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.

INTRODUCTION

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

As approaches to identify genetic risk are difficult in humans, research has been facilitated by studies in animal models, with a focus on genomic analysis of inbred and selectively-bred, naturally occurring genetic strains (8). This type of study produces quantitative trait loci (QTL) maps of multiple loci, each with a small impact on the phenotype. However, as with human studies, it is inherently difficult to identify relevant genes from QTL maps, as the overall phenotype cannot be predicted by individual genotypes. Mutagenesis studies in animal model systems can overcome these limitations: e.g. N-ethyl-N-nitrosourea (ENU) mutagenesis introduces thousands of point mutations into the genome with the potential to generate much stronger phenotypes than those occurring in a natural population thereby facilitating identification of causal mutations. Examination of phenotypic variation in ENU 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 (α2, α3, α4, α6, α7, α8, β2, β3, β4) (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.

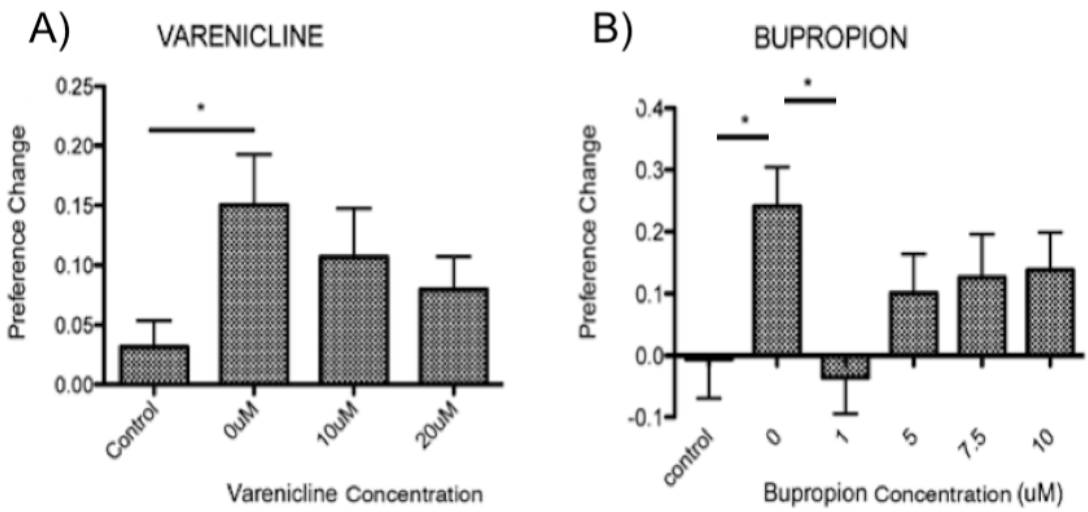
49 To evaluate the use of a behavioural CPP screen in zebrafish to predict loci affecting
50 human smoking behaviour we initially assessed 1) the ability of varenicline and
51 bupropion, pharmacological agents used to treat human nicotine addiction, to reduce
52 zebrafish nicotine-induced place preference and 2) the heritability of nicotine
53 responses in ENU-mutagenized fish. We then screened 30 families of ENU-
54 mutagenized fish to identify families with increased/decreased CPP for nicotine. For
55 two families with altered CPP response, the phenotype was confirmed following
56 independent replication with a larger number of fish. Exome sequence information
57 was used to generate a list of coding, loss of function candidate mutations affecting
58 the phenotype. One family with a mutation co-segregating with increased nicotine
59 CPP was selected for further study. Firstly, the effect of the identified gene on
60 nicotine-induced CPP was confirmed using an independent line carrying a similar
61 predicted loss of function mutation in the same gene. We then characterized the
62 mutation using gene expression analysis, immunohistochemical analysis of neuronal
63 pathways and behavioural responses to acoustic startle; a response known to be
64 modulated by serotonergic and dopaminergic signalling and, in humans, associated
65 with vulnerability to addiction (22–24). Finally, we used focused SNP analysis of
66 human cohorts to assess the predictive validity of findings in fish for human smoking
67 behaviour.
68
69 In agreement with previous studies zebrafish showed a robust CPP to nicotine.
70 Nicotine-induced CPP was abolished by varenicline and bupropion and found to be
71 heritable in fish. The screening of ENU mutagenized families identified mutations in
72 the *Slit3* gene influencing sensitivity to rewarding effects of nicotine. *Slit3* mutant
73 larvae and adult fish showed altered behavioural sensitivity to amisulpride and larvae

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

81 **RESULTS**

82 **Nicotine CPP in zebrafish is inhibited by varenicline and buproprion**

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), 10μM 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. 10μM 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

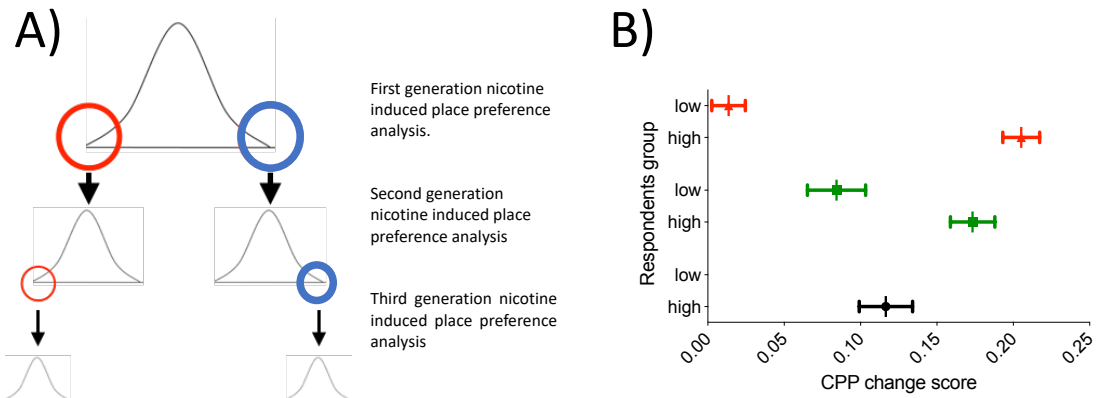
97 nicotine antagonist properties when metabolised). Bars represent mean and error bars
98 represent +SEM. Asterics (*) represents significance at $p<0.05$.

99 **Nicotine CPP is heritable in zebrafish**

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

110

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



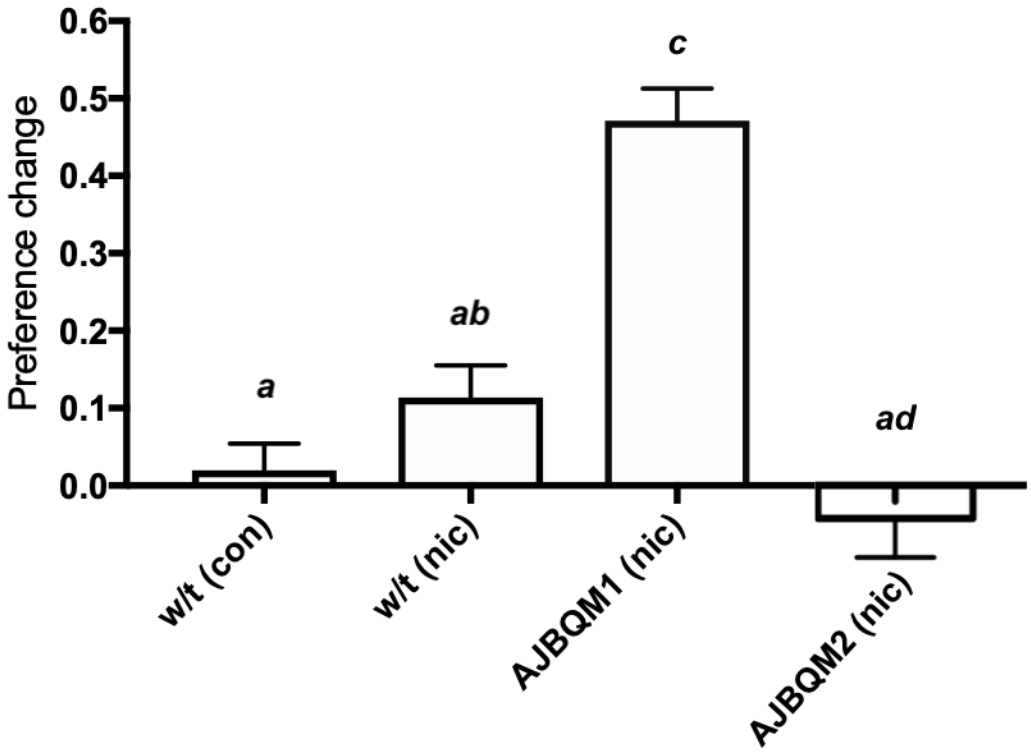
118

119 **Figure 2. A) Breeding and selection to assess heritability of nicotine-induced**
120 **place preference in ENU-mutagenized zebrafish.** To test whether nicotine
121 preference is heritable, fish in the upper and lower 10% of the change in preference
122 distribution curve were inbred and screened for CPP (Second generation CPP assay).
123 A similar approach was used for the third generation CPP assay. **B) CPP for nicotine**
124 **is heritable**. Mean preference change is increasingly distinct for the second and third
125 generation of CPP assay. Plot represents mean and ±SEM. First generation
126 (corresponding to the F_3 families used for the screen) (n=120): mean=0.11; SD=0.17.
127 Second generation: Offspring of fish from *upper* 10% of the first generation screen
128 (n=92): mean=0.17; SD=0.14. Offspring of fish from *lower* 10% of the first
129 generation screen (n=64): mean=0.08; SD=0.15. Third generation. Offspring of fish
130 from *upper* 10% of the second generation screen (n=69): mean=0.21; SD=0.10.
131 Offspring of fish from *lower* 10% of the second generation screen (n=67):
132 mean=0.01; SD=0.09.

