n=21). Black dots indicate *Slit3*<sup>sa202/+</sup> heterozygous mutant fish, white dots indicate *Slit3*<sup>sa202+/+</sup> fish. Mutations in *Slit3*<sup>sa202</sup> co-segregate with nicotine preference. Heterozygous *Slit3*<sup>+/sa202</sup> present increased place preference compared to *slit3*<sup>sa202+/+</sup> siblings (n=11).

#### Characterisation of *Slit3*<sup>sa1569</sup> mutants

SLIT3 is a member of a family of proteins with established axon guidance properties and previously suggested to be involved in dopaminergic and serotonergic pathfinding (31). Therefore we performed immunostaining of the axonal projections in three-day-old zebrafish larvae and looked at the expression patterns along the midline in the ventral forebrain, where *Slit3* is known to be expressed (32). No differences between *Slit3*<sup>sa1569</sup> mutant and wildtype larvae were observed in axonal tracts labelled by anti-acetylated tubulin antibody (Figure 5 A-A'' and B-B'') nor in catecholaminergic tracts labelled by anti-tyrosine hydroxylase antibody (Figure 5 C-C''). Staining of *Slbp*<sup>ty77e/ty77e</sup> mutant larvae, known to have fewer neurons and axonal defects (16) were used as positive control (Figure 5 D & D').



**Figure 5: Fluorescent immunohistochemistry in three-day old wild type *Slit3*<sup>sa1569+/+</sup> (A-C), heterozygous mutant *Slit3*<sup>sa1569/+</sup> (A'-C') and homozygous mutant *Slit3*<sup>sa1569/sa1569</sup> (A''-C'') larvae show no obvious differences in axon pathfinding across genotype groups along the midline in the ventral forebrain. *Slit3* loss-of-function seems not to impair overall axon pathfinding or the**

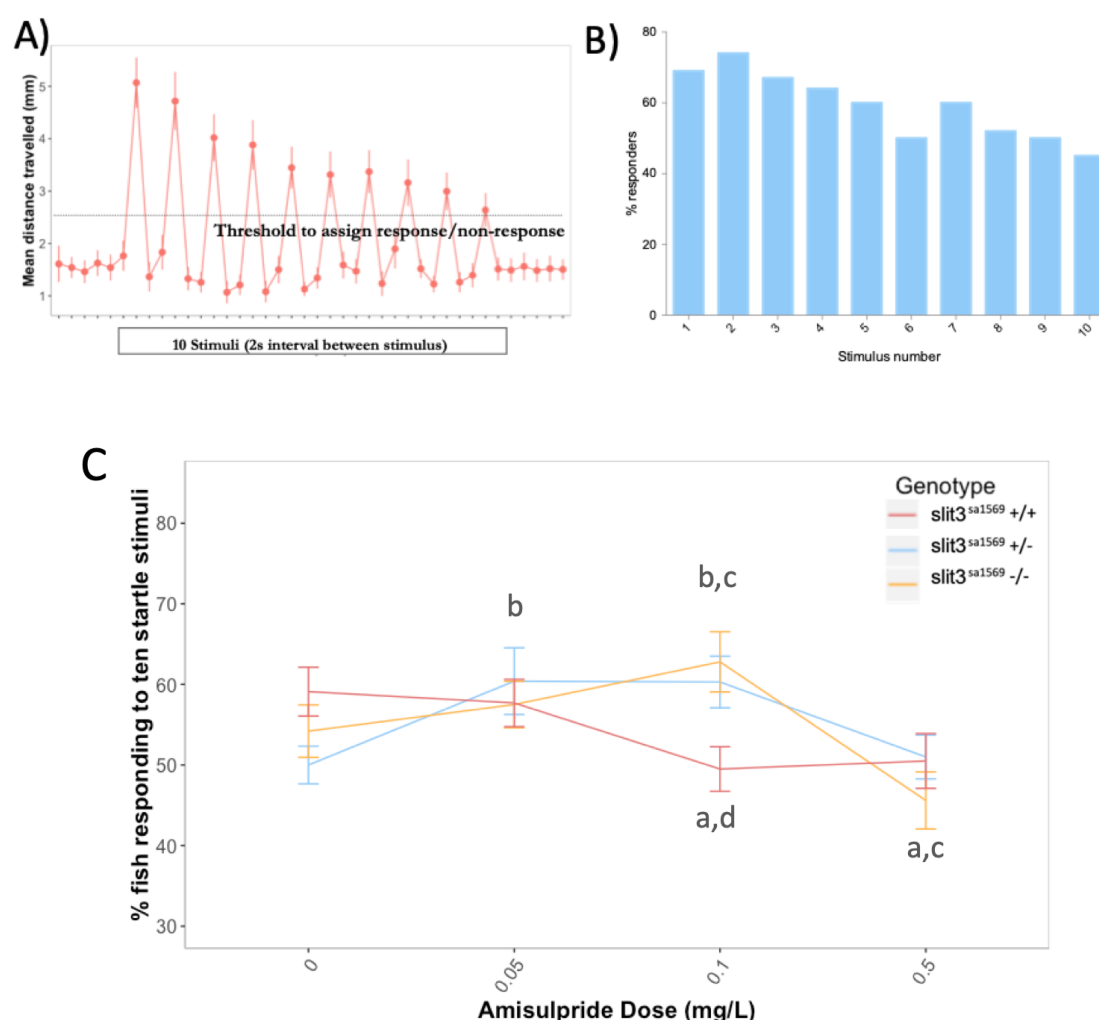
catecholaminergic axonal network. **A-A''**: Dorsal view of maximum projection anti-acetylated tubulin staining. **B-B''**: Detailed view of the ventral third of anti-acetylated tubulin stained embryos as indicated in insert above B-B''. **C-C''**: Anti-tyrosine hydroxylase staining. **D-D'**: Anti-acetylated tubulin staining for *Slbp*<sup>ty77e/ty77e</sup> mutants. *Slbp*<sup>ty77e/ty77e</sup> were used as positive control for antibody staining, as these mutants have fewer neurons and show axonal defects (33). n=10 samples per genotype group.

As subtle effects on circuit formation may not have been detected by our antibody staining, we examined the response and habituation to acoustic startle stimuli in wildtype and mutant fish. Habituation to acoustic startle is known to be sensitive to dopaminergic/serotonergic antagonists such as amisulpride (34) and, in humans, is associated with vulnerability to addiction (22–24). Five day old larvae were subjected to 10 sound/vibration stimuli over a total of 20 seconds (2 second interval between each stimulus) in the presence of 0, 0.05 mg/L, 0.1 mg/L or 0.5 mg/L amisulpride in 0.05% dimethyl sulfoxide (DMSO). The distance travelled one second after each stimulus was recorded for each fish.

Response and habituation to the stimuli was quantified as the percentage of fish moving more than 2.5 mm, which corresponds to 50% of the mean distance travelled one second after the first startle (Figure 6A). In line with the habituation response paradigm (35), a lower percentage of fish responded as the number of stimuli increased (Figure 6B). In wildtype fish higher doses of amisulpride caused a decrease in responsiveness (Main effect of 0.1 and 0.5 mg/L amisulpride across the ten taps p<0.05) (Figure 6C, red line). However *Slit3*<sup>sa1569</sup> mutants showed an inverted U-shaped response to amisulpride: a reduction of habituation at low doses and increase at the higher dose (Figure 6C, blue and orange lines). The presence of a *Slit3*<sup>sa1569</sup>

genotype by amisulpride dose interaction was confirmed by regression analysis of genotype and dose on percentage of respondents ( $p < 0.05$ ).

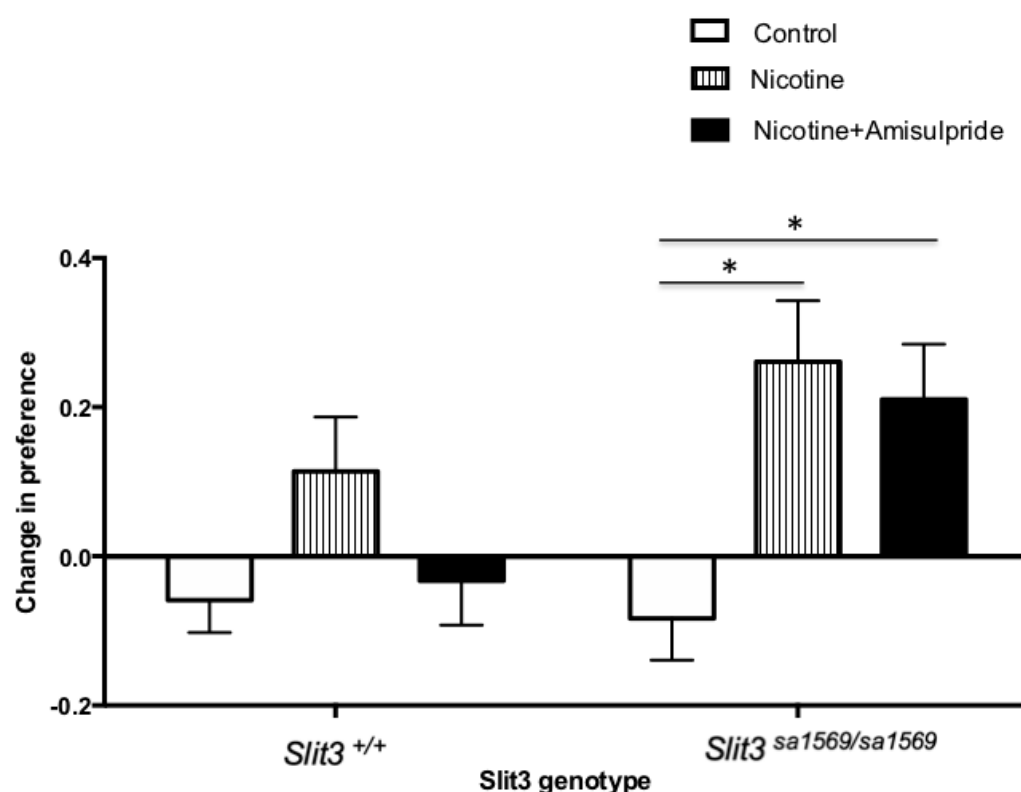
There were no significant differences in locomotion in the 15 seconds before the first startle, in magnitude of the response to the first tap stimulus, nor in total distance moved across all tap stimuli across experimental groups (Supplementary Figure 1) indicating that differences in behaviour were not confounded by differences in locomotion per se.



