bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

Alistair J. Brock[1*], Judit García-González[1*], Matthew O. Parker[2], David Jolliffe[3], Ari Sudwarts[1], Muy-Teck Teh[4], Elisabeth M. Busch-Nentwich[5], Derek L. Stemple[5], Adrian R. Martineau[3], Jaakko Kaprio[6,7], Teemu Palviainen[6], Valerie Kuan[8], Robert T. Walton[3], Caroline H. Brennan[†1]

*Authors contributed equally to this manuscript.

Affiliations: [1] School of Biological and Chemical Sciences, Queen Mary, University of London, London E1 4NS. [2] Brain and Behaviour Laboratory, School of Pharmacy and Biomedical Science, University of Portsmouth, PO1 2DT, UK. [3] Barts and The London School of Medicine and Dentistry, Blizard Institute, 58 Turner St, Whitechapel, London E1 2AB. [4] Centre for Immunobiology and Regenerative Medicine, Institute of Dentistry, Barts and The London School of Medicine and Dentistry, Blizard Building, 4 Newark St, Whitechapel, London E1 2AT. [5] Wellcome Trust Sanger Institute. [6] Institute for Molecular Medicine FIMM, HiLIFE. PO Box 20 (Tukholmankatu 8). 00014 Helsinki, Finland. [7] Department of Public Health, Faculty of Medicine, University of Helsinki. Helsinki, Finland. [8] Institute of Cardiovascular Science, University College London, London, UK.

[†]**Author for correspondence**: Caroline H. Brennan, School of Biological and Chemical Sciences, Queen Mary, University of London, London E1 4NS. c.h.brennan@qmul.ac.uk. Phone: +442078826357; Robert T. Walton, Barts and The

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

London School of Medicine and Dentistry, Blizard Institute, 58 Turner St, Whitechapel, London E1 2AB. r.walton@qmul.ac.uk. Phone: +442476574517.

Keywords: slit3, acoustic startle, smoking, conditioned place preference, gene association

Number of words in the abstract: 150 words

Number of words in the main text: 5755 words

Number of figures: 10

Number of tables: 2

Number of supplementary figures: 1

Number of supplementary tables: 7

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

RESULTS

Nicotine CPP in zebrafish is inhibited by varenicline and buproprion

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

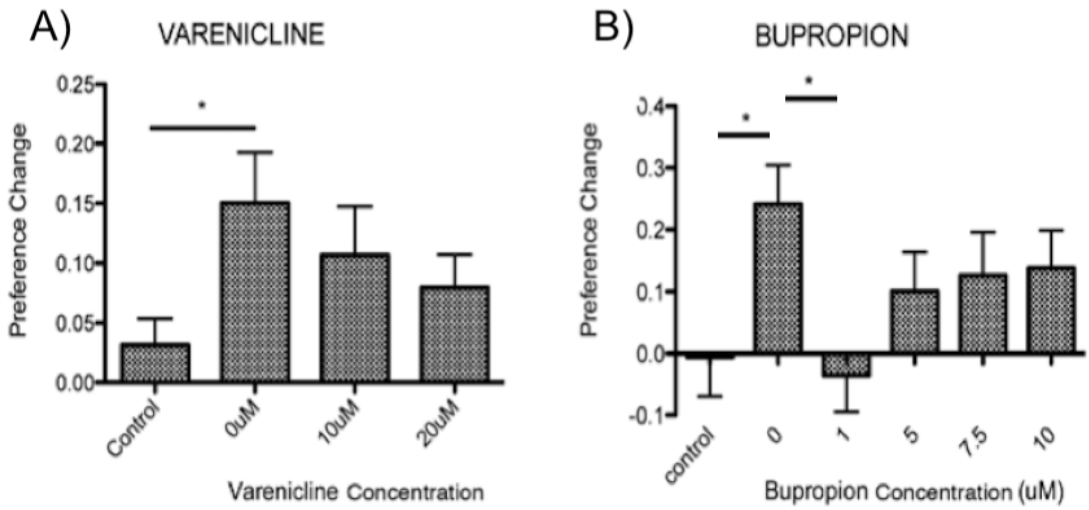


Figure 1. 10μM nicotine induced place preference in zebrafish is sensitive to inhibition by therapeutics effective in humans. A) varenicline (nicotine partial agonist) and B) bupropion (norepinephrine and dopamine reuptake inhibitor with

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

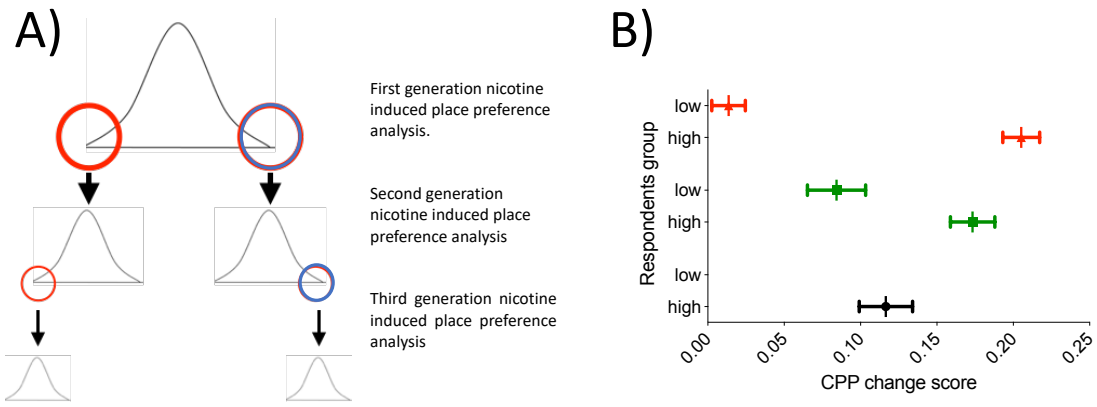


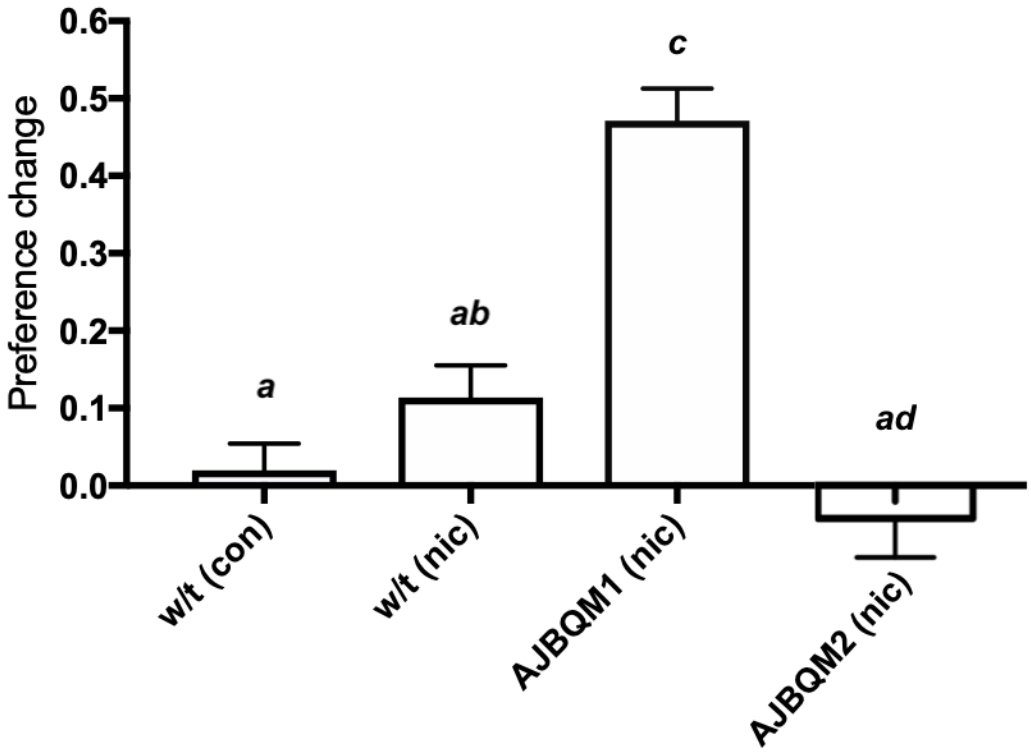
Figure 2. A) Breeding and selection to assess heritability of nicotine-induced place preference in ENU-mutagenized zebrafish. To test whether nicotine preference is heritable, fish in the upper and lower 10% of the change in preference distribution curve were inbred and screened for CPP (Second generation 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 ±SEM. First generation (corresponding to the F_3 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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

