Identifying novel genes associated with response to nicotine in a zebrafish model of drug dependence.

A thesis presented for the degree of Doctor of Philosophy

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Statement of Originality

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Abstract

Tobacco addiction is a leading preventable cause of death worldwide and places a heavy social and financial burden on society. There exists a substantial genetic variability in smoking behavior, the mechanisms of which are largely unknown. Despite significant advances in sequencing power, progress in the identification of genetic variants affecting smoking behavior based on human genome wide association studies has been slow. Thus this thesis investigates the utility of zebrafish as a model species in which to search for genetic variants affecting nicotine seeking. The work is based on the premise that as zebrafish are vertebrate with conserved neurochemical pathways and circuitry with humans, and the pathways involved in drug mediated reward and addiction are evolutionarily ancient, homologues of genes affecting zebrafish nicotine-seeking behavior will likely affect human smoking behavior. Thus results in zebrafish can be used to direct human genetic studies.

The first result chapter addresses the hypothesis that zebrafish show conserved reward responses to common drugs of abuse. A conditioned place preference assay is used to assess zebrafish reward responses to stimulants, opioids, benzodiazepines and alcohol. The results indicate that, with the exception of benzodiazepines, reward responses are conserved, supporting the use of this model in a screen for genetic variants affecting nicotine preference. The second and third results chapters describe the findings of a pilot screen of ENU-mutagenized zebrafish provided by the Sanger Institute, Cambridge. I demonstrate that nicotine preference is heritable in fish as in

humans and identify 3 mutant lines that show increased or decreased nicotine place preference. Genotyping indicated that one of the families showing increased nicotine preference carries a predicted loss of function mutation in the slit3 gene. The involvement of this gene in nicotine preference was confirmed in a separate line. Further characterization of this line using qPCR showed *slit3* mutants to have altered developmental expression of key nicotinic and dopaminergic genes.

Having identified the slit3 gene as a locus affecting nicotine seeking in fish, I then tested the hypothesis that results in fish could be used to predict loci that affect human smoking behavior. Cohorts of patients were genotyped for 20 SNPs within the slit3 locus. Results of this analysis identified 1 novel SNP in the *slit3* gene associated with smoking behavior in a cohort of individuals that were heavy smokers. This result was validated in cohorts of low and normal smoking prevalence. These data demonstrate the utility of behavioral assays in zebrafish to identify genes affecting human behavior and pave the way for the use of zebrafish to inform human studies exploring the genetic basis of drug seeking and behavioral disease.

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Chapter 1

General Introduction

The work described in this thesis uses fish to inform human research into novel alleles affecting smoking behavior. In the following section, background to smoking prevalence, the neurobiology of nicotine addiction as well as what is known of the genetic risk factors involved will be covered. In the latter sections the use of zebrafish as a model system for identifying genetic factors and molecular mechanisms of addictive disorders is described.

1.1 Worldwide smoking prevalence, health risks and associated costs

Tobacco addiction is one of the leading preventable causes of death in the world and places a huge social and financial burden on society. There are currently over 1.4 billion smokers worldwide and this figure is expected to rise to 1.9 billion in 2025 (Guindon, de Beyer, & Galbraith, 2003; Shibuya et al., 2003a). Chronic diseases directly caused by smoking include cancers, chronic lung diseases, cardiovascular disease and asthma.

Around 100 million deaths were caused by tobacco in the 20th century with the current annual global death toll from smoking being 5 million. If current trends persist tobacco-related deaths will increase to more than eight million a year by 2030 (Shafey O, Eriksen M, Ross H, & J, 2009), with 80% of those deaths predicted to occur in the developing world. All this adds up to the distinct possibility of tobacco causing up to one billion deaths in the 21st century (Shibuya et al., 2003b).

The act of quitting, even in middle age is remarkably effective in reducing the risk of illness and death. Thus, whilst persistent smoking triples the risk of death in each decade of life, stopping smoking at age 50 years halves the risk, and a smoker who stops at age 30 years has the same life expectancy as someone who has never smoked at all (Peto et al., 2000). It is therefore abundantly clear that public awareness of the dangers of smoking and the implementation of interventions to increase people's chances of quitting has huge benefits on improving quality of life and alleviating the burden smoking exerts on health services.