133 **Identification of Slit3 mutations affecting nicotine place preference in zebrafish**

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

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



144

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

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

153 Of the 25 coding, predicted loss of function mutations in AJBQM1 and AJBQM2
154 (Listed in Supplementary Table 1), only *Slit3*$^{sa1569/+}$ (exon 7 splice acceptor site
155 disruption at amino acid position 176), segregated with nicotine preference (Figure
156 4A & Supplementary Table 5A). None of the coding, predicted loss of function
157 mutations in AJBQM2 segregated with nicotine preference and this line was not
158 examined further (Supplementary Table 5B).

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

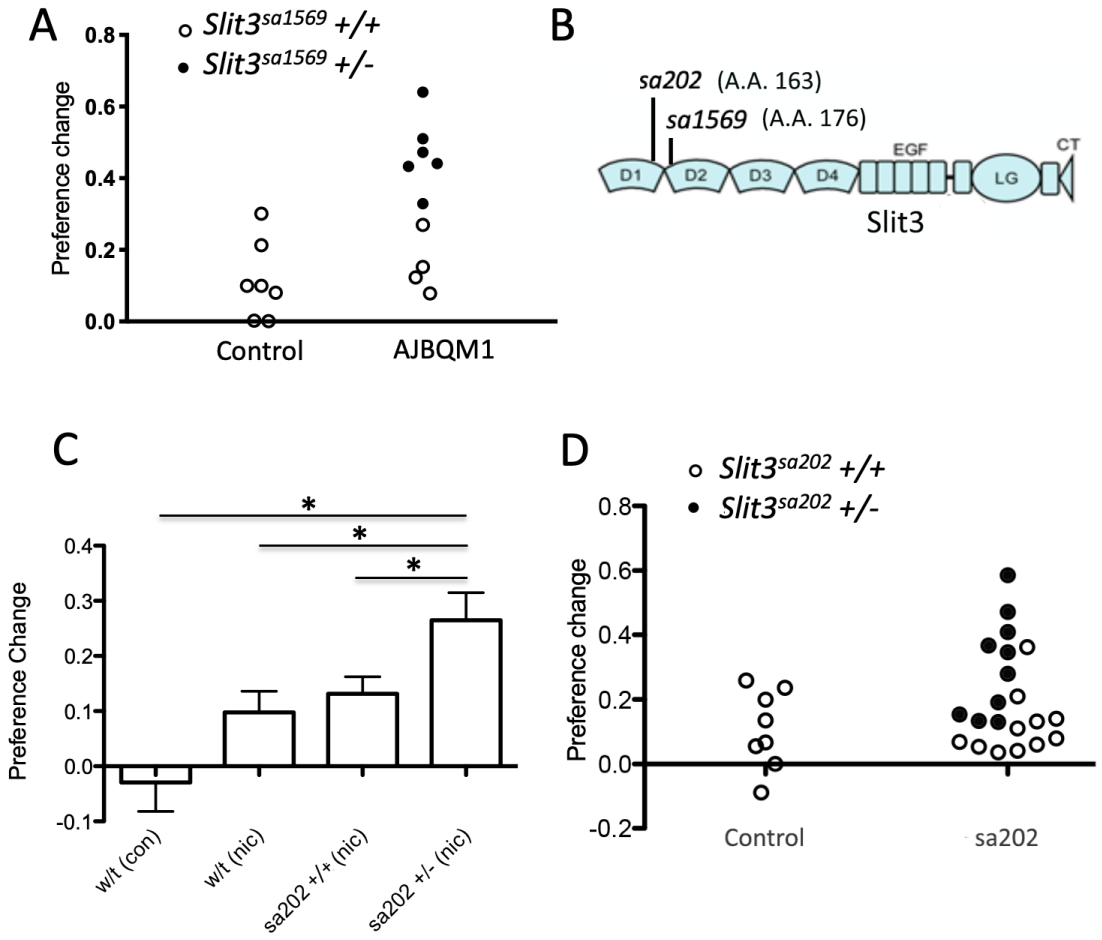
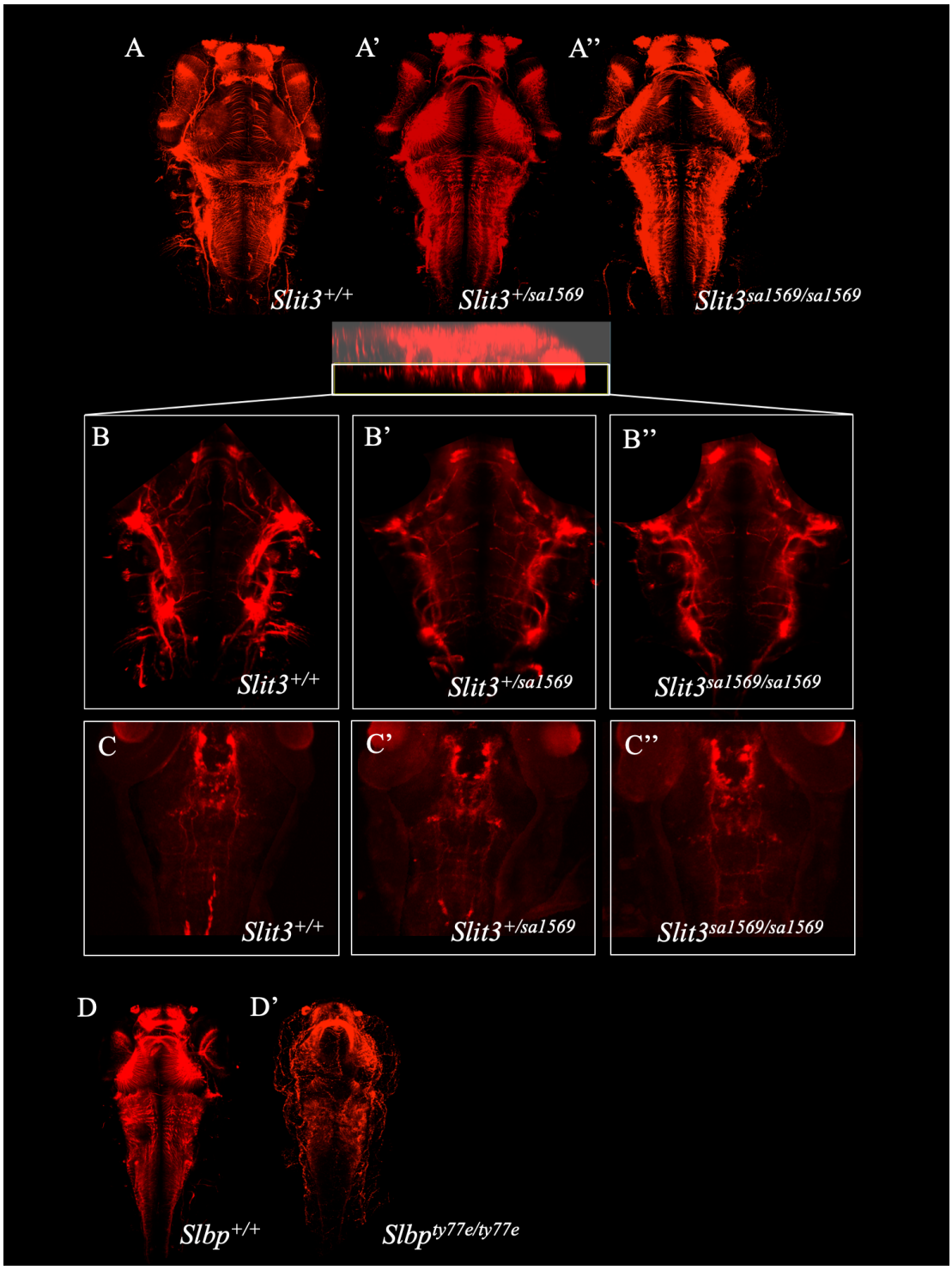


Figure 4. **A. Segregation of *Slit3*^{sa1569} mutation with nicotine seeking.** CPP change scores for individual un-mutagenized TLF wildtype fish (n=7) and AJBQM1 fish (n=10). Following CPP analysis, fish were genotyped for 25 loss of function mutations contained within the family. Black dots indicate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

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

187 **Characterisation of *Slit3*^{sa1569} mutants**

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



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

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

210 As subtle effects on circuit formation may not have been detected by our antibody
211 staining, we examined the response and habituation to acoustic startle stimuli in
212 wildtype and mutant fish. Habituation to acoustic startle is known to be sensitive to
213 dopaminergic/serotonergic antagonists such as amisulpride (34) and, in humans, is
214 associated with vulnerability to addiction (22–24). Five day old larvae were subjected
215 to 10 sound/vibration stimuli over a total of 20 seconds (2 second interval between
216 each stimulus) in the presence of 0, 0.05 mg/L, 0.1 mg/L or 0.5 mg/L amisulpride in
217 0.05% dimethyl sulfoxide (DMSO). The distance travelled one second after each
218 stimulus was recorded for each fish.