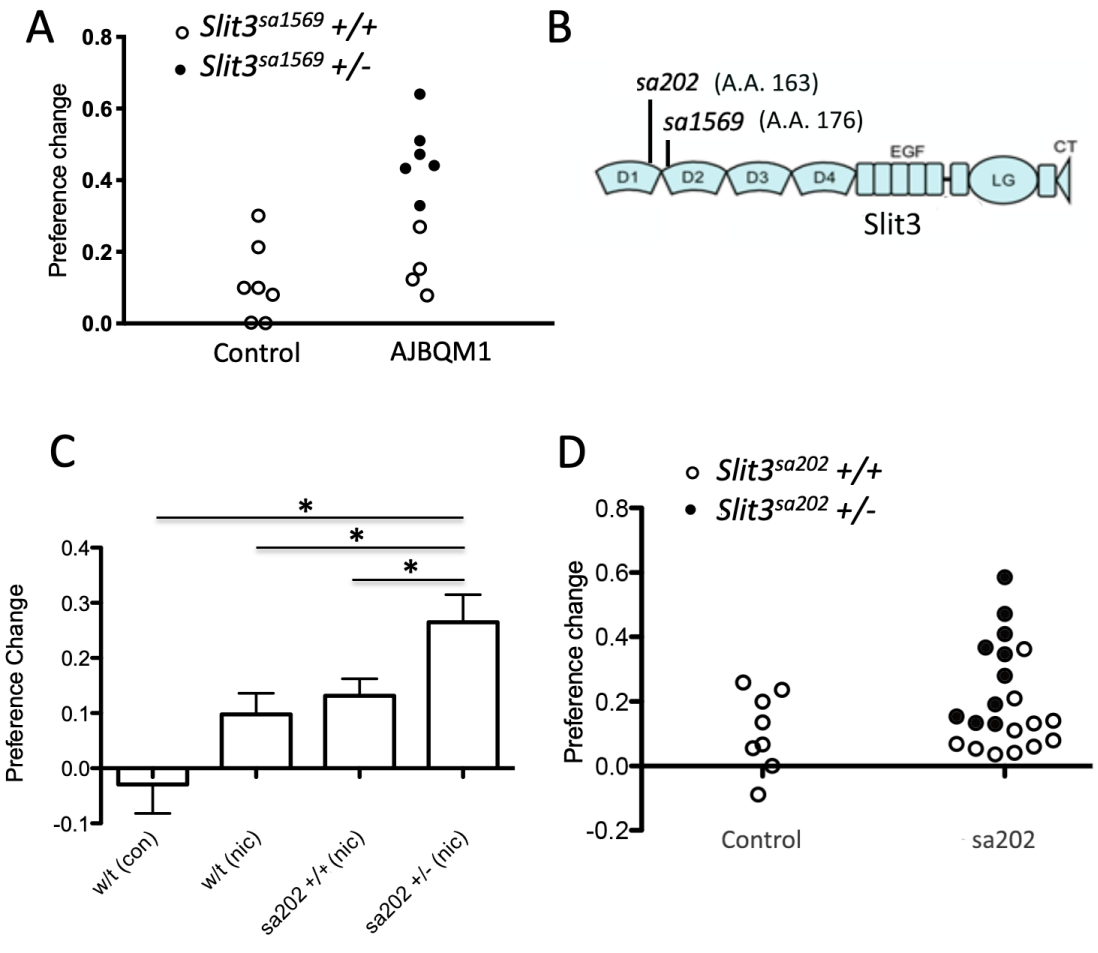
bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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).

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



167

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

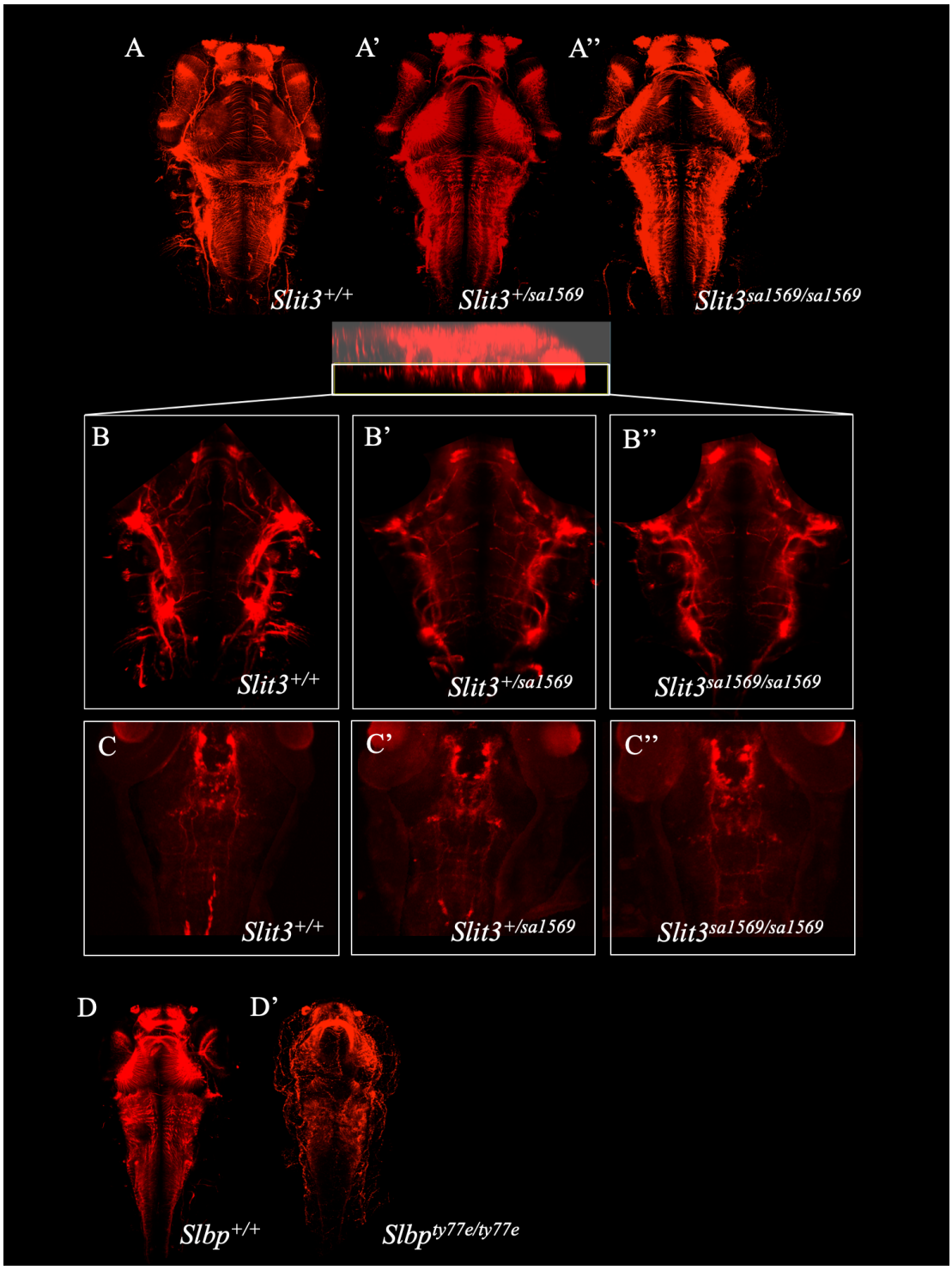
bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

Characterisation of *Slit3*^{sa1569} 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*^{sa1569} 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*$^{ty77e/ty77e}$ mutant larvae, known to have fewer neurons and axonal defects (16) were used as positive control (Figure 5 D & D').

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



198

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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.

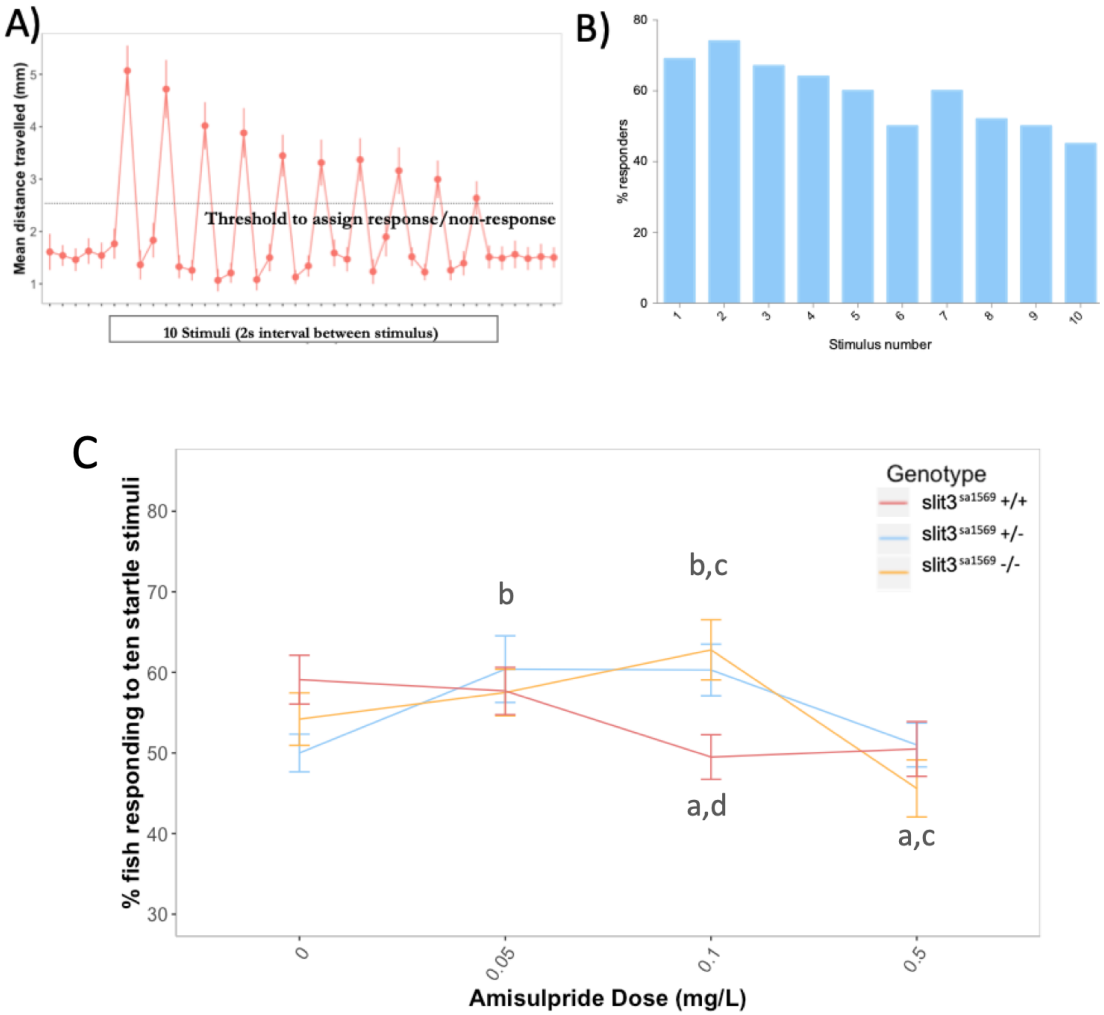
210 As subtle effects on circuit formation may not have been detected by our antibody
211 staining, we examined the response and habituation to acoustic startle stimuli in
212 wildtype and mutant fish. Habituation to acoustic startle is known to be sensitive to
213 dopaminergic/serotonergic antagonists 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}