1.2 The neuro-circuitry underlying addiction

In recent years addiction has become recognized as a disease of memory and learning affecting evolutionarily ancient neural pathways. These pathways will have originally arisen as a means aiding rapid establishment of self-preserving patterns of motor behavior. With excessive exposure to drugs of abuse, these pathways can be hijacked and allow destructive, drug-associated behavior to gain pathological importance. Although the mechanisms are not fully understood, and other systems are undoubtedly involved, it is currently thought that coordinated signaling within dopaminergic and glutamatergic systems integrates the main neurological processes thought to be involved in addictive disorders like motivation, memory and learning (Bonci & Malenka, 1999; Nestler, 2001). Glutamatergic neurotransmission plays a fundamental role in regulating synaptic plasticity (the reconfiguring of neural networks) underlying memory and learning, and dopaminergic neurotransmission within the mesolimbic system underlies reward and motivational drive. Co-ordinated activation of glutamatergic and dopaminergic neurotransmission integrates these two systems and enhances the motivational value of memories thus reinforcing associated patterns of behavior commonly seen in addicts. Repeated stimulation of the mesolimbic reward pathway by drugs of abuse (including nicotine) leads to adaptive changes in gene expression and synaptic organization in the central nervous system. These adaptive changes reinforce drug taking and underlie long-term changes in behavior and dependence (L. J. Kily et al., 2008; Le Foll, Gallo, Le Strat, Lu, & Gorwood, 2009).

The mesolimbic pathway transmits dopamine from the ventral tegmental area (VTA) to the nucleus accumbens. Since the mesolimbic pathway is shown to be associated with feelings of reward and desire, this pathway has been heavily implicated in most neurobiological theories of addiction. Under normal circumstances the brain uses these dopaminergic and glutamate systems to optimize responses that enhance survival. Drugs of abuse, act predominantly by either directly or indirectly increasing dopaminergic transmission in the mesolimbic system. By repeatedly activating these pathways, they are able to induce very long term, or even permanent, alterations in motivational networks, ultimately leading to changes in behavioral control (i.e. addiction) (Berke & Hyman, 2000; Hyman, Malenka, & Nestler, 2006; Kelley, 2004).

Much of the recent progress in identifying lasting neuro-adaptations that are associated with such addiction-related behaviors have come in animal models (Kalivas, 2004; Shaham & Hope, 2005; Weiss et al., 2001). For instance, drug-seeking phenotypes have predominantly been associated with altered basal levels or sensitivity of dopaminergic, serotonergic and glutamate neurotransmission (Kalivas, 2004; Tupala & Tiihonen, 2004; Weiss et al., 2001). Similarly, expression analysis has identified components of a number of other neurotransmitter pathways involved in drug addiction such as the mono-aminergic, glutamatergic and cannabinoid pathways. Several other pathways including 5-hydroxytyramine (5-HT), opioid, gamma-Aminobutyric acid (GABA) and benzodiazepine receptor pathways have also been implicated through altered levels of expression in the nucleus accumbens (NAc), pre-frontal cortex (PFC) or amygdala (Nestler, 2004; O'Brien & Gardner, 2005; Ohkuma, Katsura, & Tsujimura, 2001; Rhodes & Crabbe, 2005). Components of signal transduction pathways such as ERK (extracellularly regulated kinase) and PI3K

(phosphatidylinositol 3-kinase) have been shown to be altered in their expression levels in the brains of animals demonstrating drug dependency ((M. D. Li, Kane, Wang, & Ma, 2004; Lu, Koya, Zhai, Hope, & Shaham, 2006; Pollock, 2002; Rhodes & Crabbe, 2005; Yuferov, Nielsen, Butelman, & Kreek, 2005). Orexins are hypothalamic neuropeptides more traditionally implicated in behaviors like feeding, sleep and arousal. More recent evidence from animal models suggests a role for orexins in reward processing and drug addiction though their interaction with the mesocorticolimbic reward pathway (Sharf, Sarhan, & Dileone, 2010).

All these changes in neurotransmission and gene expression have been proposed to contribute to altered sensitivity to the rewarding effects of drugs of abuse. For these changes to occur however, there needs to be exposure to the drugs that activate these pathways. Nicotine is the active ingredient (although some other compounds involved) in tobacco responsible for its addictive quality. Activation of the mesolimbic pathway via nicotinic acetylcholine receptors underlies its reinforcing properties. Repeated stimulation leads to these permanent changes in neurocircuitry and is why people often persist with compulsive smoking behaviors despite the deleterious effects on health.

1.3 Pharmacodynamics of nicotine & cessation treatments

Nicotine from tobacco is rapidly absorbed through the lungs and buccal mucosa and then transported through the bloodstream and across the blood-brain barrier, reaching

the brain within 10–20 seconds after inhalation (Le Houezec, 2003). The elimination half-life of nicotine in the body is around two hours (Le Houezec, 2003). Nicotine crosses the blood–brain barrier where a variety of neural substrates, especially nicotinic acetylcholine receptors, mediate the reinforcing effects of nicotine and the development of nicotine dependence (D'Souza & Markou, 2011).