219 Response and habituation to the stimuli was quantified as the percentage of fish
220 moving more than 2.5 mm, which corresponds to 50% of the mean distance travelled
221 one second after the first startle (Figure 6A). In line with the habituation response
222 paradigm (35), a lower percentage of fish responded as the number of stimuli
223 increased (Figure 6B). In wildtype fish higher doses of amisulpride caused a decrease
224 in responsiveness (Main effect of 0.1 and 0.5 mg/L amisulpride across the ten taps
225 $p<0.05$) (Figure 6C, red line). However *Slit3*^{sa1569} mutants showed an inverted U-
226 shaped response to amisulpride: a reduction of habituation at low doses and increase
227 at the higher dose (Figure 6C, blue and orange lines). The presence of a *Slit3*^{sa1569}

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.

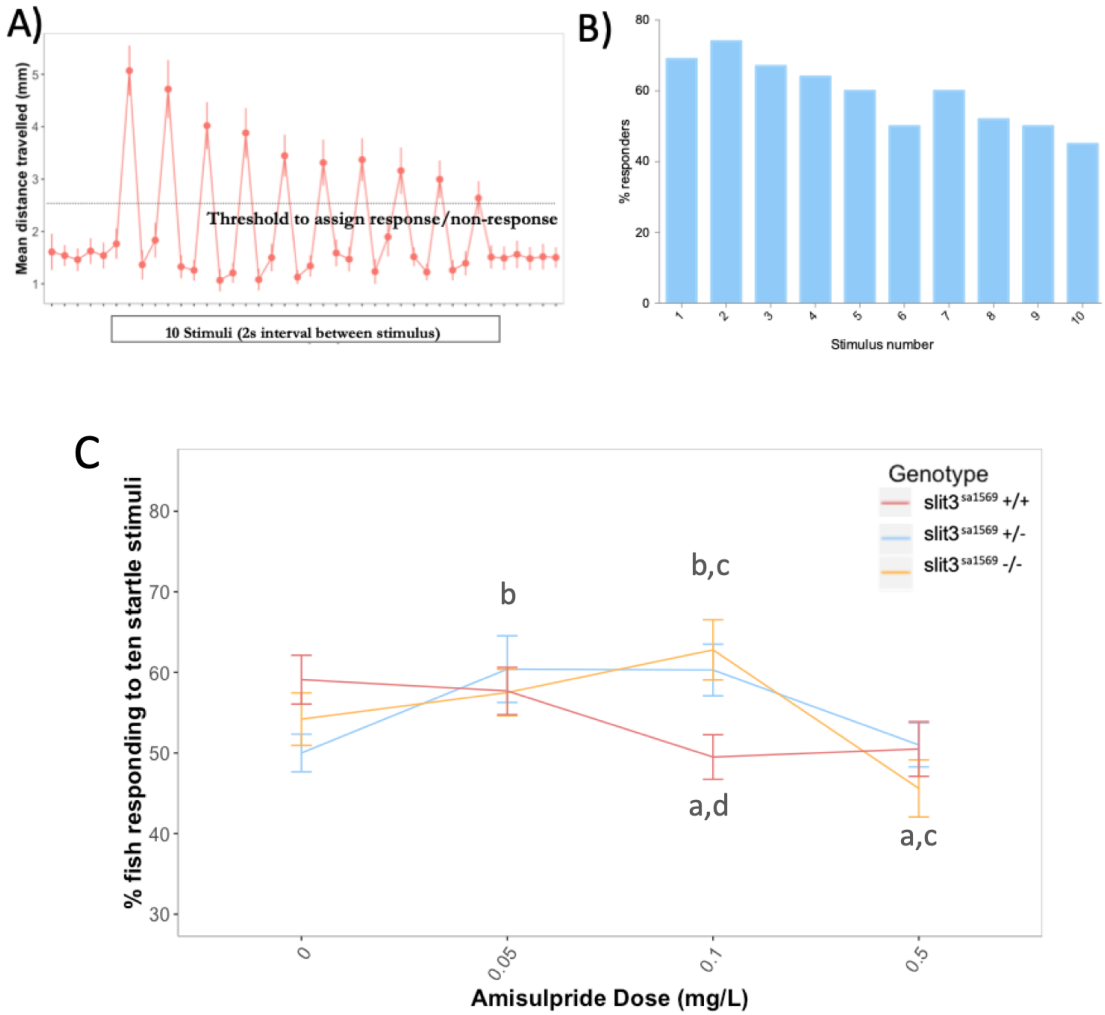
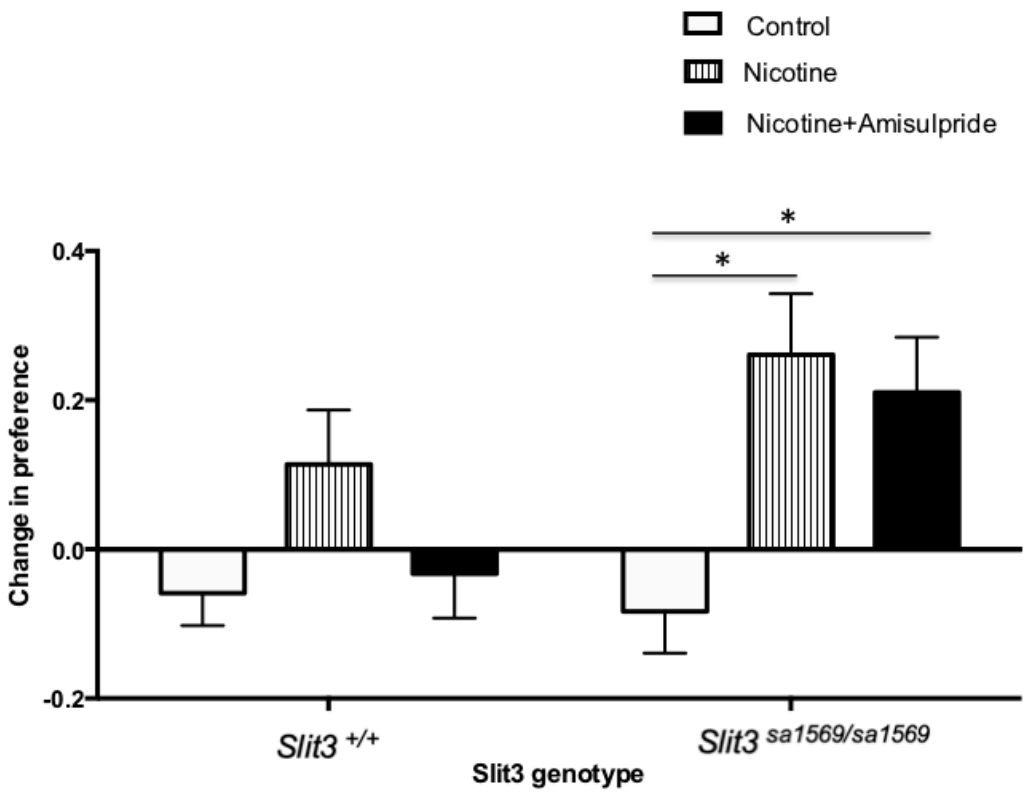


Figure 6. Habituation response in the presence and absence of amisulpride. A: Response and habituation to 10 stimuli with two seconds interval between stimuli