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

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



235

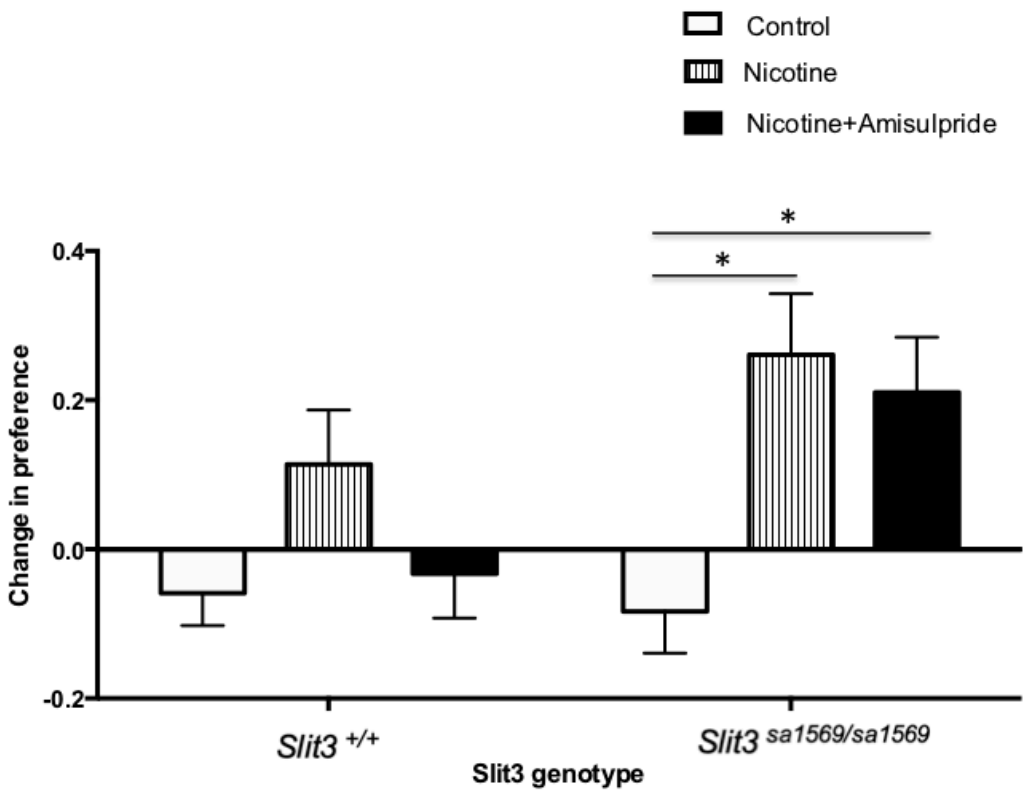
236 **Figure 6. Habituation response in the presence and absence of amisulpride. A:**
237 **Response and habituation to 10 stimuli with two seconds interval between stimuli**

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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 (±SEM), stratified by *Slit3*^{sa1569} genotype and amisulpride dose**. The effect of amisulpride on habituation varies by genotype: *Slit3*^{sa1569} mutants show a U-shaped response to amisulpride, in contrast with wildtype fish (Genotype by amisulpride dose interaction ($p<0.05$)). 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* $^{sa1569/+}$ and *(c) to* Slit3 $^{sa1569/\ sa1569}$. Letter *(d)* indicates significant genotype effect between *Slit3* wildtype and *Slit3* ^{sa1569} mutants at 0.1 mg/L amisulpride. n=42-48 fish per experimental group.

Adult Slit3 $^{sa1569/\ sa1569}$ 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 5μM nicotine in wild type fish was prevented by pre-exposure to 0.5mg/L amisulpride. Nicotine-induced CPP in *Slit3*^{sa1569} homozygous mutants was not affected (Figure 7).

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



257

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).

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

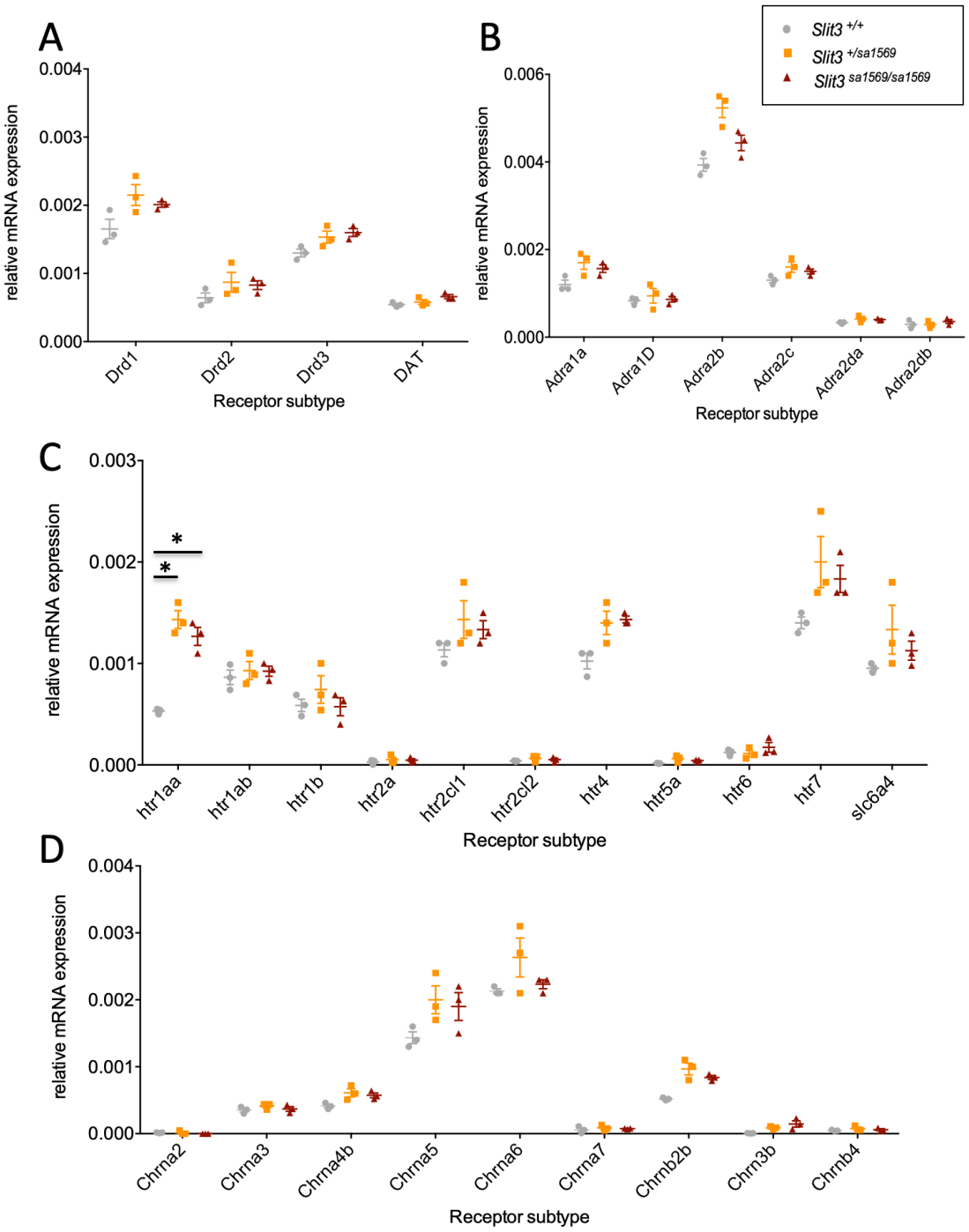


Figure 8. Quantitative real-time pcr analysis of five day old wildtype *Slit3*$^{sa1569+/+}$, *Slit3*$^{sa1569/+}$ heterozygous and *Slit3*$^{sa1569/sa1569}$ homozygous mutant larvae (Total n=30, 3 samples per experimental group with n=10 embryos per sample). Only *Htr1aa* ([F(2,6)=44], p=0.0003) showed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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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).

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

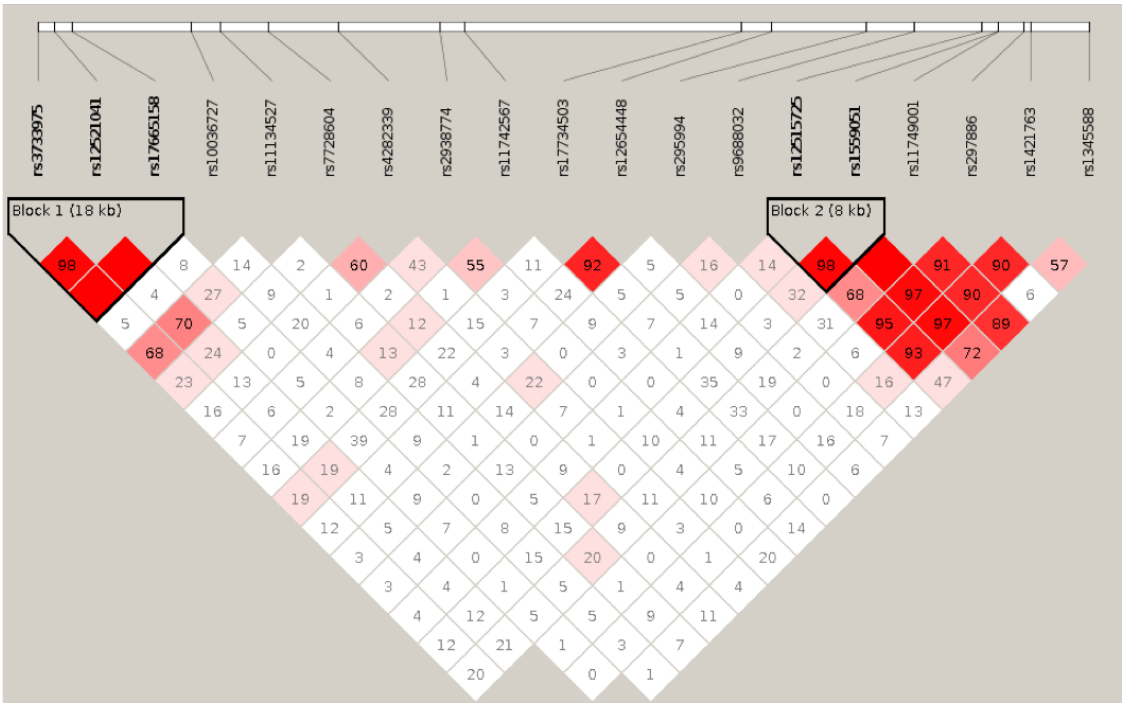


Figure 9: Linkage disequilibrium (LD) plot of *SLIT3* SNPs in human smoking association analysis. Numbers within each square indicate D' values (white: D' < 1, LOD < 2; blue: D' = 1, LOD < 2; pink: D' < 1, LOD ≥ 2; and bright red: D' = 1, LOD ≥ 2.)