**Figure 6. Habituation response in the presence and absence of amisulpride. A: Response and habituation to 10 stimuli with two seconds interval between stimuli**

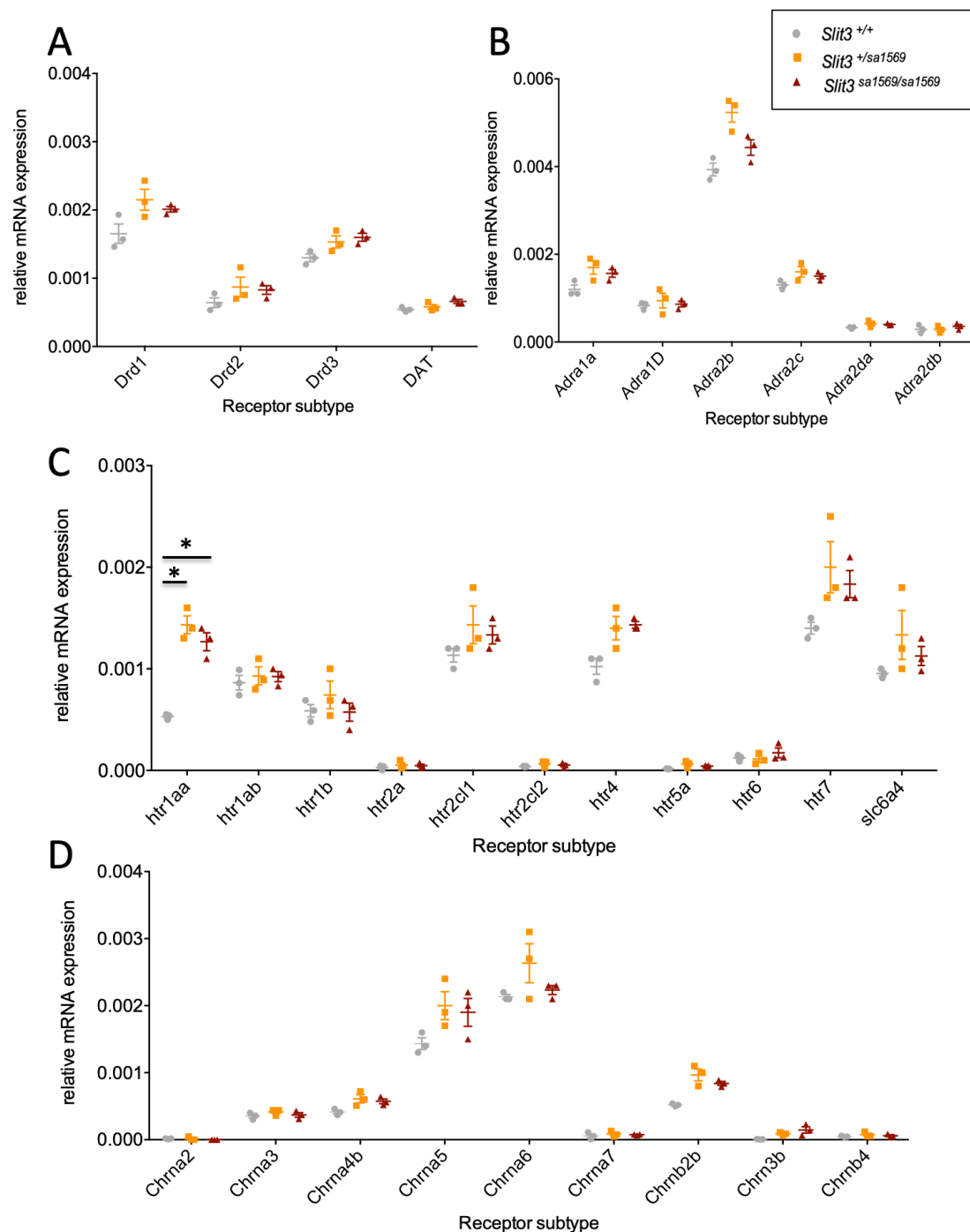
**in wildtype, drug free zebrafish.** Mean distances travelled were measured in one second time bins. Line indicates 2.5 mm, which corresponds to 50% of the mean distance travelled one second after the first stimulus. **B: Habituation response in wildtype zebrafish:** The percentage of fish responding to the stimuli decreases with stimulus/tap number (Main effects of tap number  $p < 0.05$ ). Respondents are defined as fish moving more than 2.5 mm. **C: Mean percentage of responders across the ten stimuli ( $\pm$ SEM), stratified by *Slit3*<sup>sa1569</sup> genotype and amisulpride dose.** The effect of amisulpride on habituation varies by genotype: *Slit3*<sup>sa1569</sup> mutants show a U-shaped response to amisulpride, in contrast with wildtype fish (Genotype by amisulpride dose interaction ( $p < 0.05$ )). Letters (a)-(c) indicate amisulpride doses that significantly differed from the carrier control within each genotype group ( $p < 0.5$  as per Tukey test). (a) corresponds to *Slit3* wildtype, (b) to *Slit3*<sup>sa1569/+</sup> and (c) to *Slit3*<sup>sa1569/sa1569</sup>. Letter (d) indicates significant genotype effect between *Slit3* wildtype and *Slit3*<sup>sa1569</sup> mutants at 0.1 mg/L amisulpride. n=42-48 fish per experimental group.

Adult *Slit3*<sup>sa1569/sa1569</sup> mutant zebrafish showed a qualitatively different response to inhibition of CPP by amisulpride compared to wildtype siblings, consistent with a persistent difference in sensitivity to this drug. The minimal CPP induced by 5 $\mu$ M nicotine in wild type fish was prevented by pre-exposure to 0.5mg/L amisulpride. Nicotine-induced CPP in *Slit3*<sup>sa1569</sup> homozygous mutants was not affected (Figure 7).



**Figure 7. CPP induced by 5 $\mu$ M nicotine is blocked by 0.5mg/L dopamine/serotonin antagonist amisulpride in wildtype *Slit3*<sup>sa1569+/+</sup> fish but not in *Slit3*<sup>sa1569</sup> homozygous mutants.** Bars represent mean (+SEM). (n=11-14 fish per group). \*Two-way ANOVA followed by post-hoc Tukey tests (p < 0.05).

As *Slit3*<sup>sa1569</sup> homozygous mutant fish showed altered sensitivity to nicotine and amisulpride, we examined whether expression of dopamine, serotonin, adrenergic or nicotinic receptor mRNA was dis-regulated in *Slit3*<sup>sa1569</sup> mutant larvae using quantitative real-time pcr (qpcr). Only *Htr1aa* ([F(2,6)=44], p=0.0003) showed a significant difference across genotypes after correcting for multiple testing (Figure 8).



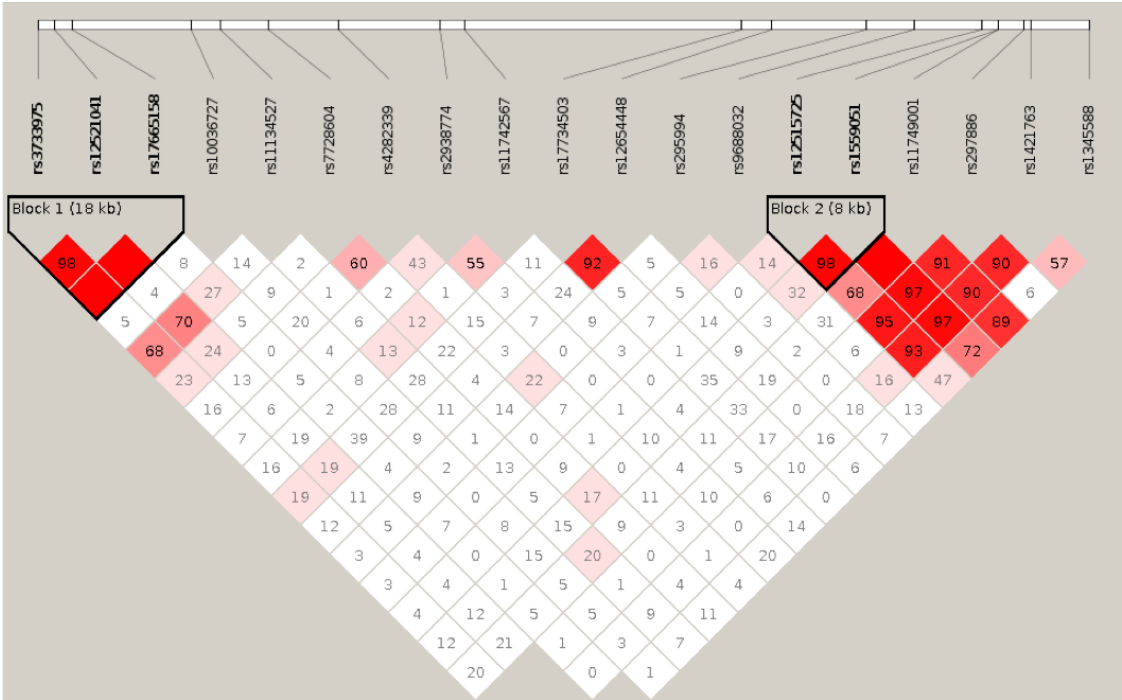
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269 **Figure 8. Quantitative real-time pcr analysis of five day old wildtype *Slit3*<sup>sa1569+/+</sup>,**  
 270 ***Slit3*<sup>sa1569/+</sup> heterozygous and *Slit3*<sup>sa1569/sa1569</sup> homozygous mutant larvae (Total**  
 271 **n=30, 3 samples per experimental group with n=10 embryos per sample). Only**  
 272 ***Htr1aa* ([F(2,6)=44], p=0.0003) showed a significant difference across genotypes**

after correcting for multiple testing. \*Two-way ANOVA followed by post-hoc Tukey test ( $p < 0.05$ ).

### **Variations at the *SLIT3* locus predict smoking behaviour in human samples**

We next examined associations between 19 single nucleotide polymorphisms (SNPs) in the human *SLIT3* gene and smoking behaviour in two London cohorts. Two SNPs, rs12654448 and rs17734503 in high linkage disequilibrium (Figure 9) 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 ( $p=0.004715$ ). The SNP rs11742567 was associated with cigarette consumption in light smokers ( $<20$  cigarettes per day,  $p=0.003909$ ) and with quitting. Associations are reported in Table 1. No other *SLIT3* polymorphisms were associated with smoking initiation, persistent smoking or cessation (Supplementary Tables 6 & 7).



**Figure 9: Linkage disequilibrium (LD) plot of *SLIT3* SNPs in human smoking association analysis.** Numbers within each square indicate  $D'$  values (white:  $D' < 1$ ,  $\text{LOD} < 2$ ; blue:  $D' = 1$ ,  $\text{LOD} < 2$ ; pink:  $D' < 1$ ,  $\text{LOD} \geq 2$ ; and bright red:  $D' = 1$ ,  $\text{LOD} \geq 2$ .)