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

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



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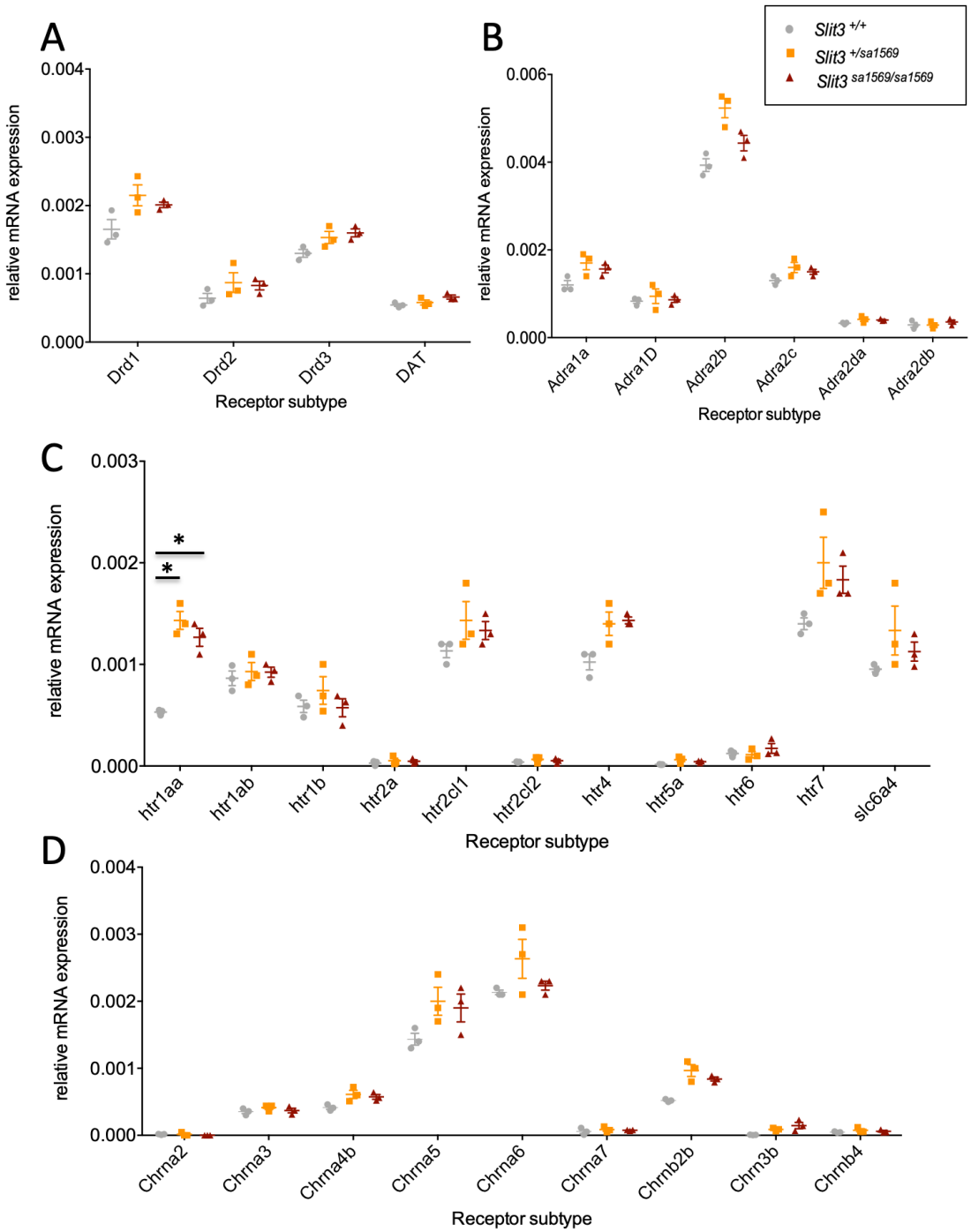
258 **Figure 7. CPP induced by 5μM nicotine is blocked by 0.5mg/L**
259 **dopamine/serotonin antagonist amisulpride in wildtype *Slit3*$^{sa1569+/+}$ fish but not**
260 **in *Slit3*^{sa1569} homozygous mutants.** Bars represent mean (+SEM). (n=11-14 fish per
261 group). *Two-way ANOVA followed by post-hoc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

262

263 As *Slit3*^{sa1569} homozygous mutant fish showed altered sensitivity to nicotine and
264 amisulpride, we examined whether expression of dopamine, serotonin, adrenergic or
265 nicotinic receptor mRNA was dis-regulated in *Slit3*^{sa1569} mutant larvae using
266 quantitative real-time pcr (qpcr). Only *Htr1aa* ([F(2,6)=44], p=0.0003) showed a
267 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*$^{sa1569+/+}$,**

270 ***Slit3*$^{sa1569/+}$ heterozygous and *Slit3*$^{sa1569/sa1569}$ 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

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

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

276 We next examined associations between 19 single nucleotide polymorphisms (SNPs)
277 in the human *SLIT3* gene and smoking behaviour in two London cohorts. Two SNPs,
278 rs12654448 and rs17734503 in high linkage disequilibrium (Figure 9) were associated
279 with level of cigarette consumption (p=0.00125 and p=0.00227). We repeated the
280 analysis on heavy smokers: rs12654448 (p=0.0003397) and rs17734503
281 (p=0.0008575) were again associated with cigarette consumption together with
282 rs11742567 (p=0.004715). The SNP rs11742567 was associated with cigarette
283 consumption in light smokers (<20 cigarettes per day, p=0.003909)) and with
284 quitting. Associations are reported in Table 1. No other SLIT3 polymorphisms were
285 associated with smoking initiation, persistent smoking or cessation (Supplementary
286 Tables 6 & 7).

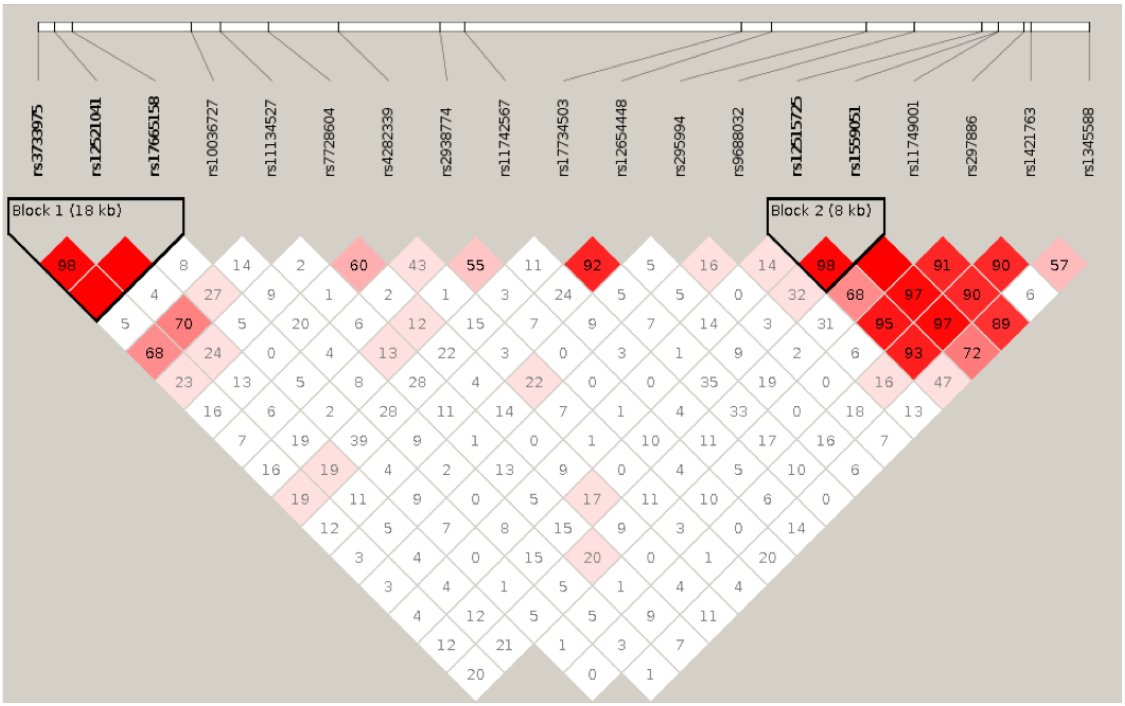


Figure 9: Linkage disequilibrium (LD) plot of *SLIT3* SNPs in human smoking association analysis. Numbers within each square indicate D' values (white: D' < 1, LOD < 2; blue: D' = 1, LOD < 2; pink: D' < 1, LOD ≥ 2; and bright red: D' = 1, LOD ≥ 2.)

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. β 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 (≥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

	Tobacco consumption				*Tobacco consumption - heavy smokers (≥20 cigs/day)*				*Tobacco consumption - light smokers (<20 cigs/day)*				*Smoking cessation*				
SNP	**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
rs11742567	0.135	-1.166	0.779	(-2.691, 0.361)	**0.005**	**-2.346**	**0.825**	**(-3.962, -0.730)**	**0.004**	**1.888**	**0.644**	**(0.6258, 3.151)**	**1.586**	**0.163**	**1.153**	**2.183**	**0.005**
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
rs12654448	**0.001**	**-4.241**	**1.307**	**(-6.803, -1.680)**	**0.0003**	**-4.830**	**1.334**	**(-7.444, -2.216)**	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
rs17734503	**0.002**	**-3.987**	**1.299**	**(-6.534, -1.441)**	**0.001**	**-4.458**	**1.325**	**(-7.055, -1.861)**	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

302 We subsequently investigated associations with more detailed smoking phenotypes in
303 the Finnish twins cohort (36) (Table 2). Associations were observed between
304 rs17734503 and DSM-IV nicotine dependence symptoms (p=0.0322) and age at onset
305 of weekly smoking (p=0.00116) and between rs12654448 and age at onset of weekly
306 smoking (p=0.00105). Associations were seen elsewhere between *SLIT3* markers and
307 Fagerström Test for Nicotine Dependence (FTND), cigarettes smoked each day,
308 sensation felt after smoking first cigarette and time to first cigarette in the morning. In
309 keeping with the London studies the minor allele was associated with a lower degree
310 of dependence and decreased cigarette consumption.

311 The SNPs rs12654448 and rs17734503 are in non-coding domains, therefore it was
312 not possible to predict loss or gain of function of SLIT3 from the SNP location. No
313 evidence of affecting gene expression was found as per GTEx database
314 (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.