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

	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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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/).

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

319

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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*^{sa1569}) line the loss of function mutation causes a truncation at amino acid 176 and in the *Slit3*^{sa202} 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.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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 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,

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

MATERIALS AND METHODS

Generation of F3 families of ENU-mutagenised fish: Wildtype and ENU-mutagenized Tubingen 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_0 males were outcrossed to create a population of F_1 fish heterozygous for ENU-induced mutations. Due to the high ENU mutation rate (1/300 kb) and homologous recombination when F_1 gametes are generated, all F_2 were heterozygous for multiple mutations. F_2 families, each generated from a separate F_1 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_2 family were inbred to generate 30 F_3 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_1 generation performed at the Sanger, each F_3 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.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

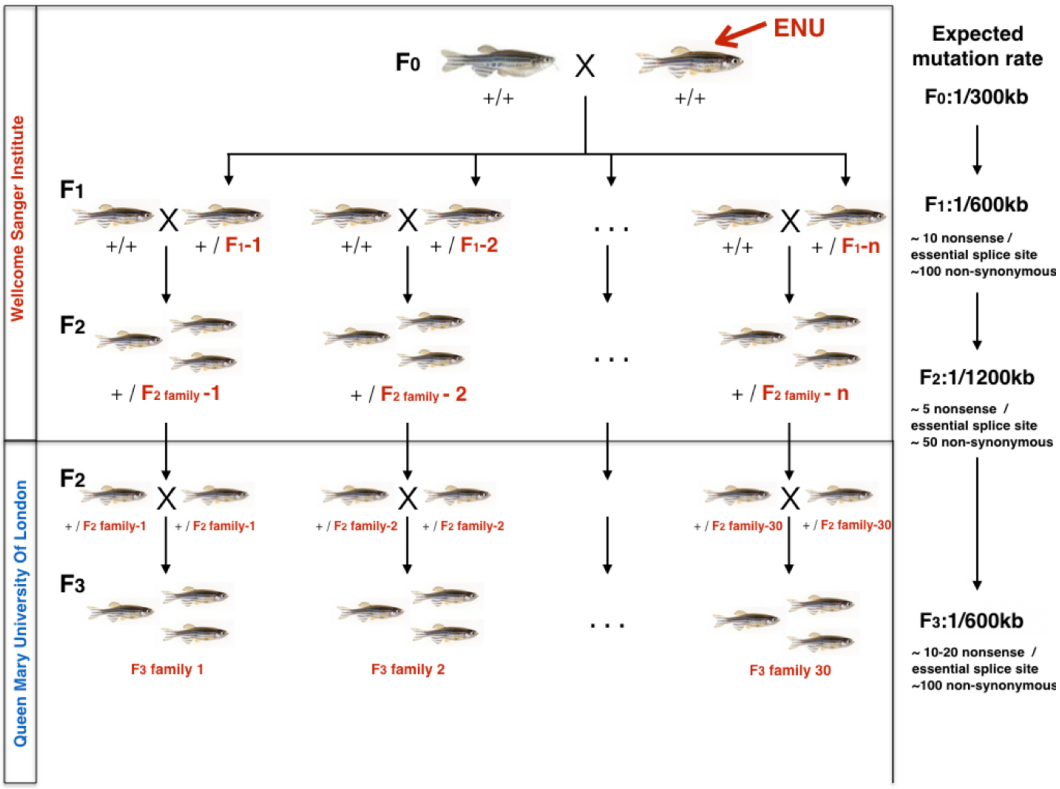


Figure 10. Zebrafish breeding scheme to generate F3 families. F_2 ENU-mutagenized zebrafish, heterozygous for multiple mutations across the entire genome were obtained from the Wellcome Sanger Institute as part of the Zebrafish Mutation Project. At Queen Mary University of London, heterozygous F_2 fish were incrossed to generate 30 F_3 families, each containing 10-20 nonsense or essential splice site mutations and about 1500 additional exonic and intronic point mutations. F_3 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 ~25–28ºC. Fish were maintained in aquarium-treated water and fed three times daily with live

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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,

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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 (0-20μM) or buproprion (0-10μM) 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 10μM 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*^{sa1569} mutant fish, fish were incubated in the presence or absence of 0.5mg/L amisulpride for 10 min before conditioning to 5μM nicotine. Two-way ANOVA was performed with genotype (*Slit3*$^{+/+}$ *and* *Slit3*$^{sa1569/sa1569}$) 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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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_1 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*^{sa202}) to confirm the association. Heterozygous *Slit3*$^{sa202/+}$ and sibling *Slit3*$^{+/+}$ 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*$^{+/+}$, heterozygous mutant *Slit3*$^{sa1569/+}$ and homozygous mutant *Slit3*$^{sa1569/sa1569}$ 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.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Acknowledgements

Funding: MRC UK, G1000403 (CHB/RW). NC3Rs G1000053 (CHB). BBSRC BB/M007863 (CHB) NIHR PGfAR RP-PG-0609-10181 (RW). NIHR PGfAR, RP-PG-0407-10398 (ARM). CHB is a member of the Royal Society Industry Fellows' College. RW is an NIHR Senior investigator (NF-SI-0515-10076). VK holds a Wellcome Trust Clinical Research Fellowship. JK is supported by the Academy of Finland (grants 308248, 312073). CHB conceived the project and directed the research. RW, AM and JK oversaw the human studies. AJB, JGG, DJ, MTT, AS, MOP, CHB conducted experiments. TP, VK analysed the human data. EMB and DS supplied the mutant lines. Some of the data in this manuscript has been published previously as a preprint: bioRxiv 453928: doi: https://doi.org/10.1101/453928

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

References

1. World Health Organization, Bloomberg Philanthropies. WHO report on the global tobacco epidemic, 2017: monitoring tobacco use and prevention policies. 2017.

2. National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health. The Health Consequences of Smoking—50 Years of Progress: A Report of the Surgeon General. Atlanta (GA): Centers for Disease Control and Prevention (US); 2014. (Reports of the Surgeon General).

3. Xu X, Bishop EE, Kennedy SM, Simpson SA, Pechacek TF. Annual healthcare spending attributable to cigarette smoking: an update. Am J Prev Med. 2015 Mar;48(3):326–33.

4. Munafò M, Clark T, Johnstone E, Murphy M, Walton R. The genetic basis for smoking behavior: a systematic review and meta-analysis. Nicotine Tob Res. 2004 Aug;6(4):583–97.

5. Batra V, Patkar AA, Berrettini WH, Weinstein SP, Leone FT. The genetic determinants of smoking. Chest. 2003 May;123(5):1730–9.

6. Liu M, Jiang Y, Wedow R, Li Y, Brazel DM, Chen F, et al. Association studies of up to 1.2 million individuals yield new insights into the genetic etiology of tobacco and alcohol use. Nat Genet. 2019 Jan 14;

7. Erzurumluoglu AM, Liu M, Jackson VE, Barnes DR, Datta G, Melbourne CA, et al. Meta-analysis of up to 622,409 individuals identifies 40 novel smoking behaviour associated genetic loci. Mol Psychiatry. 2019 Jan 7;

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

8. Darland T, Dowling JE. Behavioral screening for cocaine sensitivity in mutagenized zebrafish. Proc Natl Acad Sci USA. 2001 Sep 25;98(20):11691–6.

9. Brennan CH, Parker MO. Zebrafish (Danio rerio) models of substance abuse: Harnessing the capabilities. Behaviour. 2012;149(10–12):1037–62.

10. Engleman EA, Katner SN, Neal-Beliveau BS. Caenorhabditis elegans as a Model to Study the Molecular and Genetic Mechanisms of Drug Addiction. Prog Mol Biol Transl Sci. 2016;137:229–52.

11. Shipley AT, Imeh-Nathaniel A, Orfanakos VB, Wormack LN, Huber R, Nathaniel TI. The Sensitivity of the Crayfish Reward System to Mammalian Drugs of Abuse. Front Physiol. 2017

12. Berridge KC, Kringelbach ML. Affective neuroscience of pleasure: reward in humans and animals. Psychopharmacology (Berl). 2008 Aug;199(3):457–80.

13. Tzschentke TM. Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. Prog Neurobiol. 1998 Dec;56(6):613–72.

14. Ninkovic J, Folchert A, Makhankov YV, Neuhauss SCF, Sillaber I, Straehle U, et al. Genetic identification of AChE as a positive modulator of addiction to the psychostimulant D-amphetamine in zebrafish. Journal of Neurobiology. 2006 Apr;66(5):463–75.

15. Ackerman KM, Nakkula R, Zirger JM, Beattie CE, Boyd RT. Cloning and spatiotemporal expression of zebrafish neuronal nicotinic acetylcholine receptor alpha 6 and alpha 4 subunit RNAs. Dev Dyn. 2009 Apr;238(4):980–92.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

16. Zirger JM, Beattie CE, McKay DB, Thomas Boyd R. Cloning and expression of zebrafish neuronal nicotinic acetylcholine receptors. Gene Expression Patterns. 2003 Dec 1;3(6):747–54.