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

301

| SNP               | Tobacco consumption |               |              |                         | Tobacco consumption - heavy smokers<br>(≥20 cigs/day) |               |              |                         | Tobacco consumption - light smokers<br>(<20 cigs/day) |              |              |                        | Smoking cessation |              |              |              |              |
|-------------------|---------------------|---------------|--------------|-------------------------|-------------------------------------------------------|---------------|--------------|-------------------------|-------------------------------------------------------|--------------|--------------|------------------------|-------------------|--------------|--------------|--------------|--------------|
|                   | P value             | β             | SE           | 95% CI                  | P value                                               | β             | SE           | 95% CI                  | P value                                               | β            | SE           | 95% CI                 | OR                | SE           | L95          | U95          | P value      |
| rs10036727        | 0.629               | -0.388        | 0.802        | (-1.960, 1.183)         | 0.448                                                 | -0.653        | 0.860        | (-2.337, 1.032)         | 0.940                                                 | -0.051       | 0.686        | (-1.396, 1.293)        | 0.947             | 0.160        | 0.693        | 1.295        | 0.734        |
| rs11134527        | 0.218               | 1.014         | 0.822        | (-0.596, 2.625)         | 0.327                                                 | -0.867        | 0.883        | (-2.599, 0.864)         | 0.261                                                 | 0.795        | 0.705        | (-0.586, 2.176)        | 0.665             | 0.165        | 0.482        | 0.918        | 0.013        |
| <b>rs11742567</b> | 0.135               | -1.166        | 0.779        | (-2.691, 0.361)         | <b>0.005</b>                                          | <b>-2.346</b> | <b>0.825</b> | <b>(-3.962, -0.730)</b> | <b>0.004</b>                                          | <b>1.888</b> | <b>0.644</b> | <b>(0.6258, 3.151)</b> | <b>1.586</b>      | <b>0.163</b> | <b>1.153</b> | <b>2.183</b> | <b>0.005</b> |
| rs11749001        | 0.059               | 1.972         | 1.044        | (-0.074, 4.018)         | 0.873                                                 | 0.177         | 1.103        | (-1.985, 2.338)         | 0.206                                                 | 1.200        | 0.944        | (-0.651, 3.051)        | 0.953             | 0.206        | 0.637        | 1.426        | 0.817        |
| rs12515725        | 0.592               | -0.406        | 0.756        | (-1.888, 1.076)         | 0.488                                                 | -0.565        | 0.813        | (-2.159, 1.029)         | 0.278                                                 | -0.688       | 0.631        | (-1.925, 0.550)        | 1.028             | 0.151        | 0.765        | 1.381        | 0.855        |
| rs12521041        | 0.904               | -0.105        | 0.865        | (-1.801, 1.591)         | 0.996                                                 | -0.005        | 0.942        | (-1.851, 1.841)         | 0.059                                                 | -1.354       | 0.710        | (-2.746, 0.038)        | 1.554             | 0.178        | 1.096        | 2.205        | 0.013        |
| <b>rs12654448</b> | <b>0.001</b>        | <b>-4.241</b> | <b>1.307</b> | <b>(-6.803, -1.680)</b> | <b>0.0003</b>                                         | <b>-4.830</b> | <b>1.334</b> | <b>(-7.444, -2.216)</b> | 0.410                                                 | -1.034       | 1.251        | (-3.486, 1.417)        | 1.625             | 0.279        | 0.941        | 2.808        | 0.082        |
| rs1345588         | 0.240               | -1.268        | 1.078        | (-3.380, 0.845)         | 0.253                                                 | -1.334        | 1.164        | (-3.616, 0.948)         | 0.869                                                 | -0.150       | 0.907        | (-1.927, 1.627)        | 1.417             | 0.222        | 0.918        | 2.189        | 0.116        |
| rs1421763         | 0.272               | -0.982        | 0.894        | (-2.735, 0.770)         | 0.978                                                 | -0.027        | 0.959        | (-1.908, 1.853)         | 0.162                                                 | -1.074       | 0.764        | (-2.571, 0.424)        | 0.917             | 0.176        | 0.649        | 1.294        | 0.622        |
| rs1559051         | 0.961               | -0.040        | 0.819        | (-1.644, 1.564)         | 0.458                                                 | 0.656         | 0.882        | (-1.073, 2.384)         | 0.507                                                 | 0.455        | 0.685        | (-0.880, 1.797)        | 0.919             | 0.163        | 0.668        | 1.265        | 0.606        |
| rs17665158        | 0.131               | 1.338         | 0.884        | (-0.394, 3.070)         | 0.236                                                 | 1.114         | 0.939        | (-0.727, 2.955)         | 0.034                                                 | 1.620        | 0.758        | (0.1354, 3.106)        | 0.723             | 0.172        | 0.516        | 1.013        | 0.060        |
| <b>rs17734503</b> | <b>0.002</b>        | <b>-3.987</b> | <b>1.299</b> | <b>(-6.534, -1.441)</b> | <b>0.001</b>                                          | <b>-4.458</b> | <b>1.325</b> | <b>(-7.055, -1.861)</b> | 0.410                                                 | -1.034       | 1.251        | (-3.486, 1.417)        | 1.616             | 0.275        | 0.942        | 2.773        | 0.081        |
| rs2938774         | 0.140               | 1.101         | 0.745        | (-0.359, 2.562)         | 0.528                                                 | 0.496         | 0.786        | (-1.044, 2.036)         | 0.015                                                 | -1.655       | 0.674        | (-2.976, -0.333)       | 0.753             | 0.148        | 0.563        | 1.007        | 0.056        |
| rs295994          | 0.714               | 0.283         | 0.770        | (-1.227, 1.793)         | 0.643                                                 | 0.378         | 0.813        | (-1.215, 1.971)         | 0.238                                                 | -0.796       | 0.672        | (-2.114, 0.521)        | 0.799             | 0.154        | 0.591        | 1.082        | 0.147        |
| rs297886          | 0.620               | 0.442         | 0.890        | (-1.303, 2.187)         | 0.961                                                 | -0.048        | 0.986        | (-1.979, 1.884)         | 0.489                                                 | 0.488        | 0.704        | (-0.891, 1.867)        | 1.108             | 0.177        | 0.784        | 1.568        | 0.561        |
| rs3733975         | 0.909               | -0.099        | 0.860        | (-1.784, 1.587)         | 0.982                                                 | 0.022         | 0.934        | (-1.809, 1.852)         | 0.059                                                 | -1.354       | 0.710        | (-2.746, 0.038)        | 1.488             | 0.176        | 1.054        | 2.101        | 0.024        |
| rs4282339         | 0.669               | -0.434        | 1.013        | (-2.419, 1.552)         | 0.942                                                 | -0.080        | 1.103        | (-2.241, 2.081)         | 0.238                                                 | -1.006       | 0.849        | (-2.670, 0.658)        | 0.984             | 0.203        | 0.661        | 1.464        | 0.936        |
| rs7728604         | 0.701               | 0.286         | 0.744        | (-1.173, 1.745)         | 0.321                                                 | 0.827         | 0.832        | (-0.803, 2.457)         | 0.654                                                 | 0.262        | 0.583        | (-0.880, 1.404)        | 0.935             | 0.149        | 0.698        | 1.253        | 0.653        |
| rs9688032         | 0.948               | -0.050        | 0.766        | (-1.551, 1.451)         | 0.770                                                 | 0.246         | 0.839        | (-1.398, 1.890)         | 0.080                                                 | -1.076       | 0.610        | (-2.272, 0.119)        | 1.066             | 0.156        | 0.786        | 1.446        | 0.680        |

We subsequently investigated associations with more detailed smoking phenotypes in the Finnish twins cohort (36) (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 Fagerström Test for Nicotine Dependence (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.

The SNPs rs12654448 and rs17734503 are in non-coding domains, therefore it was not possible to predict loss or gain of function of *SLIT3* from the SNP location. No evidence of affecting gene expression was found as per GTEx database (<https://gtexportal.org/home/>).

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

| SNP               | DSM-IV ND diagnosis |        |          | DSM-IV ND symptoms |        |                 | FTND ( $\geq 4$ ) |        |          | FTND score |        |          |
|-------------------|---------------------|--------|----------|--------------------|--------|-----------------|-------------------|--------|----------|------------|--------|----------|
|                   | $\beta$             | SE     | P value  | $\beta$            | SE     | P value         | $\beta$           | SE     | P value  | $\beta$    | SE     | P value  |
| <b>rs12654448</b> | -0.0343             | 0.0262 | 0.190975 | -0.1839            | 0.0964 | 0.056728        | 0.0526            | 0.0287 | 0.066509 | 0.075      | 0.1365 | 0.58286  |
| <b>rs17734503</b> | -0.0354             | 0.0259 | 0.171821 | -0.2044            | 0.0954 | <b>0.032199</b> | 0.0474            | 0.0283 | 0.094383 | 0.0443     | 0.135  | 0.743052 |
| <b>rs11742567</b> | 0.0006              | 0.0163 | 0.97262  | -0.0359            | 0.0601 | 0.55086         | 0.0134            | 0.0179 | 0.45384  | 0.0449     | 0.0851 | 0.597682 |
| rs17665158        | 0.0117              | 0.019  | 0.538639 | 0.1536             | 0.0696 | 0.027544        | 0.0178            | 0.0207 | 0.391096 | 0.0935     | 0.0988 | 0.344157 |
| rs1345588         | -0.0031             | 0.0222 | 0.889847 | -0.0389            | 0.0817 | 0.634184        | 0.0578            | 0.0242 | 0.01708  | 0.1901     | 0.1157 | 0.100729 |
| rs7728604         | -0.0049             | 0.0162 | 0.761485 | -0.0442            | 0.0597 | 0.459743        | 0.0004            | 0.0177 | 0.980706 | -0.0261    | 0.0846 | 0.757849 |
| rs11134527        | 0.0296              | 0.0171 | 0.084576 | 0.0927             | 0.063  | 0.141369        | 0.0324            | 0.0187 | 0.083498 | 0.1376     | 0.0891 | 0.122807 |
| rs10036727        | 0.0067              | 0.0165 | 0.68406  | 0.0207             | 0.0605 | 0.732266        | 0.0022            | 0.018  | 0.903865 | 0.046      | 0.0857 | 0.591583 |
| rs1559051         | 0.0249              | 0.0193 | 0.198647 | 0.0492             | 0.0703 | 0.484353        | -0.0433           | 0.0208 | 0.037736 | -0.1011    | 0.0995 | 0.309836 |
| rs12515725        | 0.0072              | 0.0159 | 0.6502   | 0.0277             | 0.0584 | 0.635739        | 0.0586            | 0.0172 | 0.000696 | 0.2482     | 0.0824 | 0.002637 |
| rs2938774         | 0.0042              | 0.0173 | 0.80717  | 0.0096             | 0.0642 | 0.881054        | -0.0157           | 0.0191 | 0.41163  | -0.0173    | 0.0907 | 0.848978 |
| rs295994          | -0.014              | 0.0171 | 0.410864 | -0.0144            | 0.0622 | 0.816397        | -0.016            | 0.0184 | 0.38542  | -0.0985    | 0.0879 | 0.262584 |
| rs9688032         | -0.0174             | 0.0173 | 0.31299  | -0.0347            | 0.0636 | 0.585081        | 0.0234            | 0.0189 | 0.216144 | 0.0626     | 0.09   | 0.4869   |
| rs11749001        | 0.0178              | 0.0235 | 0.448278 | -0.0062            | 0.0865 | 0.942516        | 0.0224            | 0.0257 | 0.383552 | 0.0067     | 0.1222 | 0.956097 |
| rs4282339         | 0.0118              | 0.0201 | 0.557544 | 0.0526             | 0.0739 | 0.476216        | -0.0077           | 0.0219 | 0.724058 | 0.1623     | 0.1045 | 0.120641 |
| rs297886          | -0.0216             | 0.0171 | 0.207835 | -0.045             | 0.0634 | 0.478517        | -0.0256           | 0.0188 | 0.173314 | -0.1354    | 0.0897 | 0.131469 |
| rs1421763         | 0.0079              | 0.0187 | 0.671624 | 0.0178             | 0.0687 | 0.795522        | 0.0641            | 0.0203 | 0.001641 | 0.2582     | 0.0971 | 0.007892 |
| rs3733975         | -0.013              | 0.0167 | 0.436903 | -0.0798            | 0.0613 | 0.192755        | -0.0384           | 0.0181 | 0.034371 | -0.2083    | 0.0866 | 0.016295 |
| rs12521041        | -0.0098             | 0.0167 | 0.559173 | -0.0669            | 0.0613 | 0.275274        | -0.0365           | 0.0182 | 0.044962 | -0.1905    | 0.0868 | 0.028295 |