	DSM-IV ND diagnosis			DSM-IV ND symptoms			FTND (≥4)			FTND score		
SNP	**β**	**SE**	**P value**	**β**	**SE**	**P value**	**β**	**SE**	**P value**	**β**	**SE**	**P value**
rs12654448	-0.0343	0.0262	0.190975	-0.1839	0.0964	0.056728	0.0526	0.0287	0.066509	0.075	0.1365	0.58286
rs17734503	-0.0354	0.0259	0.171821	-0.2044	0.0954	**0.032199**	0.0474	0.0283	0.094383	0.0443	0.135	0.743052
rs11742567	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

	CPD			max CPD			Age of onset of weekly smoking			First time sensation			FTND time to first cigarette		
SNP	**β**	**SE**	**P value**	**β**	**SE**	**P value**	**β**	**SE**	**P value**	**β**	**SE**	**P value**	**β**	**SE**	**P value**
rs12654448	-0.3509	0.5669	0.536029	-1.0602	0.7743	0.171106	0.7826	0.2384	**0.001051**	-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	**0.001156**	-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

320 **DISCUSSION**

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

331 We screened 30 ENU-mutagenized zebrafish families followed by sibling re-screen to
332 identify families of fish showing altered nicotine preference. This proof of principle
333 study indicates the relevance of zebrafish for human studies and emphasizes the
334 advantage of first using a screen with a low number of individuals per family to
335 increase efficiency. The classic three generation forward genetic screen examines
336 phenotypes in groups of 20 or more individuals from each family (37). Logistical
337 considerations make it difficult to apply such an approach to adult behavioural
338 screens. Our approach increases efficiency by initially screening a small number of
339 individuals from a large number of families and only selecting those families that
340 occur at the extremes of the distribution for further analysis. Although in this study
341 we were able to confirm phenotypes using a relatively small population of siblings,
342 re-screening of a larger number would increase the power of the analysis and allow
343 more subtle phenotypes to be identified.

344 We identified a loss of function mutation in the zebrafish *Slit3* gene associated with
345 increased nicotine place preference and confirmed the phenotype in an independent
346 line. SLIT molecules bind to ROBO receptors through a highly conserved leucine-rich
347 repeat (LRR) domain (38). In the AJBQM1 (*Slit3*^{sa1569}) line the loss of function
348 mutation causes a truncation at amino acid 176 and in the *Slit3*^{sa202} line at amino acid
349 163. These are immediately adjacent to the LRR2 domain responsible for SLIT3's
350 functional interaction with ROBO proteins (38) and would therefore be predicted to
351 lead to formation of non-functional proteins. Initially identified as a family of axon
352 guidance molecules, SLIT proteins are known to be expressed in a range of tissues
353 and, by regulating cell polarity, to play major roles in many developmental process
354 including cell migration, proliferation, adhesion, neuronal topographic map formation
355 and dendritic spine remodelling (39). *In vitro* SLIT proteins bind promiscuously to
356 ROBO receptors suggesting that the proteins may co-operate *in vivo* in areas in which
357 they overlap. However, their restricted spatial distributions, particularly of SLIT3 in
358 the central nervous system (40) suggest the individual proteins play subtly different
359 roles in vivo.

360 Despite its neuronal expression, the most prominent phenotype seen in SLIT3
361 deficient mice is postnatal diaphragmatic hernia (41,42) with no obvious neuronal or
362 axon pathfinding defects having been reported. Similarly, we did not detect any major
363 differences in axon pathfinding in *Slit3* mutant zebrafish larvae. As suggested
364 previously (43) it may be that overlap of expression with other SLIT molecules
365 compensates for loss of SLIT3 in the brain preventing gross neuronal pathfinding
366 defects. However, subtle differences in circuit formation and/or axon branching may
367 have escaped our analysis.

368 *Slit3*^{sa1569} 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*^{sa1569} 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*^{sa1569} 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 raphé 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,

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

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

406 Whilst we confirmed the translational effects of *Slit3* gene variants in a human study
407 and the association was validated in another independent cohort, there are limitations
408 to our findings: larger studies would be necessary to obtain greater precision on
409 estimates of the effect size. Further studies are also required to determine the effects
410 of genetic variation in *SLIT3* on anatomical pathways in the human brain and their
411 functioning with view to identifying people who are at high risk of developing
412 dependence. This could be achieved by using imaging techniques to study brain
413 activation in response to smoking related cues in smokers who have the *SLIT3*
414 polymorphisms linked to smoking (particularly rs12654448).
415 To our knowledge, this is the first report of a forward behavioural genetic screen in
416 adult zebrafish successfully predicting a novel human coding genetic region involved
417 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

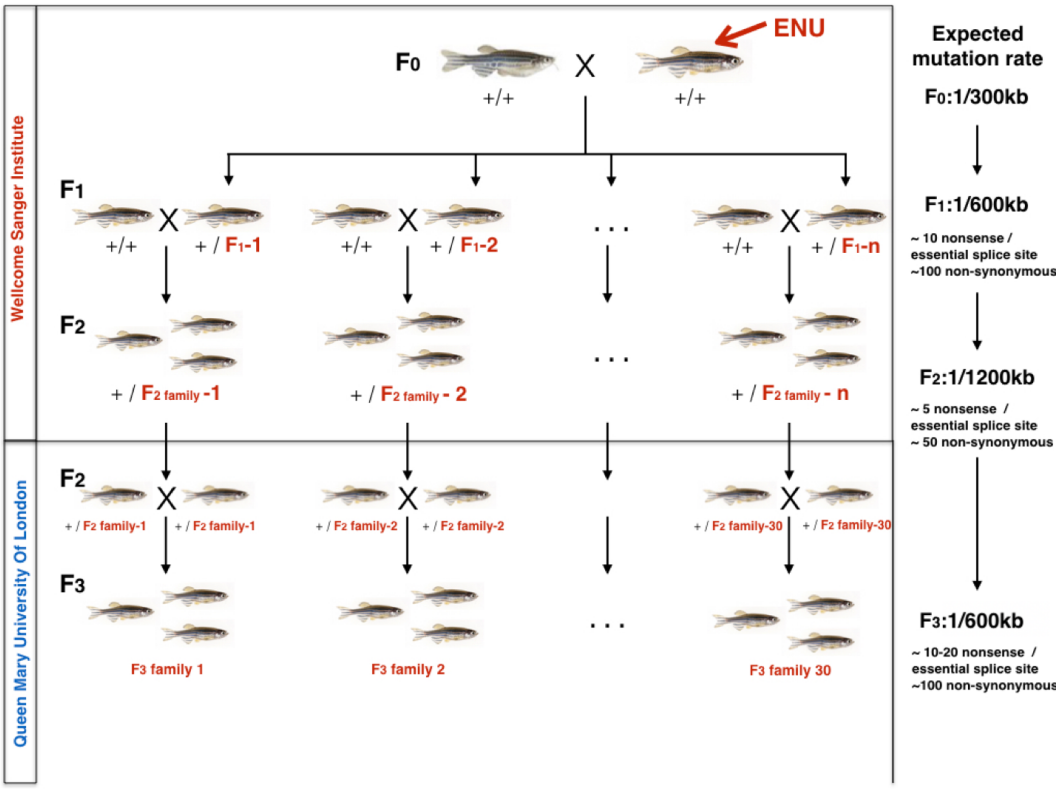
424 **MATERIALS AND METHODS**

425

426 ***Generation of F3 families of ENU-mutagenised fish:*** Wildtype and ENU-
427 mutagenized Tubingen longfin (TLF) fish were obtained from the Sanger Institute, as
428 part of the Zebrafish Mutation Project which aimed to create a knockout allele in
429 every zebrafish protein-coding gene
430 [https://www.sanger.ac.uk/resources/zebrafish/zmp/]. At the Sanger, ENU-
431 mutagenized TLF F_0 males were outcrossed to create a population of F_1 fish
432 heterozygous for ENU-induced mutations. Due to the high ENU mutation rate (1/300
433 kb) and homologous recombination when F_1 gametes are generated, all F_2 were
434 heterozygous for multiple mutations. F_2 families, each generated from a separate F_1
435 fish, were imported from the Sanger Institute to Queen Mary University of London
436 (QMUL).

437

438 At QMUL a single male and female fish from each F_2 family were inbred to generate
439 30 F_3 families that would be 25% wildtype, 50% heterozygous and 25% homozygous
440 mutant for any single mutation, assuming Mendelian genetics. Based on exome
441 sequencing data from the F_1 generation performed at the Sanger, each F_3 family
442 contained 10-20 known predicted loss of function exonic mutations, approximately
443 100 non-synonymous coding mutations and approximately 1500 unknown mutations
444 in non-coding domains across the entire genome (29). Breeding scheme is detailed in
445 Figure 10.



446

447 **Figure 10. Zebrafish breeding scheme to generate F3 families**. F_2 ENU-
448 mutagenized zebrafish, heterozygous for multiple mutations across the entire genome
449 were obtained from the Wellcome Sanger Institute as part of the Zebrafish Mutation
450 Project. At Queen Mary University of London, heterozygous F_2 fish were incrossed to
451 generate 30 F_3 families, each containing 10-20 nonsense or essential splice site
452 mutations and about 1500 additional exonic and intronic point mutations. F_3 Families
453 were arbitrarily numbered 1-30. Expected mutation rate and type of mutations in
454 coding regions are specified on the right hand side.