17. Papke RL, Ono F, Stokes C, Urban J, Boyd RT. The nicotinic acetylcholine receptors of zebrafish and an evaluation of pharmacological tools used for their study. Biochem Pharmacol. 2012 Aug 1;84(3):352–65.

18. Ponzoni L, Braida D, Pucci L, Andrea D, Fasoli F, Manfredi I, et al. The cytisine derivatives, CC4 and CC26, reduce nicotine-induced conditioned place preference in zebrafish by acting on heteromeric neuronal nicotinic acetylcholine receptors. Psychopharmacology (Berl). 2014 Dec;231(24):4681–93.

19. Kily LJM, Cowe YCM, Hussain O, Patel S, McElwaine S, Cotter FE, et al. Gene expression changes in a zebrafish model of drug dependency suggest conservation of neuro-adaptation pathways. J Exp Biol. 2008 May;211(Pt 10):1623–34.

20. Brock AJ, Goody SMG, Mead AN, Sudwarts A, Parker MO, Brennan CH. Assessing the Value of the Zebrafish Conditioned Place Preference Model for Predicting Human Abuse Potential. J Pharmacol Exp Ther. 2017 Oct;363(1):66–79.

21. Kedikian X, Faillace MP, Bernabeu R. Behavioral and Molecular Analysis of Nicotine-Conditioned Place Preference in Zebrafish. PLOS ONE. 2013 Jul 24;8(7):e69453.

22. Loeber S, Croissant B, Nakovics H, Zimmer A, Georgi A, Klein S, et al. The startle reflex in alcohol-dependent patients: changes after cognitive-behavioral therapy and predictive validity for drinking behavior. A pilot study. Psychother Psychosom. 2007;76(6):385–90.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

23. Vrana SR, Calhoun PS, Dennis MF, Kirby AC, Beckham JC. Acoustic startle and prepulse inhibition predict smoking lapse in posttraumatic stress disorder. J Psychopharmacol. 2015 Oct 1;29(10):1070–6.

24. Kumari V, Gray JA. Smoking withdrawal, nicotine dependence and prepulse inhibition of the acoustic startle reflex. Psychopharmacology. 1999 Jan 1;141(1):11–5.

25. Hall BJ, Slade S, Wells C, Rose JE, Levin ED. Bupropion–varenicline interactions and nicotine self-administration behavior in rats. Pharmacology Biochemistry and Behavior. 2015 Mar 1;130:84–9.

26. O'Connor EC, Parker D, Rollema H, Mead AN. The α4β2 nicotinic acetylcholine-receptor partial agonist varenicline inhibits both nicotine self-administration following repeated dosing and reinstatement of nicotine seeking in rats. Psychopharmacology. 2010 Feb 1;208(3):365–76.

27. Le Foll B, Chakraborty-Chatterjee M, Lev-Ran S, Barnes C, Pushparaj A, Gamaleddin I, et al. Varenicline decreases nicotine self-administration and cue-induced reinstatement of nicotine-seeking behaviour in rats when a long pretreatment time is used. Int J Neuropsychopharmacol. 2012 Oct 1;15(9):1265–74.

28. Brennan CH, Parmar A, Kily LKM, Ananthathevan A, Doshi A, Patel S. Conditioned Place Preference Models of Drug Dependence and Relapse to Drug Seeking: Studies with Nicotine and Ethanol. In: Kalueff AV, Cachat JM, editors. Zebrafish Models in Neurobehavioral Research. Totowa, NJ: Humana Press; 2011. p. 163–79. (Neuromethods).

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

29. Kettleborough RNW, Busch-Nentwich EM, Harvey SA, Dooley CM, Bruijn E de, Eeden F van, et al. A systematic genome-wide analysis of zebrafish protein-coding gene function. Nature. 2013 Apr;496(7446):494–7.

30. Brose K, Bland KS, Wang KH, Arnott D, Henzel W, Goodman CS, et al. Slit Proteins Bind Robo Receptors and Have an Evolutionarily Conserved Role in Repulsive Axon Guidance. Cell. 1999 Mar 19;96(6):795–806.

31. Smidt MP, Burbach JPH. How to make a mesodiencephalic dopaminergic neuron. Nat Rev Neurosci. 2007 Jan;8(1):21–32.

32. Miyasaka N, Sato Y, Yeo S-Y, Hutson LD, Chien C-B, Okamoto H, et al. Robo2 is required for establishment of a precise glomerular map in the zebrafish olfactory system. Development. 2005 Mar 15;132(6):1283–93.

33. Turner KJ, Hoyle J, Valdivia LE, Cerveny KL, Hart W, Mangoli M, et al. Abrogation of Stem Loop Binding Protein (Slbp) function leads to a failure of cells to transition from proliferation to differentiation, retinal coloboma and midline axon guidance deficits. PLOS ONE. 2019 Jan 29;14(1):e0211073.

34. Quednow BB, Wagner M, Westheide J, Beckmann K, Bliesener N, Maier W, et al. Sensorimotor gating and habituation of the startle response in schizophrenic patients randomly treated with amisulpride or olanzapine. Biol Psychiatry. 2006 Mar 15;59(6):536–45.

35. Rankin CH, Abrams T, Barry RJ, Bhatnagar S, Clayton D, Colombo J, et al. Habituation Revisited: An Updated and Revised Description of the Behavioral Characteristics of Habituation. Neurobiol Learn Mem. 2009 Sep;92(2):135–8.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

36. Kaprio J. Twin Studies in Finland 2006. Twin Research and Human Genetics. 2006 Dec;9(6):772–7.

37. Lawson ND, Wolfe SA. Forward and Reverse Genetic Approaches for the Analysis of Vertebrate Development in the Zebrafish. Developmental Cell. 2011 Jul 19;21(1):48–64.

38. Morlot C, Thielens NM, Ravelli RBG, Hemrika W, Romijn RA, Gros P, et al. Structural insights into the Slit-Robo complex. PNAS. 2007 Sep 18;104(38):14923–8.

39. Blockus H, Chédotal A. The multifaceted roles of Slits and Robos in cortical circuits: from proliferation to axon guidance and neurological diseases. Current Opinion in Neurobiology. 2014 Aug 1;27:82–8.

40. Marillat V, Cases O, Nguyenf-Ba-Charvet KT, Tessier-Lavigne M, Sotelo C, Chédotal A. Spatiotemporal expression patterns of slit and robo genes in the rat brain. Journal of Comparative Neurology. 2002 Jan 7;442(2):130–55.

41. Yuan W, Rao Y, Babiuk RP, Greer J, Wu JY, Ornitz DM. A genetic model for a central (septum transversum) congenital diaphragmatic hernia in mice lacking Slit3. PNAS. 2003 Apr 29;100(9):5217–22.

42. Liu J, Zhang L, Wang D, Shen H, Jiang M, Mei P, et al. Congenital diaphragmatic hernia, kidney agenesis and cardiac defects associated with Slit3-deficiency in mice. Mechanisms of Development. 2003 Sep 1;120(9):1059–70.

43. Long H, Sabatier C, Le Ma, Plump A, Yuan W, Ornitz DM, et al. Conserved Roles for Slit and Robo Proteins in Midline Commissural Axon Guidance. Neuron. 2004 Apr 22;42(2):213–23.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

44. Halberstadt AL, Geyer MA. Habituation and sensitization of acoustic startle: opposite influences of dopamine D1 and D2-family receptors. Neurobiol Learn Mem. 2009 Sep;92(2):243–8.

45. Burgess HA, Granato M. Sensorimotor gating in larval zebrafish. J Neurosci. 2007 May 2;27(18):4984–94.

46. Fletcher PJ, Lê AD, Higgins GA. Serotonin receptors as potential targets for modulation of nicotine use and dependence. In: Di Giovann G, Di Matteo V, Esposito E, editors. Progress in Brain Research. Elsevier; 2008 [cited 2019 Jul 16]. p. 361–83. (Serotonin–Dopamine Interaction: Experimental Evidence and Therapeutic Relevance; vol. 172).

47. Olausson P, Engel JA, Söderpalm B. Involvement of serotonin in nicotine dependence: Processes relevant to positive and negative regulation of drug intake. Pharmacology Biochemistry and Behavior. 2002 Apr 1;71(4):757–71.

48. Roberts DCS, Loh EA, Baker GB, Vickers G. Lesions of central serotonin systems affect responding on a progressive ratio schedule reinforced either by intravenous cocaine or by food. Pharmacology Biochemistry and Behavior. 1994 Sep 1;49(1):177–82.

49. LeMarquand D, Pihl RO, Benkelfat C. Serotonin and alcohol intake, abuse, and dependence: Findings of animal studies. Biological Psychiatry. 1994 Sep 15;36(6):395–421.

50. Opitz K, Weischer ML. Volitional oral intake of nicotine in tupaias: drug-induced alterations. Drug Alcohol Depend. 1988 May;21(2):99–104.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

51. Seth P, Cheeta S, Tucci S, File SE. Nicotinic–serotonergic interactions in brain and behaviour. Pharmacology Biochemistry and Behavior. 2002 Apr 1;71(4):795–805.

52. Rasmussen K, Kallman MJ, Helton DR. Serotonin-1A antagonists attenuate the effects of nicotine withdrawal on the auditory startle response. Synapse. 1997 Oct;27(2):145–52.

53. Rasmussen K, Calligaro DO, Czachura JF, Dreshfield-Ahmad LJ, Evans DC, Hemrick-Luecke SK, et al. The novel 5-Hydroxytryptamine(1A) antagonist LY426965: effects on nicotine withdrawal and interactions with fluoxetine. J Pharmacol Exp Ther. 2000 Aug;294(2):688–700.

54. Harrison AA, Liem YT, Markou A. Fluoxetine combined with a serotonin-1A receptor antagonist reversed reward deficits observed during nicotine and amphetamine withdrawal in rats. Neuropsychopharmacology. 2001 Jul;25(1):55–71.