| SNP        | CPD     |        |          | max CPD |        |          | Age of onset of weekly smoking |        |                 | First time sensation |        |          | FTND time to first cigarette |        |          |
|------------|---------|--------|----------|---------|--------|----------|--------------------------------|--------|-----------------|----------------------|--------|----------|------------------------------|--------|----------|
|            | $\beta$ | SE     | P value  | $\beta$ | SE     | P value  | $\beta$                        | SE     | P value         | $\beta$              | SE     | P value  | $\beta$                      | SE     | P value  |
| rs12654448 | -0.3509 | 0.5669 | 0.536029 | -1.0602 | 0.7743 | 0.171106 | 0.7826                         | 0.2384 | <b>0.001051</b> | -0.0861              | 0.1423 | 0.545206 | 0.0047                       | 0.0802 | 0.953291 |
| rs17734503 | -0.479  | 0.5608 | 0.393086 | -1.2329 | 0.7657 | 0.107544 | 0.7689                         | 0.2362 | <b>0.001156</b> | -0.1039              | 0.1406 | 0.460344 | 0.0188                       | 0.0795 | 0.812682 |
| rs11742567 | 0.0179  | 0.3532 | 0.959588 | -0.4621 | 0.4823 | 0.338159 | 0.0965                         | 0.1493 | 0.518066        | -0.1003              | 0.0884 | 0.256427 | -0.0216                      | 0.05   | 0.665265 |
| rs17665158 | 0.8135  | 0.4096 | 0.047191 | 1.5424  | 0.5587 | 0.005828 | 0.0562                         | 0.1732 | 0.745385        | 0.2476               | 0.1027 | 0.01603  | -0.0884                      | 0.058  | 0.12787  |
| rs1345588  | 0.294   | 0.4805 | 0.54066  | 0.3968  | 0.6562 | 0.54542  | 0.0989                         | 0.2031 | 0.626431        | -0.0618              | 0.1204 | 0.607606 | -0.1303                      | 0.068  | 0.055678 |
| rs7728604  | -0.0772 | 0.3511 | 0.825888 | 0.0691  | 0.4795 | 0.885363 | -0.0261                        | 0.1486 | 0.860643        | -0.029               | 0.0875 | 0.740682 | -0.0193                      | 0.0497 | 0.6978   |
| rs11134527 | 0.1831  | 0.3705 | 0.621187 | 0.7441  | 0.5057 | 0.141392 | -0.2347                        | 0.1563 | 0.133517        | 0.1142               | 0.093  | 0.21965  | -0.1089                      | 0.0523 | 0.037681 |
| rs10036727 | 0.1482  | 0.3557 | 0.67697  | 0.4246  | 0.4858 | 0.382161 | -0.0456                        | 0.1507 | 0.762197        | 0.0289               | 0.0896 | 0.74711  | -0.0639                      | 0.0504 | 0.205061 |
| rs1559051  | -0.4816 | 0.413  | 0.243693 | -0.4779 | 0.5641 | 0.397066 | 0.1437                         | 0.175  | 0.411533        | -0.0289              | 0.1045 | 0.782381 | 0.0731                       | 0.0586 | 0.212174 |
| rs12515725 | 0.5491  | 0.3429 | 0.10948  | 0.7708  | 0.4684 | 0.100032 | -0.1629                        | 0.1452 | 0.26192         | -0.0368              | 0.0865 | 0.670165 | -0.1385                      | 0.0485 | 0.00434  |
| rs2938774  | -0.2796 | 0.377  | 0.45835  | 0.1945  | 0.5149 | 0.70567  | -0.0575                        | 0.1598 | 0.718862        | 0.043                | 0.0933 | 0.645221 | -0.0136                      | 0.0533 | 0.797909 |
| rs295994   | -0.2793 | 0.3651 | 0.444276 | -0.2585 | 0.4988 | 0.604451 | 0.1543                         | 0.1548 | 0.318928        | 0.0625               | 0.0926 | 0.499881 | 0.0527                       | 0.0517 | 0.307869 |
| rs9688032  | 0.2452  | 0.3738 | 0.511921 | 0.4211  | 0.5107 | 0.409766 | -0.1142                        | 0.1584 | 0.471283        | -0.2227              | 0.0937 | 0.01755  | -0.0517                      | 0.0531 | 0.329867 |
| rs11749001 | -0.0301 | 0.5078 | 0.952789 | 0.0574  | 0.6939 | 0.934054 | -0.1994                        | 0.2147 | 0.353064        | 0.1497               | 0.1274 | 0.240255 | 0.0075                       | 0.0718 | 0.916823 |
| rs4282339  | 0.4086  | 0.434  | 0.346592 | 0.3083  | 0.593  | 0.603197 | 0.0952                         | 0.1836 | 0.603958        | -0.0394              | 0.1084 | 0.716204 | -0.0524                      | 0.0614 | 0.394221 |
| rs297886   | -0.1375 | 0.3727 | 0.712273 | -0.4782 | 0.5091 | 0.347622 | 0.1262                         | 0.1575 | 0.423104        | 0.0519               | 0.0928 | 0.576255 | 0.0632                       | 0.0527 | 0.230861 |
| rs1421763  | 0.5585  | 0.4037 | 0.166723 | 0.6702  | 0.5515 | 0.224417 | -0.1481                        | 0.1706 | 0.385475        | -0.0799              | 0.1018 | 0.432497 | -0.1269                      | 0.0571 | 0.026442 |
| rs3733975  | -0.7784 | 0.3597 | 0.030606 | -1.0555 | 0.4911 | 0.031758 | 0.0902                         | 0.1521 | 0.553335        | -0.2932              | 0.0896 | 0.001085 | 0.1373                       | 0.0509 | 0.007035 |
| rs12521041 | -0.7312 | 0.3602 | 0.042534 | -0.8864 | 0.492  | 0.071805 | 0.0522                         | 0.1523 | 0.731943        | -0.3129              | 0.0897 | 0.000499 | 0.1257                       | 0.051  | 0.01373  |

## DISCUSSION

The aim of this study was to use forward genetic screening in zebrafish to identify loci affecting human smoking behaviour. 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 two 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 that may affect propensity to develop tobacco dependence.

We screened 30 ENU-mutagenized zebrafish families followed by sibling re-screen to identify families of fish showing altered nicotine preference. This proof of principle study indicates the relevance of zebrafish for human studies and emphasizes the advantage of first using a screen with a low number of individuals per family to increase efficiency. The classic three generation forward genetic screen examines phenotypes in groups of 20 or more individuals from each family (37). Logistical considerations make it difficult to apply such an approach to adult behavioural screens. Our approach increases efficiency by initially screening a small number of individuals from a large number of families and only selecting those families that occur at the extremes of the distribution for further analysis. Although in this study 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 a loss of function mutation in the zebrafish *Slit3* gene associated with increased nicotine place preference and confirmed the phenotype in an independent line. SLIT molecules bind to ROBO receptors through a highly conserved leucine-rich repeat (LRR) domain (38). In the AJBQM1 (*Slit3*<sup>sa1569</sup>) line the loss of function mutation causes a truncation at amino acid 176 and in the *Slit3*<sup>sa202</sup> line at amino acid 163. These are immediately adjacent to the LRR2 domain responsible for SLIT3's functional interaction with ROBO proteins (38) 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 remodelling (39). *In vitro* SLIT proteins bind promiscuously to ROBO receptors suggesting that the proteins may co-operate *in vivo* in areas in which they overlap. However, their restricted spatial distributions, particularly of SLIT3 in the central nervous system (40) 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 (41,42) 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 (43) 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.

368 *Slit3*<sup>sa1569</sup> mutants showed altered sensitivity of the startle response to amisulpride.  
369 Acoustic startle is sensitive to modulation of dopaminergic and serotonergic  
370 signalling in all species studied (34,44,45). Our finding that amisulpride increased  
371 habituation to acoustic startle in wildtype fish is in agreement with the effect of  
372 amisulpride in humans (34). In contrast to results in wildtype fish, *Slit3*<sup>sa1569</sup> mutant  
373 larvae showed decreased habituation in the presence of low dose amisulpride. During  
374 adulthood, amisulpride inhibited nicotine-induced CPP in wildtype fish but not in  
375 *Slit3*<sup>sa1569</sup> mutants. These findings are consistent with a disrupted dopaminergic or  
376 serotonergic system caused by *Slit3* loss of function.

377 Although gene expression analyses revealed subtle upregulation in several receptors  
378 in *Slit3* mutants, significant difference was only seen for the *5ht1aa* receptor subtype.  
379 The observation of increased *5htr1aa* expression in *Slit3* mutants is of interest:  
380 Serotonergic signalling has been previously linked to drug reward processes including  
381 nicotine use and dependence (46,47). Manipulations which decrease brain serotonin  
382 neurotransmission (e.g., a neurotoxic serotonin depletion or a lasting serotonin  
383 synthesis inhibition) elevate self-administration of several different drugs including  
384 nicotine in rats (47–49). Compounds that facilitate serotonin neurotransmission, like  
385 selective serotonin reuptake inhibitors, decrease nicotine intake (50). Nicotine  
386 increases serotonin release in the striatum, hippocampus, cortex, dorsal raphe nucleus  
387 (DRN), spinal cord and hypothalamus (51). The effects in the cortex, hippocampus,  
388 and DRN involve stimulation of *5htr1a* receptors, and in the striatum, *5htr3* receptors.  
389 In the DRN, *5htr1a* receptors play a role in mediating the anxiolytic effects of  
390 nicotine. In contrast, in the dorsal hippocampus and lateral septum, these same  
391 receptors mediate its anxiogenic effects. Although it is possible that an anxiolytic  
392 effect of nicotine contributed to the increased nicotine-induced place preference,

preliminary assessment of anxiety-like responses (tank diving) in *Slit3* mutants, where mutants show decreased anxiety-like behaviour (n.s), argue against this.

Pharmacological studies in rodents have shown that the *5htr1a* receptor antagonists WAY100635 and LY426965 alleviate the behavioural responses induced by nicotine withdrawal (52–54). WAY100635 has also been reported to block the nicotine enhancement of cocaine and methamphetamine self-administration in adolescent rats (55). Our findings of an increase in *5ht1aa* expression, altered sensitivity to amisulpride and altered nicotine CPP support a role for *Slit3* signalling in the formation of serotonergic pathways involved in responses to nicotine. Whilst speculative, it is also potentially of interest that variants in both *5htr1a* and *Slit3* are associated with psychiatric disorders such as schizophrenia (56–58), known to involve serotonergic pathways. Further analyses, out of the scope of this study, are required in order to tease out the exact brain regions and 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).

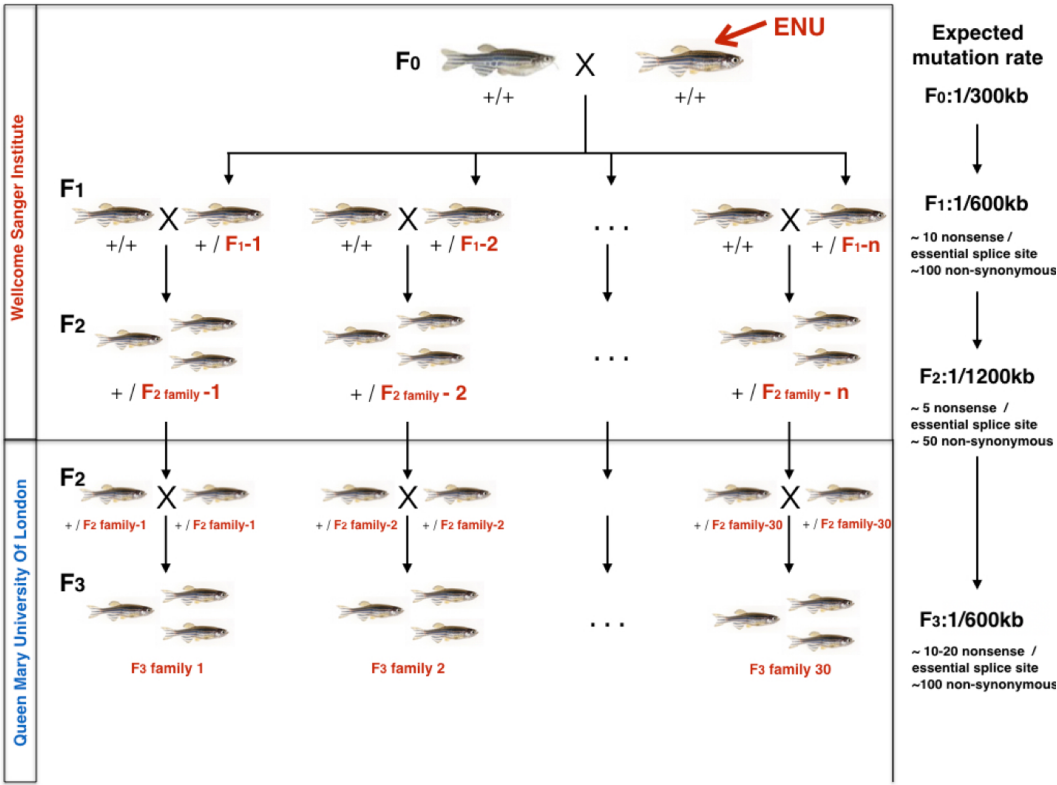
To our knowledge, this is the first report of a forward behavioural genetic screen in adult zebrafish successfully predicting a novel human coding genetic region involved in a complex human behavioural trait. Taken together, these results provide evidence

418 for a role for *SLIT3* in regulating smoking behaviour in humans and confirm adult  
 419 zebrafish as a translationally relevant animal model for exploration of addiction-  
 420 related behaviours. Further work analysing the cellular processes affected as a result  
 421 of the *Slit3* mutation may provide useful targets when designing tailored treatments to  
 422 aid smoking cessation.  
 423

## MATERIALS AND METHODS

***Generation of F3 families of ENU-mutagenised fish:*** Wildtype and ENU-mutagenized Tübingen longfin (TLF) fish were obtained from the Sanger Institute, as part of the Zebrafish Mutation Project which aimed to create a knockout allele in every zebrafish protein-coding gene [<https://www.sanger.ac.uk/resources/zebrafish/zmp/>]. At the Sanger, ENU-mutagenized TLF F<sub>0</sub> males were outcrossed to create a population of F<sub>1</sub> fish heterozygous for ENU-induced mutations. Due to the high ENU mutation rate (1/300 kb) and homologous recombination when F<sub>1</sub> gametes are generated, all F<sub>2</sub> were heterozygous for multiple mutations. F<sub>2</sub> families, each generated from a separate F<sub>1</sub> fish, were imported from the Sanger Institute to Queen Mary University of London (QMUL).