455

456 ***Fish maintenance:***

457 Fish were housed in a recirculating system (Techniplast, UK) on a 14h:10h light:dark
458 cycle (0830–2230). The housing and testing rooms were maintained at ~25–28ºC.
459 Fish were maintained in aquarium-treated water and fed three times daily with live

460 artemia (twice daily) and flake food (once). All procedures were carried out under
461 license in accordance with the Animals (Scientific Procedures) Act, 1986 and under
462 guidance from the local animal welfare and ethical review board at Queen Mary
463 University of London.
464
465 ***Conditioned place preference (CPP):*** All fish were age and weight matched for all
466 behavioural analysis and were approximately 5 months old, weighing 0.2-0.25g at the
467 start of testing. Following habituation and determination of basal preference, animals
468 were conditioned to 5μM nicotine (Sigma, Gillingham, UK Catalogue number:
469 N1019) over three consecutive days and assessed for a change in place preference the
470 following day. 5μM nicotine was used because it was predicted to induce a minimum
471 detectable change in place preference based on results of previous studies (19,21).
472 This minimal effective dose was used to avoid possible ceiling effects if using a
473 higher concentration. CPP was assessed as described previously (19,20,59): The
474 testing apparatus was an opaque 3 L rectangular tank that could be divided in half
475 with a Perspex divider. Each end of the tank had distinct visual cues (1.5cm diameter
476 black spots versus vertical 0.5cm wide black and white stripes, matched for
477 luminosity). After habituation to the apparatus and handling, we determined the basal
478 preference for each fish: individual fish were placed in the tank for 10 min and the
479 time spent at either end determined using a ceiling mounted camera and Ethovision
480 tracking software (Noldus, Wageningen, NL). Any fish showing >70% preference for
481 either end was excluded from further analysis. Fish were then conditioned with
482 nicotine in the least preferred environment for 20 min, on 3 consecutive days: Each
483 day each fish was restricted first to its preferred side for 20 min in fish water and then
484 to its least preferred side with nicotine or, if a control fish, vehicle (fish water) added,

485 for another 20 min. After 20 min in the nicotine (or vehicle)-paired environment each
486 fish was returned to its home tank. After 3 days of conditioning, on the following day,
487 fish were subject to a probe trial whereby each fish was placed in the conditioning
488 tank in the absence of divider and the time spent at either end of the tank over a 10
489 min period was determined as for assessment of basal preference. The change in place
490 preference was determined as the proportion time spent in the nicotine-paired zone
491 during the probe trial minus the proportion time spent in the nicotine-paired zone
492 during basal testing. The CPP procedure has been used and validated previously with
493 nicotine as well as other drugs (19,20,59) .
494
495 Data analysis: Change in preference scores were calculated as proportion time spent
496 in drug paired stimulus after conditioning minus proportion time spent in drug paired
497 stimulus before conditioning. Population means between generations were compared
498 using independent two-sample t-tests, and effect-sizes ascertained using Cohen's d
499 (60). For the rescreen of outlier sibling families and *Slit3*^{sa202} line, mutant lines were
500 compared with wildtype controls using an independent two-sample t-test.
501
502 ***CPP in the presence or absence of antagonists*****:** To assess the ability of compounds
503 (varenicline (Sigma, Gillingham, UK, PZ0004), buproprion (Sigma, Gillingham, UK,
504 B1277) or amisulpride (Tocris, Bristol, UK, C2132) to inhibit subjective effects of
505 nicotine a modified version of the CPP procedure was used (20). In this modified
506 version, following habituation and establishment of basal preference, each day each
507 fish was restricted first to its preferred side for 20 min in fish water and then removed
508 from the conditioning tank and transferred to a tank containing the appropriate
509 concentration of test compound or fish water (plus carrier where required) for 10min.
510 After 10min the fish was returned to its least preferred side in the conditioning tank
511 with nicotine or, if a control fish, vehicle (fish water) for another 20 min. After 20
512 min in the nicotine (or vehicle)-paired environment, each fish was returned to its

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

522 To test the effect of amisulpride on nicotine -induced CPP in wildtype and *Slit3*^{sa1569}
523 mutant fish, fish were incubated in the presence or absence of 0.5mg/L amisulpride
524 for 10 min before conditioning to 5μM nicotine. Two-way ANOVA was performed
525 with genotype (*Slit3*$^{+/+}$ *and Slit3*$^{sa1569/sa1569}$) and treatment (control, nicotine,
526 nicotine+amisulpride) as independent variables. Values of $p<0.05$ were considered
527 significant.

528

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

537

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

541 tested clustered together at one or other extreme of the change in preference
542 distribution curve. To confirm the genetic effect on the CPP phenotype in candidate
543 families, all remaining siblings of that family were assessed for nicotine induced CPP,
544 along with non-mutagenized TLF control fish, to confirm the genetic effect on the
545 phenotype.
546
547 Candidate mutations, obtained from exome sequencing on F_1 fish, were assessed for
548 co-segregation with behaviour using site specific pcr (61) (See Supplementary
549 Methods). Once a co-segregating candidate mutation was identified, larvae from an
550 independent line carrying a predicted loss of function allele in the same gene were
551 obtained from the Sanger Institute (*Slit3*^{sa202}) to confirm the association.
552 Heterozygous *Slit3*$^{sa202/+}$ and sibling *Slit3*$^{+/+}$ larvae were reared to adulthood and
553 assessed for nicotine-induced CPP as described above. All fish were fin clipped and
554 genotyped following CPP.
555
556 ***Characterization of larvae:***
557 ***Antibody staining*:** In order to visualize axonal pathways, fluorescent
558 immunohistochemistry was carried out in three day old embryos from wildtype
559 *Slit3*$^{+/+}$, heterozygous mutant *Slit3*$^{sa1569/+}$ and homozygous mutant *Slit3*$^{sa1569/sa1569}$ in-
560 crosses. To prevent skin pigmentation, embryos were incubated in 0.2mM of 1-phenyl
561 2-thiourea (Sigma, Gillingham, UK) from 24 hours after fertilization. At three days,
562 they were fixed in 4% paraformaldehyde (Sigma, Gillingham, UK) to avoid tissue
563 degradation. For the immunostaining, rabbit polyclonal anti-tyrosine hydroxylase
564 primary antibody (1:200; Sigma, Gillingham, AB152) and mouse anti-acetylated
565 tubulin monoclonal antibody (1:1000; Sigma Gillingham, UK, T6793) were used.

566 Both primary antibodies were detected with Alexa 546-conjugated secondary
567 antibodies (1:400; Fisher Scientific, Loughborough, UK A11010). Whole-mount
568 immunohistochemistry and mounting was performed as described previously (62).
569
570 ***Confocal microscopy imaging and analysis**:* Images were acquired using a Leica SP5
571 confocal microscope. Confocal z-stacks were recorded under the same conditions
572 using diode laser and images were processed under ImageJ environment.
573
574 ***Startle response in the presence or absence of amisulpride*:** Five day old larvae,
575 generated from adult *Slit3* wildtype and homozygous mutant (*Slit3*$^{sa1569/sa1569}$) fish as
576 for quantitative pcr, were individually placed in 24 well plates. In the drug-free
577 condition, each well contained 300µl system water and 0.05% of dimethyl sulfoxide
578 (DMSO, Sigma, Gillingham, UK). In the pharmacological conditions, serial dilutions
579 of the dopaminergic and serotonergic antagonist amisulpride (Tocris, Bristol, UK,
580 71675-86-9) were prepared to give final concentrations of 0.05 mg/L, 0.1 mg/L or 0.5
581 mg/L amisulpride in 0.05% DMSO. Amisulpride concentrations were chosen based
582 on previous studies in zebrafish (63) and correspond to 50, 100 and 500 times its Ki
583 value for the D2 receptor in mammals (64,65). To ensure that larvae were exposed to
584 the drug for the same amount of time, amisulpride was added 15 minutes before
585 undertaking the experiment. Care was taken regarding the distribution of
586 concentrations and genotypes to ensure that experimental groups were randomly
587 distributed in the plates. Plates were placed in a custom-made filming tower with a
588 tapping device that applied 10 sound/vibration stimuli with two seconds interval
589 between them. The setup for this device has been described elsewhere (66). Larval

590 movement was recorded using Ethovision XT software (Noldus Information
591 Technology, Wageningen, NL) and data were outputted in one second time-bins.
592
593 For each fish, distance travelled (mm) during one second after each tap was recorded.
594 Linear mixed models were calculated to assess differences in baseline distance
595 moved, distance moved one second after the first stimulus and distance moved during
596 all the stimuli across groups. To assess response and habituation to the stimuli, we
597 calculated the percentage of fish exhibiting a response ≥ 50% of the mean startle
598 response to the first tap stimulus (set to 2.5mm because this was 50% of the mean
599 distance travelled across all groups at tap one, and response to the first stimulus was
600 not significantly different across experimental groups (n.s.)).
601
602 The percentage of fish responding to stimulus together with amisulpride dose,
603 stimulus number and genotype group were modelled in a beta regression conducted
604 using the R package "betareg". To determine whether stimulus number, dose or
605 genotype variables are significant, likelihood ratio tests for nested regression models
606 were performed. Results of all statistical analyses were reported with respect to a
607 type-1 error rate of α=0.05. Post-hoc tests were conducted using Tukey's HSD.
608
609 ***Real-time quantitative pcr:*** Adult *Slit3* wildtype and *Slit3*^{sa1569} homozygous mutant
610 fish, generated from a *Slit3*$^{sa1569/+}$ heterozygous in cross, were bred to generate
611 homozygous wildtype, heterozygous mutant and homozygous mutant larvae. Embryos
612 were carefully staged at 1, 24 and 48 hour and at five day post fertilisation to ensure,
613 based on morphological criteria, there were no differences in development between
614 groups. mRNA from 3 samples of five day old embryos (n=10 pooled embryos per