55. Dao JM, McQuown SC, Loughlin SE, Belluzzi JD, Leslie FM. Nicotine alters limbic function in adolescent rat by a 5-HT1A receptor mechanism. Neuropsychopharmacology. 2011 Jun;36(7):1319–31.

56. Švob Štrac D, Pivac N, Mück-Šeler D. The serotonergic system and cognitive function. Transl Neurosci. 2016 May 9;7(1):35–49.

57. Carrard A, Salzmann A, Malafosse A, Karege F. Increased DNA methylation status of the serotonin receptor 5HTR1A gene promoter in schizophrenia and bipolar disorder. J Affect Disord. 2011 Aug;132(3):450–3.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

58. Shi Y, Zhao X, Yu L, Tao R, Tang J, La Y, et al. Genetic structure adds power to detect schizophrenia susceptibility at SLIT3 in the Chinese Han population. Genome Res. 2004 Jul;14(7):1345–9.

59. Parker MO, Evans AM-D, Brock AJ, Combe FJ, Teh M-T, Brennan CH. Moderate alcohol exposure during early brain development increases stimulus-response habits in adulthood. Addict Biol. 2016 Jan;21(1):49–60.

60. Cohen J. A power primer. Psychol Bull. 1992 Jul;112(1):155–9.

61. Hamajima N, Saito T, Matsuo K, Kozaki K, Takahashi T, Tajima K. Polymerase chain reaction with confronting two-pair primers for polymorphism genotyping. Jpn J Cancer Res. 2000 Sep;91(9):865–8.

62. Driever W, Rath M, Nitschke R, Filippi A, Ronneberger O, Driever W. Generation of high quality multi-view confocal 3D datasets of zebrafish larval brains suitable for analysis using Virtual Brain Explorer (ViBE-Z) software. Protocol Exchange. 2012 Jun 22 [cited 2018 Apr 26];

63. Tran S, Nowicki M, Muraleetharan A, Gerlai R. Differential effects of dopamine D1 and D 2/3 receptor antagonism on motor responses. Psychopharmacology (Berl). 2015 Feb;232(4):795–806.

64. Perrault G, Depoortere R, Morel E, Sanger DJ, Scatton B. Psychopharmacological profile of amisulpride: an antipsychotic drug with presynaptic D2/D3 dopamine receptor antagonist activity and limbic selectivity. J Pharmacol Exp Ther. 1997 Jan;280(1):73–82.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

65. Schoemaker H, Claustre Y, Fage D, Rouquier L, Chergui K, Curet O, et al. Neurochemical characteristics of amisulpride, an atypical dopamine D2/D3 receptor antagonist with both presynaptic and limbic selectivity. J Pharmacol Exp Ther. 1997 Jan;280(1):83–97.

66. Parker M, Brennan C. Translational Pharmacology of a Putative Measure of Motor Impulsivity in Larval Zebrafish. Current Psychopharmacology. 2016;5(2):73-84.

67. Tang R, Dodd A, Lai D, McNabb WC, Love DR. Validation of zebrafish (Danio rerio) reference genes for quantitative real-time RT-PCR normalization. Acta Biochim Biophys Sin (Shanghai). 2007 May;39(5):384–90.

68. Collier AD, Echevarria DJ. The utility of the zebrafish model in conditioned place preference to assess the rewarding effects of drugs. Behavioural Pharmacology. 2013 Sep;24(5 and 6):375.

69. Martineau AR, James WY, Hooper RL, Barnes NC, Jolliffe DA, Greiller CL, et al. Vitamin D3 supplementation in patients with chronic obstructive pulmonary disease (ViDiCO): a multicentre, double-blind, randomised controlled trial. Lancet Respir Med. 2015 Feb;3(2):120–30.

70. Martineau AR, MacLaughlin BD, Hooper RL, Barnes NC, Jolliffe DA, Greiller CL, et al. Double-blind randomised placebo-controlled trial of bolus-dose vitamin D3 supplementation in adults with asthma (ViDiAs). Thorax. 2015 May;70(5):451–7.

71. Martineau AR, Hanifa Y, Witt KD, Barnes NC, Hooper RL, Patel M, et al. Double-blind randomised controlled trial of vitamin D3 supplementation for the prevention of acute respiratory infection in older adults and their carers (ViDiFlu). Thorax. 2015 Oct;70(10):953–60.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

72. Loukola A, Wedenoja J, Keskitalo-Vuokko K, Broms U, Korhonen T, Ripatti S, et al. Genome-wide association study on detailed profiles of smoking behavior and nicotine dependence in a twin sample. Mol Psychiatry. 2014 May;19(5):615–24.

73. Loukola A, Broms U, Maunu H, Widén E, Heikkilä K, Siivola M, et al. Linkage of nicotine dependence and smoking behavior on 10q, 7q and 11p in twins with homogeneous genetic background. Pharmacogenomics J. 2008 Jun;8(3):209–19.

74. Broms U, Wedenoja J, Largeau MR, Korhonen T, Pitkäniemi J, Keskitalo-Vuokko K, et al. Analysis of detailed phenotype profiles reveals CHRNA5-CHRNA3-CHRNB4 gene cluster association with several nicotine dependence traits. Nicotine Tob Res. 2012 Jun;14(6):720–33.

75. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988 Feb 11;16(3):1215.

76. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007 Sep;81(3):559–75.

77. Zhou X, Stephens M. Genome-wide Efficient Mixed Model Analysis for Association Studies. Nat Genet. 2012 Jun 17;44(7):821–4.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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)

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

31

32

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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).

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

			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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

- ***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 (≥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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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	
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	-

14

15

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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.

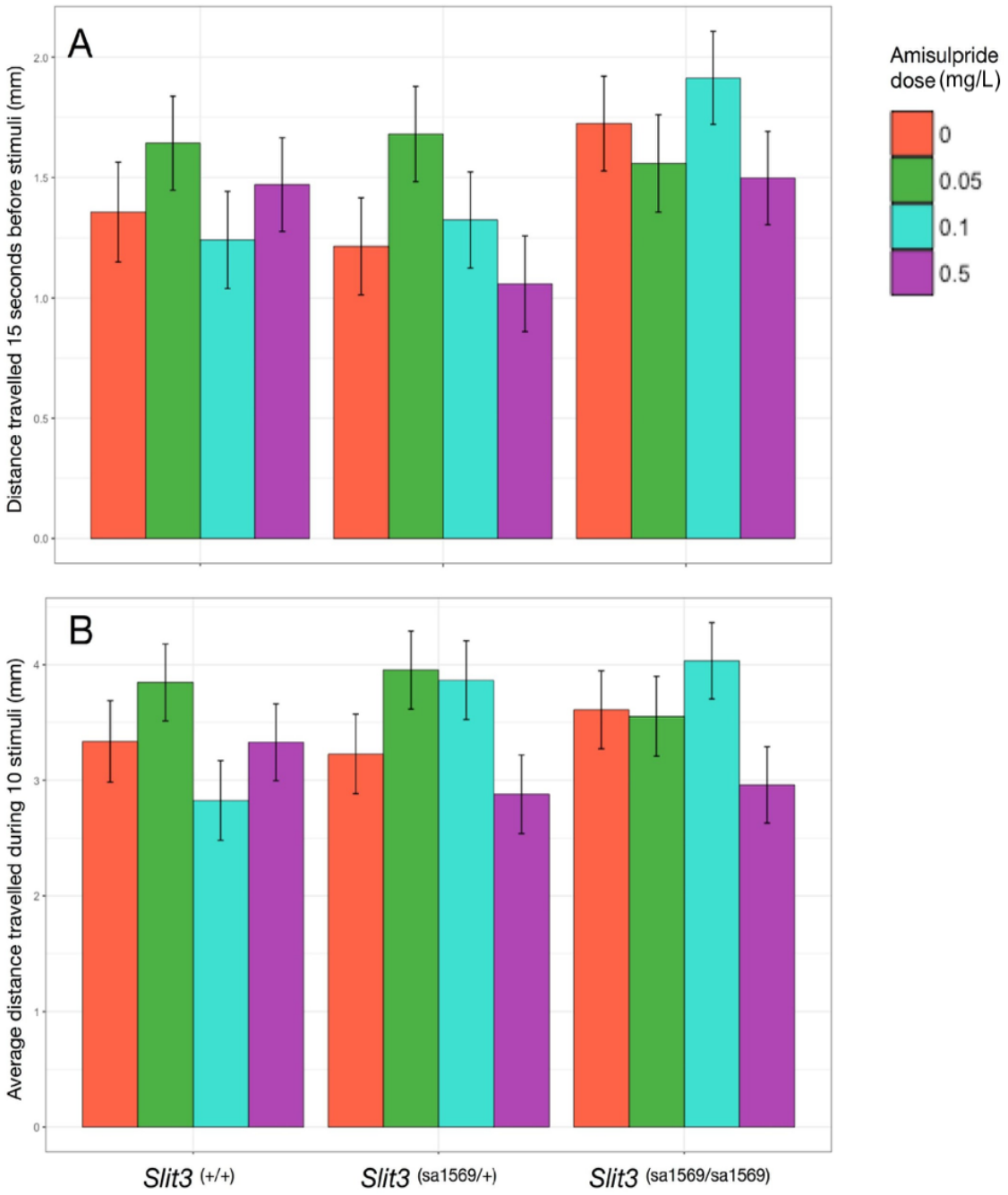
SNP	OR	SE	L95	U95	P value
rs2938774	0.7253	0.1418	0.5493	0.9578	0.02357
rs11742567	1.328	0.1538	0.9825	1.796	0.06496
rs4282339	0.7277	0.1815	0.5099	1.039	0.07991
rs297886	1.328	0.176	0.9405	1.875	0.1071
rs9688032	1.269	0.1495	0.9467	1.701	0.111
rs7728604	1.198	0.1446	0.9024	1.591	0.2112
rs1345588	0.7788	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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.



33

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*

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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).

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Supplementary references

1. World Health Organization, Bloomberg Philanthropies. WHO report on the global tobacco epidemic, 2017: monitoring tobacco use and prevention policies. 2017.