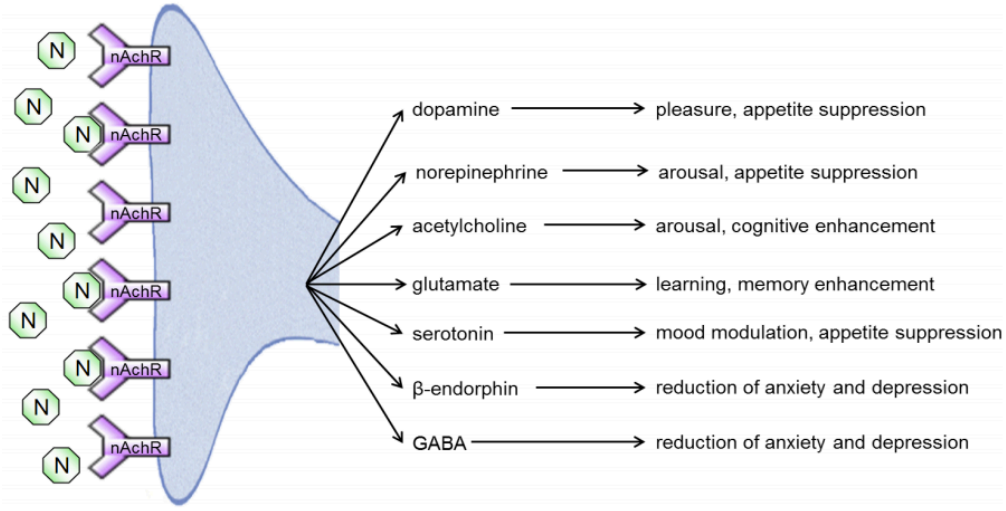


Figure 1.1: Summary of neurotransmitters (and related psychological effects) whose release are potentiated by the binding of nicotine to nAChRs in the brain accounting for the various psychoactive effects of smoking. Figure reproduced with permission (Quaak, van Schooten, & van Schayck, 2013) adapted from8 (Benowitz, 1999).

Through binding to nicotinic acetylcholine receptors (nAChRs), nicotine potentiates the release of various neurotransmitters including dopamine, serotonin noradrenaline and acetylcholine (Pomerleau & Pomerleau, 1984; Pomerleau & Rosecrans, 1989). Nicotinic acetylcholine receptors are broadly distributed in the brain but are mainly concentrated in the cortex, thalamus, hippocampus and amygdale. Many subtypes of the nAchR are known, accounting for nicotine's broad

effects, however the majority of neuronal nAchRs fall into two categories; α7-homopentamers with a low affinity to nicotine and α4β2 heteropentamers which have a high affinity to nicotine. The α4β2 receptors account for over 90% of neuronal nAchRs (Singh & Budhiraja, 2008) with studies suggesting that their presence in the nucleus accumbens plays a major role in the reinforcing effects of nicotine (Mansvelder & McGehee, 2002; McCallum et al., 2006).

NAchRs are present on the cell bodies of dopaminergic neurons in the VTA as well as on pre-synaptic terminals of excitatory glutamatergic neurons and inhibitory gabaergic neurons with which they form synapses, with different combinations of these nAchR receptor subunits being expressd on each of these cell types (Klink, de Kerchove d'Exaerde, Zoli, & Changeux, 2001). As the subunit composition influences the affinity and pharmacodynamics of channel opening and desensitization, the cells upon which the nAChR subunits reside, respond differently to nicotine depending on the particular combination of subunits. When nicotine levels increase in a smoker's blood stream, nAChRs present on the DA and glutamatergic neurons are initially activated leading to an increase in firing of the DA neurons. On top of this, the subunit combination of nAchRs present on GABAergic neurons are more sensitive to desensitization than those on DA neurons leading to a gradual decrease in GABA-ergic inhibition. This leads to an additive effect of nicotine mediated increase in DA transmission within the mesolimbic system by a combination of enhanced excitation and decreased inhibition of the DA neurons in the VTA (P. B. Clarke & Pert, 1985; Corrigall, Franklin, Coen, & Clarke, 1992; Pidoplichko, DeBiasi, Williams, & Dani, 1997).