615 sample) for each genotype was isolated using the phenol-chloroform method. cDNA
616 was generated using the ProtoScript® II First Strand cDNA Synthesis Kit (NEB (UK
617 Ltd.), Hitchen, UK). Relative qpcr assays were performed using the LightCycler 480
618 qpcr system from Roche Diagnostics, Ltd. with all reactions carried out in triplicates.
619 Reference genes for all the qpcr analyses were β-actin, ef1α and rpl13α based on
620 previous studies (59,67,68). Accession numbers and primer sequences for the genes
621 can be found in Supplementary Table 2.
622
623 Relative mRNA expression in qpcr was calculated against reference gene cycle-
624 threshold (Ct) values, and then subjected to one-way ANOVA. To account for
625 multiple testing a Bonferroni correction was applied, and significance was declared at
626 a threshold of 0.001.
627
628 ***Human Cohorts***: In London human subjects were recruited from three clinical
629 groups: patients with chronic obstructive pulmonary disease (COPD) (Cohort 1;
630 n=272); patients with asthma (Cohort 2; n=293); and residents and carers in sheltered
631 accommodation, with neither condition (Cohort 3; n=298). The methods used for
632 recruitment and definition of phenotypes are reported elsewhere (69–71). The studies
633 were approved by East London and The City Research Ethics Committee 1
634 (09/H0703/67, 09/H0703/76 and 09/H0703/112). Written informed consent was
635 obtained from all participants.
636
637 Details of the Finnish twin cohort are reported elsewhere (72–74). In brief, twin pairs
638 concordant for moderate to heavy smoking were identified from the population-based
639 Finnish Twin Cohort survey responders. The twin pairs and their siblings were invited

640 to a computer-assisted, telephone-based, structured, psychiatric interview (SSAGA)
641 (72), to yield detailed information on smoking behaviour and nicotine dependence as
642 defined by Fagerström Test for Nicotine Dependence (FTND) and DSM-IV
643 diagnoses. Human phenotypes to be investigated in relation to zebrafish nicotine
644 seeking behaviour were determined by consensus *a priori*.
645
646 Sample characteristics of the human cohorts and detailed definitions of both London
647 and Finnish phenotypes can be found in the supplementary material and
648 Supplementary Table 3.
649
650 ***Human genotyping:*** For the London cohorts, DNA from participants was extracted
651 from whole blood using the salting-out method (75) and normalized to 5ng/µl. 10ng
652 DNA was used as template for 2 µl TaqMan assays (Applied Biosystems, Foster City,
653 CA, USA) performed on the ABI 7900HT platform in 384-well format and analysed
654 with Autocaller software. Pre-developed assays were used to type all SNPs. See
655 Supplementary Table 4 for primer and reporter sequences. Typing for two SNP
656 (rs6127118 and rs11574010) failed. For the Finnish cohort, DNA was extracted from
657 whole blood and genotyping was performed at the Wellcome Trust Sanger Institute
658 (Hinxton, UK) on the Human670-QuadCustom Illumina BeadChip (Illumina, Inc.,
659 San Diego, CA, USA), as previously described (72–74).

660 ***Human association analyses:*** London cohort association analysis was performed
661 using PLINK v1.07 (76). SLIT3 SNPs that had been previously associated with
662 disease phenotype were identified and the 20 with the highest linkage disequilibrium
663 score selected for analysis. Of twenty SLIT3 SNPs, one departed from Hardy-
664 Weinberg equilibrium (rs13183458) and was excluded. Linear regression was

665 performed on average number of cigarettes smoked per day, controlling for age, sex
666 and cohort. This analysis was repeated on heavy smokers (≥ 20 cigarettes per day)
667 and light smokers (< 20 cigarettes per day) to investigate whether effects were related
668 to intake level. Smoking cessation (current *vs* ever smokers) was analysed using
669 logistic regression controlling for age, sex and cohort. All analyses were performed
670 under the additive genetic model and multiple testing was taken into account using the
671 Benjamini-Hochberg adjustment. Only individuals from European ancestry were
672 included in analyses.

673 Association analyses for the Finnish Twin Cohort were performed using GEMMA
674 v0.94 (77) with linear mixed model against the allelic dosages controlling for age and
675 sex. Sample relatedness and population stratification were taken into account by using
676 genetic relatedness matrix as random effect of the model.
677

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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
cacna1ba (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 (MOBKL2A)	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)
Serotonergic pathway			
Htr1aa	NM_001123321.1	Forward: TTCTACATCCCGCTCATCCTCA Reverse: CCTCCAAGTTTTACCCACCTCTC	180
Htr1ab	NM_001145766.1	Forward: AAACACCGAGGCGAAGAGGAA Reverse: GGCAGCCAACACAGAATGAAAGT	99
Htr2a	XM_684208.9	Forward: TACGGTGGCTGGGAACATTTTAG Reverse: GGGACACAGTGATGCAGGGAAA	187
Htr5a	NM_001126410.2	Forward: TGGATCAAAGAGGACCAACACC Reverse: CTGAAACGTCACCGTGGCAT	118
Htr2cl1	NM_001129893.1	Forward: AACTTCTTCCTCCGCTCACTCG Reverse: ATGGCACACAGGTGCATGATGG	179
Htr2cl2	XM_001339004.7	Forward: CACAACCCCACCAACTTCTTCC Reverse: ACGTCCAGAAAGATCCACAGCG	152
Htr4	XM_021481160.1 XM_009291062.3	Forward: GTTTCTTTCCAAGCGCCTC Reverse: ACTTCTTCCATCTCAGGCATC	168
Htr6	XM_009297078.3	Forward: ACTACAGTCATCAGGAGCCACC Reverse: GCCAGGCACTGAAGAATAGTCC	147
Htr7	XM_003199584.5	Forward: TGGATGTGATGTGCTGTACCGC Reverse: GCCATGCACTTTCCACTCTGTC	118
slc6a4/ SERT	NM_001039972.1	Forward: ACCAGGGGCGAAGCCAAGCA Reverse: GCCACAGGCCCCGCTGTTA	117
Htr1b	NM_001128709.1	Forward: CCTTGTCGTCAGTTCTGGGT Reverse: ATCAGAAAGTTCGCCGGTGT	112
Nicotinic pathway			
Chrna2	NC_007128.7	Forward: TGGCTGCAGATCAGTCAAAGAC Reverse: CCCTCTAACTGTCCCTTCACAA	271
Chrna3	Not found in BLAST	Forward: TGTACATCCGCCGATTACCGCT Reverse: TCCGCAGTCGGAGGGCAGTA	?
Chrna4b	ENSDART00000018614.7	Forward: TTACAAGAGGTTTGGGCGCT Reverse: ACAGACCAGTAGATCATCACTCC	90
Chrna5	NM_001017885.1	Forward: GGCTCCCAGGTCGACATTCTC Reverse: AACCCCGGTTACCAGTGGCCT	103
Chrna6	NM_001042684	Forward: AGGCTCTTTCGTCGTTTATTC Reverse: TCTCAGCCAAAGGTTTGTTTC	156
Chrna7	ENSDART00000171463.3 and ENSDART00000166391.2	Forward: ACCGTGTCACATTGTTCATTCTC Reverse: ACAGGTCTCTCCAGTGGGTTA	105

Chrnb2b	ENSDART00000041625.7 and ENSDART00000185728.1	Forward: CACAAAGTCACGCTCCGATAC Reverse: CCGTCGCTCTGAGCAGATAA	160
Chrnb3b	ENSDARG00000038508.5	Forward: CAGGAGTCAACCTCCGCTTT Reverse: TGAATCTGAACGCACTGGCT	106
Chrnb4	NC_007129.7	Forward: ATGTGAATGAATGGCGGTGTGTG Reverse: ATGCGCGTGTCAGATTTACCC	203
Dopaminergic pathway			
Drd1b	NM_001135976.2	Forward: TGGTTCCTTTCTGCAACCCA Reverse: AGTGATGAGTTCGCCCAACC	100
Drd2a	XM_009291617.3 and XM_005157501.4	Forward: TCCACAAAATCAGGAAAAGCGT Reverse: CAGCCAATGTAAACCGGCAA	106
Drd3	XM_021470111.1 and XM_005162673.4	Forward: ATCGAGTTTCGCAGAGCCTT Reverse: TCCACAGTGTCTGAAAGCCG	95
Slc6a3	NM_131755.1	Forward: GCCTGGTTTTACGGAGTGGA Reverse: GGAGGATTGAAGGTGGCGAA	66
Adrenergic pathway			
Adra1aa	NM_001324454.1	Forward: AAGAAGGCCGCCAAGACTTT Reverse: GTCCGAGGGTCTGTACGTTG	114
Adra1d	XM_691951.6	Forward: AAGCTGCTAAAACCCTCGCC Reverse: GGCTTCAGAGCTGGGAAGAAT	103
Adra2b	NM_207638.1	Forward: AAAAGCCAGGCCTCCAACTT Reverse: GGGCTTGCAGAAGGTTGTTG	92
Adra2c	NM_207639.1	Forward: CGCCGTTTTAACGAGCAGAG Reverse: AGTGTGGCCACCAGAATGTC	87
Adra2da	NM_194364.2	Forward: CATCATCCTCGTGGTGTCCC Reverse: ATCCCATGATCTCGTTGGCG	188
Adra2db	NM_194365.1	Forward: TGCCACTTTGGTCATTCCGT Reverse: AGCCAGGTAGAAAGCACACC	88

33 **Supplementary Table 3: Sample characteristics of the human cohorts.** Detailed inclusion
34 and exclusion criteria for the London cohorts can be found in https://clinicaltrials.gov.
35 Further details about recruitment and definition of medical phenotypes can be found
36 elsewhere (69–71). **Differences in sample size for Finnish cohorts was due to hard-call
37 genotype probability threshold. DSM-IV nicotine dependence symptoms, Fagerström scores
38 and cigarettes smoked each day (N = 1715). Sensation felt after smoking first cigarette (N =
39 1915). Time to first cigarette in the morning (N= 1726).