At QMUL a single male and female fish from each F<sub>2</sub> family were inbred to generate 30 F<sub>3</sub> families that would be 25% wildtype, 50% heterozygous and 25% homozygous mutant for any single mutation, assuming Mendelian genetics. Based on exome sequencing data from the F<sub>1</sub> generation performed at the Sanger, each F<sub>3</sub> family contained 10-20 known predicted loss of function exonic mutations, approximately 100 non-synonymous coding mutations and approximately 1500 unknown mutations in non-coding domains across the entire genome (29). Breeding scheme is detailed in Figure 10.



**Figure 10. Zebrafish breeding scheme to generate F3 families.** F<sub>2</sub> ENU-mutagenized zebrafish, heterozygous for multiple mutations across the entire genome were obtained from the Wellcome Sanger Institute as part of the Zebrafish Mutation Project. At Queen Mary University of London, heterozygous F<sub>2</sub> fish were incrossed to generate 30 F<sub>3</sub> families, each containing 10-20 nonsense or essential splice site mutations and about 1500 additional exonic and intronic point mutations. F<sub>3</sub> Families were arbitrarily numbered 1-30. Expected mutation rate and type of mutations in coding regions are specified on the right hand side.

***Fish maintenance:***

Fish were housed in a recirculating system (Techniplast, UK) on a 14h:10h light:dark cycle (0830–2230). The housing and testing rooms were maintained at ~25–28°C. 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.

***Conditioned place preference (CPP):*** All fish were age and weight matched for all behavioural analysis and were approximately 5 months old, weighing 0.2-0.25g at the start of testing. Following habituation and determination of basal preference, animals were conditioned to 5µM nicotine (Sigma, Gillingham, UK Catalogue number: N1019) over three consecutive days and assessed for a change in place preference the following day. 5µM nicotine was used because it was predicted to induce a minimum detectable change in place preference based on results of previous studies (19,21). This minimal effective dose was used to avoid possible ceiling effects if using a higher concentration. CPP was assessed as described previously (19,20,59): The testing apparatus was an opaque 3 L rectangular tank that could be divided in half with a Perspex divider. Each end of the tank had distinct visual cues (1.5cm diameter black spots versus vertical 0.5cm wide black and white stripes, matched for luminosity). After habituation to the apparatus and handling, we determined the basal preference for each fish: individual fish were placed in the tank for 10 min and the time spent at either end determined using a ceiling mounted camera and Ethovision tracking software (Noldus, Wageningen, NL). Any fish showing >70% preference for either end was excluded from further analysis. Fish were then conditioned with nicotine in the least preferred environment for 20 min, on 3 consecutive days: Each day each fish was restricted first to its preferred side for 20 min in fish water and then to its least preferred side with nicotine or, if a control fish, vehicle (fish water) added,

for another 20 min. After 20 min in the nicotine (or vehicle)-paired environment each fish was returned to its home tank. After 3 days of conditioning, on the following day, fish were subject to a probe trial whereby each fish was placed in the conditioning tank in the absence of divider and the time spent at either end of the tank over a 10 min period was determined as for assessment of basal preference. The change in place preference was determined as the proportion time spent in the nicotine-paired zone during the probe trial minus the proportion time spent in the nicotine-paired zone during basal testing. The CPP procedure has been used and validated previously with nicotine as well as other drugs (19,20,59) .

Data analysis: Change in preference scores were calculated as proportion time spent in drug paired stimulus after conditioning minus proportion time spent in drug paired stimulus before conditioning. Population means between generations were compared using independent two-sample t-tests, and effect-sizes ascertained using Cohen's d (60). For the rescreen of outlier sibling families and *Slit3*<sup>sa202</sup> line, mutant lines were compared with wildtype controls using an independent two-sample t-test.

**CPP in the presence or absence of antagonists:** To assess the ability of compounds (varenicline (Sigma, Gillingham, UK, PZ0004), bupropion (Sigma, Gillingham, UK, B1277) or amisulpride (Tocris, Bristol, UK, C2132) to inhibit subjective effects of nicotine a modified version of the CPP procedure was used (20). In this modified version, following habituation and establishment of basal preference, each day each fish was restricted first to its preferred side for 20 min in fish water and then removed from the conditioning tank and transferred to a tank containing the appropriate concentration of test compound or fish water (plus carrier where required) for 10min. After 10min the fish was returned to its least preferred side in the conditioning tank with nicotine or, if a control fish, vehicle (fish water) for another 20 min. After 20 min in the nicotine (or vehicle)-paired environment, each fish was returned to its

home tank. After three days of conditioning to nicotine in the presence or absence of test compound, on the following day, fish were subject to a probe trial whereby each fish was placed in the conditioning tank in the absence of divider and the time spent at either end of the tank over a 10 min period determined. To assess the ability of varenicline (0-20 $\mu$ M) or bupropion (0-10 $\mu$ M) to inhibit subjective effects of nicotine in wildtype fish, fish were incubated in the presence and absence of increasing doses of test compound (or vehicle) for 10 min before conditioning to 10 $\mu$ M nicotine. Statistical analysis was performed using a univariate analysis of variance (ANOVA), followed by Tukey's post hoc test.

To test the effect of amisulpride on nicotine -induced CPP in wildtype and *Slit3*<sup>sa1569</sup> mutant fish, fish were incubated in the presence or absence of 0.5mg/L amisulpride for 10 min before conditioning to 5 $\mu$ M nicotine. Two-way ANOVA was performed with genotype (*Slit3*<sup>+/+</sup> and *Slit3*<sup>sa1569/sa1569</sup>) and treatment (control, nicotine, nicotine+amisulpride) as independent variables. Values of p<0.05 were considered significant.

### ***Breeding and selection to assess heritability of nicotine-induced place preference:***

To test whether nicotine preference is heritable, fish falling in the upper and lower deciles of the 'change in place preference' distribution were kept for analysis and further breeding. Individuals were bred (in-cross of fish from the upper decile and in-cross of fish from the lower decile done separately) and their offspring screened for CPP (Second Generation CPP analysis). The same approach was repeated again: fish at the extremes of the Second Generation CPP distribution curve were selected and in-crossed, and their offspring were used to perform a Third Generation CPP analysis.

***Identification of ENU-induced mutations influencing nicotine place preference:*** To investigate whether ENU-induced mutations affect fish sensitivity to the rewarding effects of nicotine, candidate families were selected when the 3-4 fish from a family

tested clustered together at one or other extreme of the change in preference distribution curve. To confirm the genetic effect on the CPP phenotype in candidate families, all remaining siblings of that family were assessed for nicotine induced CPP, along with non-mutagenized TLF control fish, to confirm the genetic effect on the phenotype.

Candidate mutations, obtained from exome sequencing on F<sub>1</sub> fish, were assessed for co-segregation with behaviour using site specific pcr (61) (See Supplementary Methods). Once a co-segregating candidate mutation was identified, larvae from an independent line carrying a predicted loss of function allele in the same gene were obtained from the Sanger Institute (*Slit3*<sup>sa202</sup>) to confirm the association. Heterozygous *Slit3*<sup>sa202/+</sup> and sibling *Slit3*<sup>+/+</sup> larvae were reared to adulthood and assessed for nicotine-induced CPP as described above. All fish were fin clipped and genotyped following CPP.

### ***Characterization of larvae:***

***Antibody staining:*** In order to visualize axonal pathways, fluorescent immunohistochemistry was carried out in three day old embryos from wildtype *Slit3*<sup>+/+</sup>, heterozygous mutant *Slit3*<sup>sa1569/+</sup> and homozygous mutant *Slit3*<sup>sa1569/sa1569</sup> in-crosses. To prevent skin pigmentation, embryos were incubated in 0.2mM of 1-phenyl 2-thiourea (Sigma, Gillingham, UK) from 24 hours after fertilization. At three days, they were fixed in 4% paraformaldehyde (Sigma, Gillingham, UK) to avoid tissue degradation. For the immunostaining, rabbit polyclonal anti-tyrosine hydroxylase primary antibody (1:200; Sigma, Gillingham, AB152) and mouse anti-acetylated tubulin monoclonal antibody (1:1000; Sigma Gillingham, UK, T6793) were used.

Both primary antibodies were detected with Alexa 546-conjugated secondary antibodies (1:400; Fisher Scientific, Loughborough, UK A11010). Whole-mount immunohistochemistry and mounting was performed as described previously (62).

**Confocal microscopy imaging and analysis:** Images were acquired using a Leica SP5 confocal microscope. Confocal z-stacks were recorded under the same conditions using diode laser and images were processed under ImageJ environment.

**Startle response in the presence or absence of amisulpride:** Five day old larvae, generated from adult *Slit3* wildtype and homozygous mutant (*Slit3*<sup>sa1569/sa1569</sup>) fish as for quantitative pcr, were individually placed in 24 well plates. In the drug-free condition, each well contained 300µl system water and 0.05% of dimethyl sulfoxide (DMSO, Sigma, Gillingham, UK). In the pharmacological conditions, serial dilutions of the dopaminergic and serotonergic antagonist amisulpride (Tocris, Bristol, UK, 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 (63) and correspond to 50, 100 and 500 times its Ki value for the D2 receptor in mammals (64,65). 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 (66). Larval

movement was recorded using Ethovision XT software (Noldus Information Technology, Wageningen, NL) and data were outputted in one second time-bins.

For each fish, distance travelled (mm) during one second after each tap was recorded. 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 response and habituation to the stimuli, we calculated the percentage of fish exhibiting a response  $\geq 50\%$  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.)).

The percentage of fish responding to stimulus together with amisulpride dose, stimulus number and genotype group were modelled in a beta regression conducted using the R package “betareg”. To determine whether stimulus number, dose or genotype variables are significant, likelihood ratio tests for nested regression models were performed. Results of all statistical analyses were reported with respect to a type-1 error rate of  $\alpha=0.05$ . Post-hoc tests were conducted using Tukey's HSD.

**Real-time quantitative pcr:** Adult *Slit3* wildtype and *Slit3*<sup>sa1569</sup> homozygous mutant fish, generated from a *Slit3*<sup>sa1569/+</sup> heterozygous in cross, were bred to generate homozygous wildtype, heterozygous mutant and homozygous mutant larvae. Embryos were carefully staged at 1, 24 and 48 hour and at five day post fertilisation to ensure, based on morphological criteria, there were no differences in development between groups. mRNA from 3 samples of five day old embryos (n=10 pooled embryos per

sample) for each genotype was isolated using the phenol-chloroform method. cDNA was generated using the ProtoScript® II First Strand cDNA Synthesis Kit (NEB (UK Ltd.), Hitchen, UK). Relative qpcr assays were performed using the LightCycler 480 qpcr system from Roche Diagnostics, Ltd. with all reactions carried out in triplicates. Reference genes for all the qpcr analyses were  $\beta$ -actin, *efl $\alpha$*  and *rpl13 $\alpha$*  based on previous studies (59,67,68). Accession numbers and primer sequences for the genes can be found in Supplementary Table 2.

Relative mRNA expression in qpcr was calculated against reference gene cycle-threshold (Ct) values, and then subjected to one-way ANOVA. To account for multiple testing a Bonferroni correction was applied, and significance was declared at a threshold of 0.001.

**Human Cohorts:** In London human subjects 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 (69–71). The studies were approved by East London and The City Research Ethics Committee 1 (09/H0703/67, 09/H0703/76 and 09/H0703/112). Written informed consent was obtained from all participants.