In addition to nicotine, tobacco smoke contains the monoaminoxidase inhibitors harman and norharman. Monoamine oxidase enzymes break down monoaminergic neurotransmitters like dopamine, serotonin and norepinephrine. It is thought that inhibition of this process in addition to dopamine mediated nicotine reinforcement are the key additive components contributing to the addictive properties of smoking tobacco.

1.4 Types of smoking cessation treatment and their efficacy

Through public awareness and better education, the risks involved in smoking and the health benefits of cessation are well known, and nearly 40 percent of current smokers try to quit each year. Despite this, only approximately 5 percent of quit attempts will still be successful after 6 to 12 months, with the majority failing within the first 8 days (J. R. Hughes, 2004). Professionally administered smoking-cessation therapy improves the odds of a successful quit attempt after 12 months marginally to 10.2% (Fiore & Jaen, 2008), however it is thought that the effectiveness of smoking cessation treatments is limited by the lack of effective medications.

To date, the only smoking-cessation medications approved by the Food and Drug Administration (FDA) are nicotine replacement therapy (NRT), bupropion (Wellbutrin/Zyban) and varenicline (Chantix). NRT is available in several formulations, including transdermal patch, gum, nasal spray, inhaler, and lozenge. Of these FDA approved treatments, varenicline appears to be most effective, yielding

abstinence rates of approximately 22 percent at the end of 1 year (Gonzales et al., 2006). The effectiveness of the three treatments according to the most recent 2013 Cochrane Review on smoking cessation treatments is surmised in Table 1.

Treatment	Odds Ratio* (95% CI)†
NRT: All forms, pooled (meta-analysis of 123 studies with ≥6 mo follow-up)	1.84 (1.66–1.88)
Gum	1.66 (1.52–1.81)
Patch	1.81 (1.63–2.02)
Inhaler	2.14 (1.44–3.18)
Lozenge	2.05 (1.62–2.59)
Nasal spray	2.35 (1.63–3.38)
Bupropion (meta-analysis of 19 trials with ≥6 mo follow-up)	2.06 (1.77–2.40)
Varenicline	2.88 (2.40-3.47)

* = Of abstinence when compared with control
† CI = Confidence interval.

Table 1.1: Odds ratios of abstinence compared to placebo for the three main smoking cessation treatments. Values derived from 2013 Cochrane Review on pharmacological interventions for smoking cessation treatment (Cahill, Stevens, Perera, & Lancaster, 2013).

1.4.1 Nicotine replacement therapy (NRT)

The principal mechanism of action of NRT is to partially replace the nicotine normally obtained through smoking tobacco, thus alleviating the severity of withdrawal symptoms and cravings, increasing the chances of successful cessation attempts (Gross & Stitzer, 1989). Differences in formulations may have an impact on the efficacy for some of these effects. For example, the more rapid delivery of nicotine obtained with the nasal spray appears to provide faster relief of withdrawal

symptoms. Furthermore, the inhaler formulation provides an alternative coping mechanism for the behavioral aspects of smoking by imitating the hand-to-mouth motion.

1.4.2 Bupropion

Bupropion is a cessation aid generally marketed with the trade name Zyban by GlaxoSmithKline. It is a non-tricyclic antidepressant and affects multiple neurotransmitter systems. Primarily it acts as a dopamine reuptake inhibitor whilst also being a mild noradrenaline reuptake inhibitor and nicotinic acetylcholine receptor antagonist. It is hypothesized that smokers have artificially elevated dopamine levels due to the presence of monoamine oxidase inhibitors. On stopping smoking, MAO are removed and the dopamine levels fall, leading to feelings of depression as well as nicotine withdrawal (Ascher et al., 1995). Bupropion is thought to alleviate these symptoms of depression while also, through its nAChR antagonist action, reducing the reinforcing spike of dopamine after smoking a cigarette.

Bupropion is licensed for smoking cessation in both the UK and US and is recommended as a first line agent for smoking cessation. Patients themselves set a target quit date, typically 2 weeks hence, and are prescribed 150 mg daily for 6 days followed by 150 mg twice daily for 7 to 9 weeks. The first prescription for bupropion should last approximately 4 weeks and take the patient beyond the target quit date.

Those who return following successful cessation are given the remainder of the treatment course.

Approximately 1 in 5 smokers successfully stop and remain non-smoking at one year with bupropion therapy. Trials reporting the efficacy of Bupropion as a cessation treatment have shown comparably significant one-year abstinence rates of 23% vs 12% (Hurt et al., 1997) and 30% vs 16% (Jorenby et al., 1999) for bupropion therapy when compared with placebo. A recent Cochrane systematic review of 19 randomized trials, showed that bupropion doubled the odds of smoking cessation when compared to placebo (Odds ratio 2.06, 95% confidence intervals 1.77 to 2.40 (R. A. Hughes, Jewitt, & Swan, 2004)).