Cohort name	*N*	*Country*	*Cohort description*	*ClinicalTrials.gov ID*	*Mean age (years)*	*% female*	*Smoking phenotypes investigated*	*N heavy smokers*
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, ≥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.

<u>*Nicotine dependence*</u>

- ***DSM-IV ND diagnosis***: Nicotine dependence by DSM-IV diagnosis (≥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 (≥4):*** Nicotine dependent if ≥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 6, 16-30 min 6, 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

of 6-30 min, and from the smoking behaviour point of view there is a significant difference whether one smokes the first cigarette within 6 minutes or 30 minutes from waking up. In this data set 22% of smokers belong to the 6-15 min and 24% to the 16-30 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
CYP3A4	rs2740574		
		Forward	CCAGGCATAGGTAAAGATCTGTAGGT
		Reverse	CTCAAGTGGAGCCATTGGCATA
		Reporters	ACAAGGGCAAGAGAG and ACAAGGGCAGGAGAG
CUBN	rs3740165		
		Forward	GCAATGAGATTAAATCTTCAGGAAACACA
		Reverse	CTGGAGGTATAGGAAGCAGTGAAG
		Reporters	CCGCCATATGGCCTG and CGCCATACGGCCTG
RXRA	rs7861779		
		Forward	TGGCCCATGCACGAGTAG
		Reverse	ACCGAGACAGGCCAAACTC
		Reporters	CAGCAGAGGTGGCCGA and 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	
Slit3	**WT**	**WT**	**WT**	**WT**	**HET**	**HET**	**HET**	**HET**	**HET**	**HET**	**7.6592×10^{-5}**
Cacne	WT	HET	WT	WT	HOM	HET	WT	WT	WT	WT	0.691
Vcan	HET	HET	WT	HET	HOM	WT	HET	HET	WT	WT	0.259
Evpl	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	-
Mob3a	HET	HET	HET	HOM	HOM	HET	WT	HET	WT	HET	0.236
Prkg1	HET	HET	HET	HET	HET	HET	HET	HET	HOM	HOM	-
Glis3	WT	HET	HET	HET	WT	WT	WT	HET	WT	HET	0.602
Tulp2	HOM	HOM	WT	HET	WT	HET	HET	HET	WT	WT	0.481
Dchs1	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	-
Flad1	HOM	HOM	WT	HET	WT	WT	WT	HET	HET	HET	0.981
Akd1	HOM	HOM	WT	HET	WT	HET	HET	HET	WT	WT	0.418
Magl2	HET	WT	HET	WT	HET	HOM	WT	WT	HOM	HET	0.73
Trimm55	WT	HET	WT	HET	WT	WT	WT	WT	HET	WT	0.51

(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	
Tspan3a	HET	HET	HET	WT	WT	WT	HET	HET	WT	WT	WT	HET	HET	HET	0.583
Raspn	WT	HOM	WT	WT	WT	HOM	HOM	HOM	WT	WT	WT	HOM	WT	HOM	0.792
A9	WT	WT	HET	WT	WT	WT	HET	WT	HET	HET	HET	HET	HET	HET	0.339
Capn3	HET	HET	HET	WT	HET	WT	WT	HET	WT	HET	WT	WT	WT	WT	0.911
Klf11a	WT	WT	WT	HET	WT	WT	WT	HET	WT	WT	HET	WT	HET	WT	0.318
Kctd4	HET	WT	WT	HET	WT	WT	HET	WT	HET	HET	HET	HET	WT	WT	0.252
Slc6a11	HET	HET	HET	WT	HET	WT	HET	HET	WT	WT	WT	WT	HET	WT	0.697
Pkhd1l1	WT	WT	WT	HET	HOM	HOM	HET	WT	WT	WT	WT	WT	WT	HOM	0.499
Slc19a3	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	-
Sv2b	WT	WT	HET	WT	WT	WT	WT	HET	HOM	HET	HOM	HOM	HET	HOM	0.269
Snrnp70	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	-
Ac10103686	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	-

16 **Supplementary Table 6:** Results of association analysis of *SLIT3* SNPs on **smoking**
17 **initiation.** Logistic regression of initiation vs non-initiation on additive genotype, controlling
18 for age, sex and cohort. OR: Odds ratio. >1 value indicates that the minor allele increases
19 odds of persistent smoking relative to the major allele, SE: standard error, L95: lower limit of
20 95% confidence interval, U95: upper limit of 95% confidence interval. Benjamini Hochberg
21 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

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24 **Supplementary Table 7:** Results of association analysis of *SLIT3* SNPs on **persistent**
25 **smoking.** Logistic regression of initiation vs non-initiation on additive genotype, controlling
26 for age, sex and cohort. OR: Odds ratio. >1 value indicates that the minor allele increases
27 odds of persistent smoking relative to the major allele, SE: standard error, L95: lower limit of
28 95% confidence interval, U95: upper limit of 95% confidence interval. Benjamini Hochberg
29 cut off at 0.1 = 0.00526.

30

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

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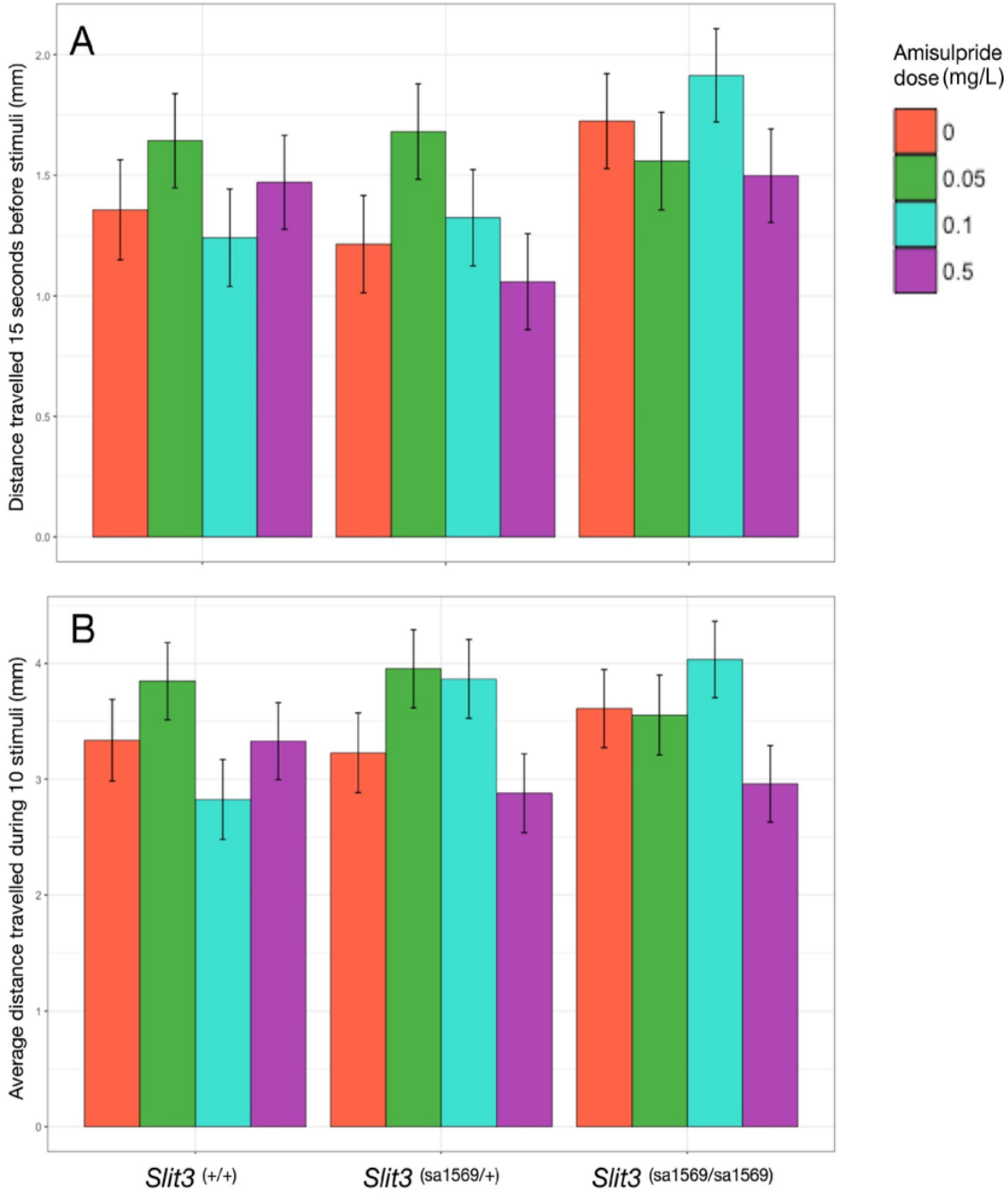

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34 **Supplementary Figure 1.** Average distance moved before (Figure 1A) and during startle
35 stimuli (Figure 1B) in wildtype and *Slit3*^{sa1569} mutant five day old zebrafish larvae. **A)**
36 Distance moved as function of amisulpride dose, fish *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*

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

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