Details of the Finnish twin cohort are reported elsewhere (72–74). 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) (72), to yield detailed information on smoking behaviour and nicotine dependence as defined by Fagerström Test for Nicotine Dependence (FTND) and DSM-IV diagnoses. Human phenotypes to be investigated in relation to zebrafish nicotine seeking behaviour were determined by consensus *a priori*.

Sample characteristics of the human cohorts and detailed definitions of both London and Finnish phenotypes can be found in the supplementary material and Supplementary Table 3.

**Human genotyping:** For the London cohorts, DNA from participants was extracted from whole blood using the salting-out method (75) 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 analysed with Autocaller software. Pre-developed assays were used to type all SNPs. See Supplementary Table 4 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 (72–74).

**Human association analyses:** London cohort association analysis was performed using PLINK v1.07 (76). SLIT3 SNPs that had been previously associated with disease phenotype 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 ( $\geq 20$  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 analysed using logistic regression controlling for age, sex and cohort. All 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 analyses.

Association analyses for the Finnish Twin Cohort were performed using GEMMA v0.94 (77) with linear mixed model against the allelic dosages controlling for age and sex. Sample relatedness and population stratification were taken into account by using genetic relatedness matrix as random effect of the model.

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## **Competing interests**

The authors of this manuscript certify that they have NO affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript

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## **Supplementary Methods**

**Zebrafish genomic DNA extraction:** Genomic DNA was extracted from fin-clips using QIAGEN DNeasy® Blood and Tissue Kit (Qiagen, Manchester, UK) according to manufacturer's instructions. Samples were eluted into distilled water and stored at -20°C until later use.

**Site Specific Polymerase Chain Reaction:** Allele-specific pcr single nucleotide polymorphism (SNP) assays were used for genotyping F3 individuals for mutations known to be present in the ENU-mutagenized F1 generation. Four primer pairs were designed to carry out pcr genotyping as previously described (61). The list of loss-of-function mutations in the AJBQM1 and AJBQM2 lines is detailed in Supplementary Table 1. For each line, a primer was designed with 3' complementary to the ENU-SNP with a second primer ~100bp downstream. The second pair had one primer with 3' complementary to the wild-type base with a second primer ~200bp upstream. The resulting pcr results in a 300bp fragment that spans the region and acts as an internal control for the pcr plus one 100bp fragment if homozygous for the mutation, 2 bands of 100bp and 200bp if heterozygous, and one 200bp fragment if homozygous wild-type. The 4-primer groups were designed with melting temperatures as close as possible using the NCBI primer design tool and were ordered from Eurofins, MWG operon (Ebersberg, DE).

**Supplementary Table 1:** List of loss-of-function mutations in the AJBQM1 (A) and AJBQM2 (B) lines. List was derived from exome sequencing and provided by the Wellcome Sanger Trust, Hinxton, Cambridge.

**A)**

| SNP Name                   | Allele Number | Location        | Description                                                              |
|----------------------------|---------------|-----------------|--------------------------------------------------------------------------|
| cacna1b (Cacna1b)          | sa1562        | Zv9:5:31016641  | voltage-dependent N-type calcium channel subunit alpha-1B                |
| vcana (VCAN)               | sa1563        | Zv9:5:48057817  | novel protein similar to vertebrate chondroitin sulfate proteoglycan 2   |
| si:ch211-157f15.1 (EVPL)   | sa1564        | Zv9:6:21645941  | envoplakin                                                               |
| mobkl2a (MOBK2A)           | sa1565        | Zv9:8:20954361  | mps one binder kinase activator-like 2A                                  |
| ENSDARG00000068026 (PRKG1) | sa1566        | Zv9:8:53199402  | protein kinase, cGMP-dependent, type I                                   |
| glis3 (GLIS3)              | sa1567        | Zv9:10:663606   | zinc finger protein GLIS3                                                |
| si:dkey-220f10.4 (TULP2)   | sa1568        | Zv9:12:21973687 | novel tub family member protein                                          |
| slit3 (SLIT3)              | sa1569        | Zv9:14:25591202 | slit homolog 3 protein                                                   |
| dchs1 (DCHS1)              | sa1570        | Zv9:15:31441900 | dachsous 1                                                               |
| flad1 (FLAD1)              | sa1571        | Zv9:16:25049338 | Molybdenum cofactor biosynthesis protein-like region FAD synthase region |
| si:ch211-199m3.2 (AKD1)    | sa1572        | Zv9:20:33741430 | adenylate kinase domain containing 1                                     |
| si:dkey-4c23.3 (???)       | sa1573        | Zv9:22:25367694 | novel protein similar to vitellogenin 1 (Vg1)                            |
| magi2 (MAGI2)              | sa1574        | Zv9:25:21478784 | membrane associated guanylate kinase, WW and PDZ domain containing 2     |
| zgc:101050 (TRIMM55)       | sa158         | Zv9:23:17631394 | hypothetical protein LOC445187 (tripartite motif-containing 55)          |

## B)

| SNP Name                    | Allele Number | Location        | Description                                                          |
|-----------------------------|---------------|-----------------|----------------------------------------------------------------------|
| capn3 (CAPN3)               | sa150         | Zv9:17:45493087 | calpain-3                                                            |
| chrna9 (CHRNA9)             | sa975         | Zv9:1:22190803  | cholinergic receptor, nicotinic, alpha 9                             |
| snrnp70 (SNRNP70)           | sa976         | Zv9:3:32068963  | U1 small nuclear ribonucleoprotein 70 kDa                            |
| zgc:158677 (SV2B)           | sa977         | Zv9:7:16060160  | synaptic vesicle protein 2B homolog                                  |
| wu:fa96e12 (AC103686.1)     | sa978         | Zv9:7:44124381  | DNA-dependent protein kinase catalytic subunit                       |
| kctd4 (KCTD4)               | sa980         | Zv9:9:19495015  | potassium channel tetramerisation domain containing 4                |
| LOC557854 (SLC19A3)         | sa981         | Zv9:15:34443534 | solute carrier family 19, member 3                                   |
| tspan3a (TSPAN3)            | sa984         | Zv9:18:26858396 | tetraspanin 3                                                        |
| rapsn (RAPSN)               | sa985         | Zv9:18:20289900 | 43 kDa receptor-associated protein of the synapse                    |
| si:ch211-132b12.1 (SLC6A11) | sa986         | Zv9:18:38859333 | hypothetical protein LOC100034467                                    |
| pkhd1l1 (PKHD1L1)           | sa987         | Zv9:19:23349482 | polycystic kidney and hepatic disease 1 (autosomal recessive)-like 1 |
| klf11a (KLF-11)             | sa988         | Zv9:20:29529553 | kruppel-like factor 11a                                              |

### 30 **Supplementary Table 2.** Gene ID and primer sequences used in gene expression analysis.

| Gene name                   | Transcript ID                                       | Sequence (5' -> 3')                                                 | Fragment size (bp) |
|-----------------------------|-----------------------------------------------------|---------------------------------------------------------------------|--------------------|
| <i>Serotonergic pathway</i> |                                                     |                                                                     |                    |
| <i>Htr1aa</i>               | NM_001123321.1                                      | Forward: TTCTACATCCCGCTCATCCTCA<br>Reverse: CCTCCAAGTTTACCCACCTCTC  | 180                |
| <i>Htr1ab</i>               | NM_001145766.1                                      | Forward: AAACACCGAGGCGAAGAGGAA<br>Reverse: GGCAGCCAACACAGAATGAAAGT  | 99                 |
| <i>Htr2a</i>                | XM_684208.9                                         | Forward: TACGGTGGCTGGGAACATTTTAG<br>Reverse: GGGACACAGTGATGCAGGGAAA | 187                |
| <i>Htr5a</i>                | NM_001126410.2                                      | Forward: TGGATCAAAGAGGACCAACACC<br>Reverse: CTGAAACGTCACCGTGGCAT    | 118                |
| <i>Htr2cl1</i>              | NM_001129893.1                                      | Forward: AACTTCTTCCTCCGCTCACTCG<br>Reverse: ATGGCACACAGGTGCATGATGG  | 179                |
| <i>Htr2cl2</i>              | XM_001339004.7                                      | Forward: CACAACCCACCAACTTCTTCC<br>Reverse: ACGTCCAGAAAGATCCACAGCG   | 152                |
| <i>Htr4</i>                 | XM_021481160.1<br>XM_009291062.3                    | Forward: GTTTCCTTCCAAGCGCCTC<br>Reverse: ACTTCTTCCATCTCAGGCATC      | 168                |
| <i>Htr6</i>                 | XM_009297078.3                                      | Forward: ACTACAGTCATCAGGAGCCACC<br>Reverse: GCCAGGCACTGAAGAATAGTCC  | 147                |
| <i>Htr7</i>                 | XM_003199584.5                                      | Forward: TGGATGTGATGTGCTGTACCGC<br>Reverse: GCCATGCACTTTCCACTCTGTCT | 118                |
| <i>slc6a4/<br/>SERT</i>     | NM_001039972.1                                      | Forward: ACCAGGGGCGAAGCCAAGCA<br>Reverse: GCCACAGGCCCGCTGTTA        | 117                |
| <i>Htr1b</i>                | NM_001128709.1                                      | Forward: CCTTGTCGTCAGTTCTGGGT<br>Reverse: ATCAGAAAGTTCCCGGTGT       | 112                |
| <i>Nicotinic pathway</i>    |                                                     |                                                                     |                    |
| <i>Chrna2</i>               | NC_007128.7                                         | Forward: TGGCTGCAGATCAGTCAAAGAC<br>Reverse: CCCTCTAACTGTCCCTTCACAA  | 271                |
| <i>Chrna3</i>               | Not found in BLAST                                  | Forward: TGTACATCCGCCGATTACCGCT<br>Reverse: TCCGCAGTCGGAGGGCAGTA    | ?                  |
| <i>Chrna4b</i>              | ENSDART00000018614.7                                | Forward: TTACAAGAGGTTTGGGCGCT<br>Reverse: ACAGACCAGTAGATCATCACTCC   | 90                 |
| <i>Chrna5</i>               | NM_001017885.1                                      | Forward: GGCTCCCAGGTCGACATTCTC<br>Reverse: AACCCCGGTTACCAGTGGCCT    | 103                |
| <i>Chrna6</i>               | NM_001042684                                        | Forward: AGGCTCTTTCGTCGTTTATTC<br>Reverse: TCTCAGCCAAAGGTTTGTTC     | 156                |
| <i>Chrna7</i>               | ENSDART00000171463.3<br>and<br>ENSDART00000166391.2 | Forward: ACCGTGTCACATTGTTCACTCTC<br>Reverse: ACAGGTCTCTCCAGTGGGTTA  | 105                |

|                             |                                                       |                                                                      |     |
|-----------------------------|-------------------------------------------------------|----------------------------------------------------------------------|-----|
| <i>Chrb2b</i>               | ENS DART00000041625.7<br>and<br>ENS DART00000185728.1 | Forward: CACAAAGTCACGCTCCGATAC<br>Reverse: CCGTCGCTCTGAGCAGATAA      | 160 |
| <i>Chrb3b</i>               | ENS DARG00000038508.5                                 | Forward: CAGGAGTCAACCTCCGCTTT<br>Reverse: TGAATCTGAACGCACTGGCT       | 106 |
| <i>Chrb4</i>                | NC_007129.7                                           | Forward: ATGTGAATGAATGGCGGTGTGTG<br>Reverse: ATGCGCGTGTGTCAGATTTACCC | 203 |
| <i>Dopaminergic pathway</i> |                                                       |                                                                      |     |
| <i>Drd1b</i>                | NM_001135976.2                                        | Forward: TGGTTCCTTTCTGCAACCCA<br>Reverse: AGTGATGAGTTCGCCCCAACC      | 100 |
| <i>Drd2a</i>                | XM_009291617.3 and<br>XM_005157501.4                  | Forward: TCCACAAAATCAGGAAAAGCGT<br>Reverse: CAGCCAATGTAAACCGGCAA     | 106 |
| <i>Drd3</i>                 | XM_021470111.1 and<br>XM_005162673.4                  | Forward: ATCGAGTTTTCGAGAGCCTT<br>Reverse: TCCACAGTGTCTGAAAGCCG       | 95  |
| <i>Slc6a3</i>               | NM_131755.1                                           | Forward: GCCTGGTTTTACGGAGTGGA<br>Reverse: GGAGGATTGAAGGTGGCGAA       | 66  |
| <i>Adrenergic pathway</i>   |                                                       |                                                                      |     |
| <i>Adra1a</i>               | NM_001324454.1                                        | Forward: AAGAAGGCCGCAAGACTTT<br>Reverse: GTCCGAGGGTCTGTACGTTG        | 114 |
| <i>Adra1d</i>               | XM_691951.6                                           | Forward: AAGCTGCTAAAACCCTCGCC<br>Reverse: GGCTTCAGAGCTGGGAAGAAT      | 103 |
| <i>Adra2b</i>               | NM_207638.1                                           | Forward: AAAAGCCAGGCCTCCAACCTT<br>Reverse: GGGCTTGCAGAAGGTTGTTG      | 92  |
| <i>Adra2c</i>               | NM_207639.1                                           | Forward: CGCCGTTTTAACGAGCAGAG<br>Reverse: AGTGTGGCCACCAGAATGTC       | 87  |
| <i>Adra2da</i>              | NM_194364.2                                           | Forward: CATCATCCTCGTGGTGTCCC<br>Reverse: ATCCCATGATCTCGTTGGCG       | 188 |
| <i>Adra2db</i>              | NM_194365.1                                           | Forward: TGCCACTTTGGTCATTCCGT<br>Reverse: AGCCAGGTAGAAAGCACACC       | 88  |