2. National Center for Chronic Disease Prevention and Health Promotion (US) Office on Smoking and Health. The Health Consequences of Smoking—50 Years of Progress: A Report of the Surgeon General [Internet]. Atlanta (GA): Centers for Disease Control and Prevention (US); 2014 [cited 2018 Jul 20]. (Reports of the Surgeon General). Available from: http://www.ncbi.nlm.nih.gov/books/NBK179276/

3. Xu X, Bishop EE, Kennedy SM, Simpson SA, Pechacek TF. Annual healthcare spending attributable to cigarette smoking: an update. Am J Prev Med. 2015 Mar;48(3):326–33.

4. Munafò M, Clark T, Johnstone E, Murphy M, Walton R. The genetic basis for smoking behavior: a systematic review and meta-analysis. Nicotine Tob Res. 2004 Aug;6(4):583–97.

5. Batra V, Patkar AA, Berrettini WH, Weinstein SP, Leone FT. The genetic determinants of smoking. Chest. 2003 May;123(5):1730–9.

6. Liu M, Jiang Y, Wedow R, Li Y, Brazel DM, Chen F, et al. Association studies of up to 1.2 million individuals yield new insights into the genetic etiology of tobacco and alcohol use. Nat Genet. 2019 Jan 14;

7. Erzurumluoglu AM, Liu M, Jackson VE, Barnes DR, Datta G, Melbourne CA, et al. Meta-analysis of up to 622,409 individuals identifies 40 novel smoking behaviour associated genetic loci. Mol Psychiatry. 2019 Jan 7;

8. Darland T, Dowling JE. Behavioral screening for cocaine sensitivity in mutagenized zebrafish. Proc Natl Acad Sci USA. 2001 Sep 25;98(20):11691–6.

9. Brennan CH, Parker MO. Zebrafish (Danio rerio) models of substance abuse: Harnessing the capabilities. Behaviour. 2012;149(10–12):1037–62.

10. Engleman EA, Katner SN, Neal-Beliveau BS. Caenorhabditis elegans as a Model to Study the Molecular and Genetic Mechanisms of Drug Addiction. Prog Mol Biol Transl Sci. 2016;137:229–52.

11. Shipley AT, Imeh-Nathaniel A, Orfanakos VB, Wormack LN, Huber R, Nathaniel TI. The Sensitivity of the Crayfish Reward System to Mammalian Drugs of Abuse. Front Physiol [Internet]. 2017 [cited 2019 Jul 29];8. Available from: https://www.frontiersin.org/articles/10.3389/fphys.2017.01007/full

12. Berridge KC, Kringelbach ML. Affective neuroscience of pleasure: reward in humans and animals. Psychopharmacology (Berl). 2008 Aug;199(3):457–80.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

13. Tzschentke TM. Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. Prog Neurobiol. 1998 Dec;56(6):613–72.

14. Ninkovic J, Folchert A, Makhankov YV, Neuhauss SCF, Sillaber I, Straehle U, et al. Genetic identification of AChE as a positive modulator of addiction to the psychostimulant D-amphetamine in zebrafish. Journal of Neurobiology. 2006 Apr;66(5):463–75.

15. Ackerman KM, Nakkula R, Zirger JM, Beattie CE, Boyd RT. Cloning and spatiotemporal expression of zebrafish neuronal nicotinic acetylcholine receptor alpha 6 and alpha 4 subunit RNAs. Dev Dyn. 2009 Apr;238(4):980–92.

16. Zirger JM, Beattie CE, McKay DB, Thomas Boyd R. Cloning and expression of zebrafish neuronal nicotinic acetylcholine receptors. Gene Expression Patterns. 2003 Dec 1;3(6):747–54.

17. Papke RL, Ono F, Stokes C, Urban J, Boyd RT. The nicotinic acetylcholine receptors of zebrafish and an evaluation of pharmacological tools used for their study. Biochem Pharmacol. 2012 Aug 1;84(3):352–65.

18. Ponzoni L, Braida D, Pucci L, Andrea D, Fasoli F, Manfredi I, et al. The cytisine derivatives, CC4 and CC26, reduce nicotine-induced conditioned place preference in zebrafish by acting on heteromeric neuronal nicotinic acetylcholine receptors. Psychopharmacology (Berl). 2014 Dec;231(24):4681–93.

19. Kily LJM, Cowe YCM, Hussain O, Patel S, McElwaine S, Cotter FE, et al. Gene expression changes in a zebrafish model of drug dependency suggest conservation of neuro-adaptation pathways. J Exp Biol. 2008 May;211(Pt 10):1623–34.

20. Brock AJ, Goody SMG, Mead AN, Sudwarts A, Parker MO, Brennan CH. Assessing the Value of the Zebrafish Conditioned Place Preference Model for Predicting Human Abuse Potential. J Pharmacol Exp Ther. 2017 Oct;363(1):66–79.

21. Kedikian X, Faillace MP, Bernabeu R. Behavioral and Molecular Analysis of Nicotine-Conditioned Place Preference in Zebrafish. PLOS ONE. 2013 Jul 24;8(7):e69453.

22. Loeber S, Croissant B, Nakovics H, Zimmer A, Georgi A, Klein S, et al. The startle reflex in alcohol-dependent patients: changes after cognitive-behavioral therapy and predictive validity for drinking behavior. A pilot study. Psychother Psychosom. 2007;76(6):385–90.

23. Vrana SR, Calhoun PS, Dennis MF, Kirby AC, Beckham JC. Acoustic startle and prepulse inhibition predict smoking lapse in posttraumatic stress disorder. J Psychopharmacol. 2015 Oct 1;29(10):1070–6.

24. Kumari V, Gray JA. Smoking withdrawal, nicotine dependence and prepulse inhibition of the acoustic startle reflex. Psychopharmacology. 1999 Jan 1;141(1):11–5.

25. Hall BJ, Slade S, Wells C, Rose JE, Levin ED. Bupropion–varenicline interactions and nicotine self-administration behavior in rats. Pharmacology Biochemistry and Behavior. 2015 Mar 1;130:84–9.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

26. O'Connor EC, Parker D, Rollema H, Mead AN. The α4β2 nicotinic acetylcholine-receptor partial agonist varenicline inhibits both nicotine self-administration following repeated dosing and reinstatement of nicotine seeking in rats. Psychopharmacology. 2010 Feb 1;208(3):365–76.

27. Le Foll B, Chakraborty-Chatterjee M, Lev-Ran S, Barnes C, Pushparaj A, Gamaleddin I, et al. Varenicline decreases nicotine self-administration and cue-induced reinstatement of nicotine-seeking behaviour in rats when a long pretreatment time is used. Int J Neuropsychopharmacol. 2012 Oct 1;15(9):1265–74.

28. Brennan CH, Parmar A, Kily LKM, Ananthathevan A, Doshi A, Patel S. Conditioned Place Preference Models of Drug Dependence and Relapse to Drug Seeking: Studies with Nicotine and Ethanol. In: Kalueff AV, Cachat JM, editors. Zebrafish Models in Neurobehavioral Research [Internet]. Totowa, NJ: Humana Press; 2011 [cited 2019 Apr 29]. p. 163–79. (Neuromethods). Available from: https://doi.org/10.1007/978-1-60761-922-2_7

29. Kettleborough RNW, Busch-Nentwich EM, Harvey SA, Dooley CM, Bruijn E de, Eeden F van, et al. A systematic genome-wide analysis of zebrafish protein-coding gene function. Nature. 2013 Apr;496(7446):494–7.

30. Brose K, Bland KS, Wang KH, Arnott D, Henzel W, Goodman CS, et al. Slit Proteins Bind Robo Receptors and Have an Evolutionarily Conserved Role in Repulsive Axon Guidance. Cell. 1999 Mar 19;96(6):795–806.

31. Smidt MP, Burbach JPH. How to make a mesodiencephalic dopaminergic neuron. Nat Rev Neurosci. 2007 Jan;8(1):21–32.

32. Miyasaka N, Sato Y, Yeo S-Y, Hutson LD, Chien C-B, Okamoto H, et al. Robo2 is required for establishment of a precise glomerular map in the zebrafish olfactory system. Development. 2005 Mar 15;132(6):1283–93.

33. Turner KJ, Hoyle J, Valdivia LE, Cerveny KL, Hart W, Mangoli M, et al. Abrogation of Stem Loop Binding Protein (Slbp) function leads to a failure of cells to transition from proliferation to differentiation, retinal coloboma and midline axon guidance deficits. PLOS ONE. 2019 Jan 29;14(1):e0211073.

34. Quednow BB, Wagner M, Westheide J, Beckmann K, Bliesener N, Maier W, et al. Sensorimotor gating and habituation of the startle response in schizophrenic patients randomly treated with amisulpride or olanzapine. Biol Psychiatry. 2006 Mar 15;59(6):536–45.

35. Rankin CH, Abrams T, Barry RJ, Bhatnagar S, Clayton D, Colombo J, et al. Habituation Revisited: An Updated and Revised Description of the Behavioral Characteristics of Habituation. Neurobiol Learn Mem. 2009 Sep;92(2):135–8.

36. Kaprio J. Twin Studies in Finland 2006. Twin Research and Human Genetics. 2006 Dec;9(6):772–7.

37. Lawson ND, Wolfe SA. Forward and Reverse Genetic Approaches for the Analysis of Vertebrate Development in the Zebrafish. Developmental Cell. 2011 Jul 19;21(1):48–64.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

163 38. Morlot C, Thielens NM, Ravelli RBG, Hemrika W, Romijn RA, Gros P, et al. Structural
164 insights into the Slit-Robo complex. PNAS. 2007 Sep 18;104(38):14923–8.

165 39. Blockus H, Chédotal A. The multifaceted roles of Slits and Robos in cortical circuits:
166 from proliferation to axon guidance and neurological diseases. Current Opinion in
167 Neurobiology. 2014 Aug 1;27:82–8.