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**Supplementary Table 3: Sample characteristics of the human cohorts.** Detailed inclusion and exclusion criteria for the London cohorts can be found in <https://clinicaltrials.gov>. Further details about recruitment and definition of medical phenotypes can be found elsewhere (69–71). \*\*Differences in sample size for Finnish cohorts was due to hard-call genotype probability threshold. DSM-IV nicotine dependence symptoms, Fagerström scores and cigarettes smoked each day (N = 1715). Sensation felt after smoking first cigarette (N = 1915). Time to first cigarette in the morning (N= 1726).

| <i>Cohort name</i> | <i>N</i>          | <i>Country</i> | <i>Cohort description</i>                                                                                                                         | <i>ClinicalTrials.gov ID</i> | <i>Mean age (years)</i> | <i>% female</i> | <i>Smoking phenotypes investigated</i>                                                                                 | <i>N heavy smokers</i> |
|--------------------|-------------------|----------------|---------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------|-------------------------|-----------------|------------------------------------------------------------------------------------------------------------------------|------------------------|
| ViDiCO             | 272               | UK             | Subjects with mild, moderate or severe chronic obstructive pulmonary disease (COPD) treated with the same bi-monthly 3mg vitamin D3 intervention. | NCT00977873                  | 64.6                    | 40              | Tobacco consumption; smoking cessation (current vs ever smokers)                                                       | 249                    |
| ViDiAs             | 293               | UK             | Adult patients with asthma treated with inhaled corticosteroids treated with a bi-monthly 3mg vitamin D3 intervention                             | NCT00978315                  | 47                      | 56              | Tobacco consumption; smoking cessation (current vs ever smokers)                                                       | 17                     |
| ViDiFLU            | 298               | UK             | Adults in sheltered accommodation given 10 mcg vitamin D3 daily as well as bi-monthly 3mg vitamin D3 interventions                                | NCT01069874                  | 66.8                    | 66              | Tobacco consumption; smoking cessation (current vs ever smokers)                                                       | 66                     |
| Finnish Twins      | 1915, 1715, 1726* | Finland        | Study sample ascertained from the Finnish Twin Cohort study (N=35834 adult twins) concordant for moderate to heavy smoking                        | NA                           | 55                      | 48              | DSM-IV nicotine dependence symptoms; Fagerström scores; cigarettes smoked each day; sensation felt after smoking first | NA                     |

cigarette and  
time to first  
cigarette in the  
morning

**Phenotype definitions for the London cohorts:** *Amount smoked* was defined as the average number of cigarettes smoked per day (CPD) for each participant. Participants met criteria for *smoking cessation* if they reported being ‘ever smokers’ and reported **not** smoking currently. The percentage of current smokers in the cohort was 42%, 7% and 18% for ViDiCO, ViDiAs and ViDiFLU, respectively.

**Phenotype definition for the Finnish twin cohort study:** Definitions of the phenotypes were adapted from Broms et al (74).

#### *Amount smoked*

Cigarettes per day (CPD) constitutes of eight categories: 1-2, 3-5, 6-10, 11-15, 16-19, 20-25, 26-39,  $\geq 40$  CPD. In the statistical analyses of the CPD variables, original categorical observations were replaced with class means of CPD (1.5, 3.5, 8, 13, 17.5, 22.5, 32.5, and 45 cigarettes per day, respectively). Regression coefficients can therefore be interpreted as the average change in number of cigarettes smoked per day when the number of minor allele is increased by one.

- **CPD:** Number of cigarettes smoked per day during month of heaviest smoking. Values ranged from 1 to  $>40$  with mean=19.8 cigarettes per day.
- **Maximum CPD:** Maximum number of cigarettes ever smoked during one day (24h period). Values ranged from 2 to 98 with mean=30 cigarettes per day.

#### *Smoking initiation*

- ***Age of onset of weekly smoking:*** Age (years) when started to smoke weekly (“How old were you when you first smoked a cigarette at least once a week for at least two months in a row?”). Values ranged from 6 to 54, mean=17.3 years.
- ***First time sensation.*** Sensation felt after smoking the first cigarette or first puffs. Sensation measured as: “While smoking your very first cigarettes, did you (1) like the taste or smell of the cigarette, (2) cough, (3) feel dizzy or light-headed, (4) feel more relaxed, (5) get a headache, (6) feel a pleasurable rush or buzz, (7) feel your heart racing, (8) feel nauseated, like vomiting, (9) feel your muscles tremble or become jittery, (10) feel burning in your throat”. Sum score of 10 questions (items #1, #4, and #6 were reverse-scored before summation): 0 points if answered “No”, 1 = “A little bit”, 2=“Some”, 3= “Quite a bit”, 4=“A great deal”. Cronbach’s alpha = 0.70. Values ranged from 3.6 to 15.8. Mean =10.2.

#### Nicotine dependence

- ***DSM-IV ND diagnosis:*** Nicotine dependence by DSM-IV diagnosis ( $\geq 3$  symptoms out of 7 occurring within a year). Prevalence = 53.5%.
- ***DSM-IV ND symptoms:*** Number of DSM-IV ND symptoms from 0 to 7. Mean=3
- ***FTND ( $\geq 4$ ):*** Nicotine dependent if  $\geq 4$  out of 10 points in Fagerström Test for Nicotine Dependence. Prevalence = 50.4%
- ***FTND score:*** Fagerström Test for Nicotine Dependence (FTND) score: 0 to 10 points. Mean=3.7.
- ***FTND time to first cigarette (TTF):*** Time to first cigarette in the morning (one item of the FTND scale). Five categories: 0-5 min, 6-15 min, 16-30 min, 31-60 min, >60 min. Categorization differs from original four categories (3), i.e., 6-30 minutes is split into 6-15 min and 16-30 min. In our data set 46% of smokers belong to the group

87 of 6-30 min, and from the smoking behaviour point of view there is a significant  
 88 difference whether one smokes the first cigarette within 6 minutes or 30 minutes from  
 89 waking up. In this data set 22% of smokers belong to the 6-15 min and 24% to the 16-  
 90 30 min group. Values ranged from 1 to 5 with a mean=3.1.

1 **Supplementary Table 4.** Primer and reporter sequences used for human genotyping.

| GENE          | SNP       | Sequence name | Sequence                                 |
|---------------|-----------|---------------|------------------------------------------|
| <i>CYP3A4</i> | rs2740574 | Forward       | CCAGGCATAGGTAAAGATCTGTAGGT               |
|               |           | Reverse       | CTCAAGTGGAGCCATTGGCATA                   |
|               |           | Reporters     | ACAAGGGCAAGAGAG and<br>ACAAGGGCAGGAGAG   |
|               |           |               |                                          |
| <i>CUBN</i>   | rs3740165 | Forward       | GCAATGAGATTAAATCTTCAGGAAACACA            |
|               |           | Reverse       | CTGGAGGTATAGGAAGCAGTGAAG                 |
|               |           | Reporters     | CCGCCATATGGCCTG and CGCCATACGGCCTG       |
|               |           |               |                                          |
| <i>RXRA</i>   | rs7861779 | Forward       | TGGCCCATGCACGAGTAG                       |
|               |           | Reverse       | ACCGAGACAGGCCAAACTC                      |
|               |           | Reporters     | CAGCAGAGGTGGCCGA and<br>CAGCAGAGATGGCCGA |
|               |           |               |                                          |

2

3

## Supplementary results

### Supplementary Table 5: Site specific pcr genotyping of AJBQM1 (A) and AJBQM2 (B)

**outlier siblings.** The siblings were genotyped at each of the candidate loci using site specific pcr and results compared with each individual place preference change scores. P-values result from independent two-sample t-tests comparing preference change scores between wildtype and subjects with a copy of mutant allele at each locus.

(A)

| Gene name      | CPP Change Score |      |      |      |      |      |      |      |      |     | P-value                 |
|----------------|------------------|------|------|------|------|------|------|------|------|-----|-------------------------|
|                | 0                | 0.01 | 0.07 | 0.15 | 0.32 | 0.43 | 0.44 | 0.47 | 0.51 | 0.6 |                         |
| <i>Slit3</i>   | WT               | WT   | WT   | WT   | HET  | HET  | HET  | HET  | HET  | HET | 7.6592x10 <sup>-5</sup> |
| <i>Cacne</i>   | WT               | HET  | WT   | WT   | HOM  | HET  | WT   | WT   | WT   | WT  | 0.691                   |
| <i>Vcan</i>    | HET              | HET  | WT   | HET  | HOM  | WT   | HET  | HET  | WT   | WT  | 0.259                   |
| <i>Evpl</i>    | WT               | WT   | WT   | WT   | WT   | WT   | WT   | WT   | WT   | WT  | -                       |
| <i>Mob3a</i>   | HET              | HET  | HET  | HOM  | HOM  | HET  | WT   | HET  | WT   | HET | 0.236                   |
| <i>Prkg1</i>   | HET              | HET  | HET  | HET  | HET  | HET  | HET  | HET  | HOM  | HOM | -                       |
| <i>Glis3</i>   | WT               | HET  | HET  | HET  | WT   | WT   | WT   | HET  | WT   | HET | 0.602                   |
| <i>Tulp2</i>   | HOM              | HOM  | WT   | HET  | WT   | HET  | HET  | HET  | WT   | WT  | 0.481                   |
| <i>Dchs1</i>   | WT               | WT   | WT   | WT   | WT   | WT   | WT   | WT   | WT   | WT  | -                       |
| <i>Flad1</i>   | HOM              | HOM  | WT   | HET  | WT   | WT   | WT   | HET  | HET  | HET | 0.981                   |
| <i>Akd1</i>    | HOM              | HOM  | WT   | HET  | WT   | HET  | HET  | HET  | WT   | WT  | 0.418                   |
| <i>MagI2</i>   | HET              | WT   | HET  | WT   | HET  | HOM  | WT   | WT   | HOM  | HET | 0.73                    |
| <i>Trimm55</i> | WT               | HET  | WT   | HET  | WT   | WT   | WT   | WT   | HET  | WT  | 0.51                    |