168 40. Marillat V, Cases O, Nguyenf□Ba□Charvet KT, Tessier□Lavigne M, Sotelo C,
169 Chédotal A. Spatiotemporal expression patterns of slit and robo genes in the rat brain.
170 Journal of Comparative Neurology. 2002 Jan 7;442(2):130–55.

171 41. Yuan W, Rao Y, Babiuk RP, Greer J, Wu JY, Ornitz DM. A genetic model for a central
172 (septum transversum) congenital diaphragmatic hernia in mice lacking Slit3. PNAS.
173 2003 Apr 29;100(9):5217–22.

174 42. Liu J, Zhang L, Wang D, Shen H, Jiang M, Mei P, et al. Congenital diaphragmatic
175 hernia, kidney agenesis and cardiac defects associated with Slit3-deficiency in mice.
176 Mechanisms of Development. 2003 Sep 1;120(9):1059–70.

177 43. Long H, Sabatier C, Le Ma, Plump A, Yuan W, Ornitz DM, et al. Conserved Roles for
178 Slit and Robo Proteins in Midline Commissural Axon Guidance. Neuron. 2004 Apr
179 22;42(2):213–23.

180 44. Halberstadt AL, Geyer MA. Habituation and sensitization of acoustic startle: opposite
181 influences of dopamine D1 and D2-family receptors. Neurobiol Learn Mem. 2009
182 Sep;92(2):243–8.

183 45. Burgess HA, Granato M. Sensorimotor gating in larval zebrafish. J Neurosci. 2007 May
184 2;27(18):4984–94.

185 46. Fletcher PJ, Lê AD, Higgins GA. Serotonin receptors as potential targets for modulation
186 of nicotine use and dependence. In: Di Giovann G, Di Matteo V, Esposito E, editors.
187 Progress in Brain Research [Internet]. Elsevier; 2008 [cited 2019 Jul 16]. p. 361–83.
188 (Serotonin–Dopamine Interaction: Experimental Evidence and Therapeutic Relevance;
189 vol. 172). Available from:
190 http://www.sciencedirect.com/science/article/pii/S0079612308009187

191 47. Olausson P, Engel JA, Söderpalm B. Involvement of serotonin in nicotine dependence:
192 Processes relevant to positive and negative regulation of drug intake. Pharmacology
193 Biochemistry and Behavior. 2002 Apr 1;71(4):757–71.

194 48. Roberts DCS, Loh EA, Baker GB, Vickers G. Lesions of central serotonin systems
195 affect responding on a progressive ratio schedule reinforced either by intravenous
196 cocaine or by food. Pharmacology Biochemistry and Behavior. 1994 Sep 1;49(1):177–
197 82.

198 49. LeMarquand D, Pihl RO, Benkelfat C. Serotonin and alcohol intake, abuse, and
199 dependence: Findings of animal studies. Biological Psychiatry. 1994 Sep 15;36(6):395–
200 421.

201 50. Opitz K, Weischer ML. Volitional oral intake of nicotine in tupaias: drug-induced
202 alterations. Drug Alcohol Depend. 1988 May;21(2):99–104.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

203 51. Seth P, Cheeta S, Tucci S, File SE. Nicotinic–serotonergic interactions in brain and
204 behaviour. Pharmacology Biochemistry and Behavior. 2002 Apr 1;71(4):795–805.

205 52. Rasmussen K, Kallman MJ, Helton DR. Serotonin-1A antagonists attenuate the effects
206 of nicotine withdrawal on the auditory startle response. Synapse. 1997 Oct;27(2):145–
207 52.

208 53. Rasmussen K, Calligaro DO, Czachura JF, Dreshfield-Ahmad LJ, Evans DC, Hemrick-
209 Luecke SK, et al. The novel 5-Hydroxytryptamine(1A) antagonist LY426965: effects on
210 nicotine withdrawal and interactions with fluoxetine. J Pharmacol Exp Ther. 2000
211 Aug;294(2):688–700.

212 54. Harrison AA, Liem YT, Markou A. Fluoxetine combined with a serotonin-1A receptor
213 antagonist reversed reward deficits observed during nicotine and amphetamine
214 withdrawal in rats. Neuropsychopharmacology. 2001 Jul;25(1):55–71.

215 55. Dao JM, McQuown SC, Loughlin SE, Belluzzi JD, Leslie FM. Nicotine alters limbic
216 function in adolescent rat by a 5-HT1A receptor mechanism.
217 Neuropsychopharmacology. 2011 Jun;36(7):1319–31.

218 56. Švob Štrac D, Pivac N, Mück-Šeler D. The serotonergic system and cognitive function.
219 Transl Neurosci. 2016 May 9;7(1):35–49.

220 57. Carrard A, Salzmann A, Malafosse A, Karege F. Increased DNA methylation status of
221 the serotonin receptor 5HTR1A gene promoter in schizophrenia and bipolar disorder. J
222 Affect Disord. 2011 Aug;132(3):450–3.

223 58. Shi Y, Zhao X, Yu L, Tao R, Tang J, La Y, et al. Genetic structure adds power to detect
224 schizophrenia susceptibility at SLIT3 in the Chinese Han population. Genome Res.
225 2004 Jul;14(7):1345–9.

226 59. Parker MO, Evans AM-D, Brock AJ, Combe FJ, Teh M-T, Brennan CH. Moderate
227 alcohol exposure during early brain development increases stimulus-response habits in
228 adulthood. Addict Biol. 2016 Jan;21(1):49–60.

229 60. Cohen J. A power primer. Psychol Bull. 1992 Jul;112(1):155–9.

230 61. Hamajima N, Saito T, Matsuo K, Kozaki K, Takahashi T, Tajima K. Polymerase chain
231 reaction with confronting two-pair primers for polymorphism genotyping. Jpn J Cancer
232 Res. 2000 Sep;91(9):865–8.

233 62. Driever W, Rath M, Nitschke R, Filippi A, Ronneberger O, Driever W. Generation of
234 high quality multi-view confocal 3D datasets of zebrafish larval brains suitable for
235 analysis using Virtual Brain Explorer (ViBE-Z) software. Protocol Exchange [Internet].
236 2012 Jun 22 [cited 2018 Apr 26]; Available from:
237 http://www.nature.com/protocolexchange/protocols/2408

238 63. Tran S, Nowicki M, Muraleetharan A, Gerlai R. Differential effects of dopamine D1 and
239 D 2/3 receptor antagonism on motor responses. Psychopharmacology (Berl). 2015
240 Feb;232(4):795–806.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

64. Perrault G, Depoortere R, Morel E, Sanger DJ, Scatton B. Psychopharmacological profile of amisulpride: an antipsychotic drug with presynaptic D2/D3 dopamine receptor antagonist activity and limbic selectivity. J Pharmacol Exp Ther. 1997 Jan;280(1):73–82.

65. Schoemaker H, Claustre Y, Fage D, Rouquier L, Chergui K, Curet O, et al. Neurochemical characteristics of amisulpride, an atypical dopamine D2/D3 receptor antagonist with both presynaptic and limbic selectivity. J Pharmacol Exp Ther. 1997 Jan;280(1):83–97.

66. Brennan MOP and CH. Translational Pharmacology of a Putative Measure of Motor Impulsivity in Larval Zebrafish [Internet]. Current Psychopharmacology. 2016 [cited 2018 Apr 26]. Available from: http://www.eurekaselect.com/142514/article

67. Tang R, Dodd A, Lai D, McNabb WC, Love DR. Validation of zebrafish (Danio rerio) reference genes for quantitative real-time RT-PCR normalization. Acta Biochim Biophys Sin (Shanghai). 2007 May;39(5):384–90.

68. Collier AD, Echevarria DJ. The utility of the zebrafish model in conditioned place preference to assess the rewarding effects of drugs. Behavioural Pharmacology. 2013 Sep;24(5 and 6):375.

69. Martineau AR, James WY, Hooper RL, Barnes NC, Jolliffe DA, Greiller CL, et al. Vitamin D3 supplementation in patients with chronic obstructive pulmonary disease (ViDiCO): a multicentre, double-blind, randomised controlled trial. Lancet Respir Med. 2015 Feb;3(2):120–30.

70. Martineau AR, MacLaughlin BD, Hooper RL, Barnes NC, Jolliffe DA, Greiller CL, et al. Double-blind randomised placebo-controlled trial of bolus-dose vitamin D3 supplementation in adults with asthma (ViDiAs). Thorax. 2015 May;70(5):451–7.

71. Martineau AR, Hanifa Y, Witt KD, Barnes NC, Hooper RL, Patel M, et al. Double-blind randomised controlled trial of vitamin D3 supplementation for the prevention of acute respiratory infection in older adults and their carers (ViDiFlu). Thorax. 2015 Oct;70(10):953–60.

72. Loukola A, Wedenoja J, Keskitalo-Vuokko K, Broms U, Korhonen T, Ripatti S, et al. Genome-wide association study on detailed profiles of smoking behavior and nicotine dependence in a twin sample. Mol Psychiatry. 2014 May;19(5):615–24.

73. Loukola A, Broms U, Maunu H, Widén E, Heikkilä K, Siivola M, et al. Linkage of nicotine dependence and smoking behavior on 10q, 7q and 11p in twins with homogeneous genetic background. Pharmacogenomics J. 2008 Jun;8(3):209–19.

74. Broms U, Wedenoja J, Largeau MR, Korhonen T, Pitkäniemi J, Keskitalo-Vuokko K, et al. Analysis of detailed phenotype profiles reveals CHRNA5-CHRNA3-CHRNB4 gene cluster association with several nicotine dependence traits. Nicotine Tob Res. 2012 Jun;14(6):720–33.

75. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988 Feb 11;16(3):1215.

bioRxiv preprint doi: https://doi.org/10.1101/453928; this version posted August 30, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

281 76. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR, Bender D, et al. PLINK:
282 a tool set for whole-genome association and population-based linkage analyses. Am J
283 Hum Genet. 2007 Sep;81(3):559–75.

284 77. Zhou X, Stephens M. Genome-wide Efficient Mixed Model Analysis for Association
285 Studies. Nat Genet. 2012 Jun 17;44(7):821–4.

286