13 (B)

| Gene name         | CPP Change Score |       |       |       |       |       |       |       |       |       |       |       |      |      | P-value |
|-------------------|------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|------|------|---------|
|                   | -0.38            | -0.28 | -0.27 | -0.23 | -0.21 | -0.18 | -0.17 | -0.17 | -0.12 | -0.09 | -0.09 | -0.07 | 0.04 | 0.07 |         |
| <i>Tspan3a</i>    | HET              | HET   | HET   | WT    | WT    | WT    | HET   | HET   | WT    | WT    | WT    | HET   | HET  | HET  | 0.583   |
| <i>Raspn</i>      | WT               | HOM   | WT    | WT    | WT    | HOM   | HOM   | HOM   | WT    | WT    | WT    | HOM   | WT   | HOM  | 0.792   |
| <i>A9</i>         | WT               | WT    | HET   | WT    | WT    | WT    | HET   | WT    | HET   | HET   | HET   | HET   | HET  | HET  | 0.339   |
| <i>Capn3</i>      | HET              | HET   | HET   | WT    | HET   | WT    | WT    | HET   | WT    | HET   | WT    | WT    | WT   | WT   | 0.911   |
| <i>Klf11a</i>     | WT               | WT    | WT    | HET   | WT    | WT    | WT    | HET   | WT    | WT    | HET   | WT    | HET  | WT   | 0.318   |
| <i>Kctd4</i>      | HET              | WT    | WT    | HET   | WT    | WT    | HET   | WT    | HET   | HET   | HET   | HET   | WT   | WT   | 0.252   |
| <i>Slc6a11</i>    | HET              | HET   | HET   | WT    | HET   | WT    | HET   | HET   | WT    | WT    | WT    | WT    | HET  | WT   | 0.697   |
| <i>Pkhd1l1</i>    | WT               | WT    | WT    | HET   | HOM   | HOM   | HET   | WT    | WT    | WT    | WT    | WT    | WT   | HOM  | 0.499   |
| <i>Slc19a3</i>    | WT               | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT   | WT   | -       |
| <i>Sv2b</i>       | WT               | WT    | HET   | WT    | WT    | WT    | WT    | HET   | HOM   | HET   | HOM   | HOM   | HET  | HOM  | 0.269   |
| <i>Snrnp70</i>    | WT               | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT   | WT   | -       |
| <i>Ac10103686</i> | WT               | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT    | WT   | WT   | -       |

14

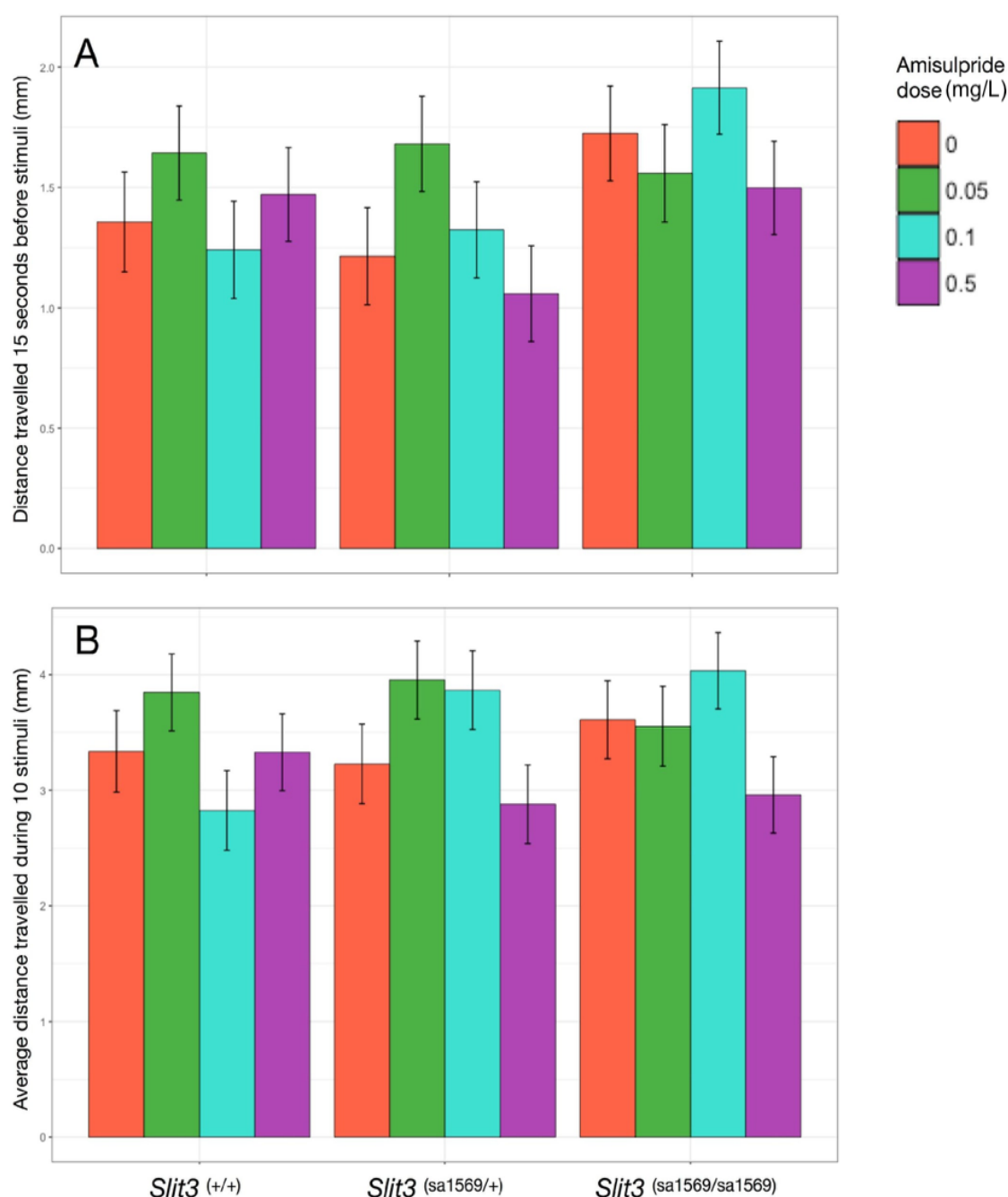
15

**Supplementary Table 6:** Results of association analysis of *SLIT3* SNPs on **smoking initiation**. Logistic regression of initiation vs non-initiation on additive genotype, controlling for age, sex and cohort. OR: Odds ratio. >1 value indicates that the minor allele increases odds of persistent smoking relative to the major allele, SE: standard error, L95: lower limit of 95% confidence interval, U95: upper limit of 95% confidence interval. Benjamini Hochberg cut off at 0.1 = 0.00526.

| SNP        | OR     | SE     | L95    | U95    | P value |
|------------|--------|--------|--------|--------|---------|
| rs2938774  | 0.7253 | 0.1418 | 0.5493 | 0.9578 | 0.02357 |
| rs11742567 | 1.328  | 0.1538 | 0.9825 | 1.796  | 0.06496 |
| rs4282339  | 0.7277 | 0.1815 | 0.5099 | 1.039  | 0.07991 |
| rs297886   | 1.328  | 0.176  | 0.9405 | 1.875  | 0.1071  |
| rs9688032  | 1.269  | 0.1495 | 0.9467 | 1.701  | 0.111   |
| rs7728604  | 1.198  | 0.1446 | 0.9024 | 1.591  | 0.2112  |
| rs1345588  | 0.7788 | 0.2046 | 0.5215 | 1.163  | 0.2218  |
| rs17734503 | 0.7362 | 0.2632 | 0.4395 | 1.233  | 0.2445  |
| rs12515725 | 0.8612 | 0.1458 | 0.6471 | 1.146  | 0.3052  |
| rs11749001 | 0.8698 | 0.1951 | 0.5933 | 1.275  | 0.4746  |
| rs3733975  | 1.116  | 0.1641 | 0.8092 | 1.54   | 0.5029  |
| rs12521041 | 1.116  | 0.1641 | 0.8092 | 1.54   | 0.5029  |
| rs11134527 | 0.9212 | 0.155  | 0.6798 | 1.248  | 0.5964  |
| rs12654448 | 0.8718 | 0.2654 | 0.5182 | 1.467  | 0.6052  |
| rs1559051  | 0.9257 | 0.1575 | 0.6799 | 1.26   | 0.6241  |
| rs17665158 | 0.9304 | 0.171  | 0.6654 | 1.301  | 0.673   |
| rs1421763  | 0.9423 | 0.1704 | 0.6748 | 1.316  | 0.7271  |
| rs295994   | 0.9732 | 0.1404 | 0.7391 | 1.281  | 0.8464  |
| rs10036727 | 1.01   | 0.1522 | 0.7491 | 1.361  | 0.9503  |

**Supplementary Table 7:** Results of association analysis of *SLIT3* SNPs on **persistent smoking**. Logistic regression of initiation vs non-initiation on additive genotype, controlling for age, sex and cohort. OR: Odds ratio. >1 value indicates that the minor allele increases odds of persistent smoking relative to the major allele, SE: standard error, L95: lower limit of 95% confidence interval, U95: upper limit of 95% confidence interval. Benjamini Hochberg cut off at 0.1 = 0.00526.

| SNP        | OR     | SE     | L95    | U95    | P value |
|------------|--------|--------|--------|--------|---------|
| rs11134527 | 1.428  | 0.1573 | 1.049  | 1.943  | 0.02359 |
| rs12521041 | 0.6871 | 0.1736 | 0.489  | 0.9655 | 0.03061 |
| rs11742567 | 0.7288 | 0.1547 | 0.5382 | 0.987  | 0.04089 |
| rs3733975  | 0.7165 | 0.1712 | 0.5123 | 1.002  | 0.05146 |
| rs17734503 | 0.6142 | 0.2631 | 0.3667 | 1.029  | 0.06398 |
| rs1345588  | 0.6786 | 0.2145 | 0.4457 | 1.033  | 0.07068 |
| rs17665158 | 1.338  | 0.163  | 0.9722 | 1.842  | 0.07393 |
| rs12654448 | 0.6225 | 0.2671 | 0.3688 | 1.051  | 0.07597 |
| rs2938774  | 1.232  | 0.1394 | 0.9373 | 1.619  | 0.1348  |
| rs295994   | 1.214  | 0.1443 | 0.9147 | 1.61   | 0.1796  |
| rs7728604  | 1.152  | 0.1434 | 0.8699 | 1.526  | 0.3232  |
| rs1559051  | 1.115  | 0.1549 | 0.8231 | 1.511  | 0.4821  |
| rs12515725 | 0.9108 | 0.1456 | 0.6846 | 1.212  | 0.521   |
| rs297886   | 0.9342 | 0.1726 | 0.6661 | 1.31   | 0.6932  |
| rs4282339  | 0.9391 | 0.19   | 0.6471 | 1.363  | 0.7411  |
| rs10036727 | 0.9596 | 0.1516 | 0.713  | 1.292  | 0.7857  |
| rs1421763  | 1.029  | 0.1671 | 0.7417 | 1.428  | 0.8633  |
| rs9688032  | 1.013  | 0.1511 | 0.7531 | 1.362  | 0.9328  |
| rs11749001 | 0.9943 | 0.1969 | 0.676  | 1.463  | 0.977   |



33

34 **Supplementary Figure 1.** Average distance moved before (Figure 1A) and during startle

35 stimuli (Figure 1B) in wildtype and *Slit3*<sup>sa1569</sup> mutant five day old zebrafish larvae. **A)**

36 Distance moved as function of amisulpride dose, fish *Slit3* genotype and their interaction.

37 The effect of dose and genotype was tested in a linear mixed model. Timepoint, well where

38 the fish were placed and plate were also included as fixed factors and the fish ID as random

39 factor. **B)** Distance moved during taps as function of amisulpride dose and fish *Slit3*

40 genotype. Drug and genotype effects were examined in a linear mixed model including  
 41 stimulus number, well, plate used and distance moved before stimuli as fixed factors and Fish  
 42 ID as random factor. Zebrafish larvae did not differ in the distance travelled before or during  
 43 startle stimuli as a function of amisulpride dose nor genotype ( $p > 0.05$ ). Bars represent  
 44 estimated marginal means  $\pm$  SEM (n=42-48 fish per group).  
 45

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