1.4.3 Varenicline

The most recent treatment to be approved by the FDA for the treatment of smoking cessation is varenicline. It is marketed by Pfizer under the trade name 'Chantix' in the USA and 'Champix' Europe, and usually comes in the form of varenicline tartrate. Varenicline has been approved by the FDA for a treatment course of up to twelve weeks. In the event smoking cessation is not achieved, treatment can be continued for another twelve weeks (Reus et al., 2007).

Varenicline is a partial agonist of the $\alpha4\beta2$ subtype of the nicotinic acetylcholine receptor while a full agonism has been displayed on $\alpha7$-receptors (Mihalak, Carroll, & Luetje, 2006). Acting as a partial agonist varenicline binds to,

and partially stimulates, the α4β2 receptor without producing a full effect like nicotine. Thus varenicline does not greatly increase the downstream release of dopamine. Due to its competitive binding on these receptors, varenicline blocks the ability of nicotine to bind and stimulate the mesolimbic dopamine system, akin to the action of buprenorphine in the treatment of opioid addiction (Rollema et al., 2007).

A randomized controlled trial has demonstrated that after one year the rate of continuous abstinence was 10% for placebo, 15% for bupropion and 23% for varenicline (Jorenby et al., 2006). A meta-analysis of 101 studies found varenicline to be more effective than both bupropion (odds ratio 1.40) and NRTs (odds ratio 1.56) (Mills, Wu, Spurden, Ebbert, & Wilson, 2009). A Cochrane systematic review concluded that varenicline trebled the likelihood of successfully quitting smoking relative to pharmacologically unassisted attempts (Odds ratio 3.98, 95% confidence interval 2.01 to 7.87 (Cahill, Stead, & Lancaster, 2012)).

1.5 Genetic factors that influence smoking behavior

Both genetic and environmental factors contribute to smoking behavior. What has become apparent is that genetic determinants account for more of this behavior than first thought. Based on twin studies, genetic factors have been estimated to account for 40-70% of the variance in smoking maintenance, 50% of the variance in cessation success and 30-50% of the variance in withdrawal symptoms (Batra, Patkar,

Berrettini, Weinstein, & Leone, 2003; Heath et al., 1993; Heath & Martin, 1993; Kendler et al., 1999; Sullivan & Kendler, 1999; True et al., 1997).

Two broad classes of genes may contribute to the variation in nicotine dependence, the first being genes that influence the response to nicotine directly, such as genes involved in nicotine metabolism and the different subtypes of nicotinic receptors for which nicotine acts as a substrate. The other types of genes effect key neurotransmitter pathways, such as the dopamine pathway, and predispose addictive behavior.

This is reflected in human genome-wide association studies (GWAS) that have identified genetic polymorphisms associated with smoking behaviors. Several genes involved in nicotine-related pathways have been identified in the recent GWAS and are summarized in table 2. The majority of the genes showing high statistical associations with smoking behaviors are those in the cholinergic receptor gene clusters, which seem to predominantly affect the level of tobacco consumption, albeit with a relatively small effect size. A closer examination of these clusters has led to the identification of specific independent loci responsible for these associations (Saccone, Saccone, et al., 2009; Saccone, Wang, et al., 2009) as well as haplotypes associated with the effects in Europeans (W. Berrettini et al., 2008). Further research has demonstrated that markers in acetylcholine synthetic pathways may also contribute to nicotine dependence (W. Berrettini et al., 2008). One gene involved in dopamine metabolism and noradrenalin synthesis (dopamine-b hydroxylase) has been linked to spontaneous cessation in GWAS studies and interestingly this gene has also demonstrated effects on response to treatment with NRT in one randomized controlled trial (Johnstone et al., 2004). Owing to the requirement for large numbers

in GWAS, only genes involved in nicotine dependence have been identified in studies to date. Thus, this technique has not been applied to date to identify genetic determinants of response to treatment. In general, randomized trials of smoking cessation have fewer than 1000 participants, half of whom will receive a placebo., Random trials, therefore, provide insufficient numbers of participants for genome-wide analysis. As a consequence, conventional candidate gene studies undertaken within smoking cessation trials currently provide the best available evidence for pharmacogenetic effects on smoking cessation.

			OxGSK (n=41,150) Fine mapped 100 genomes		**TAG** (n=74,053) Partnered with ENGAGE & OxGSK		**Thorgeirsson** (meta analysis n=30,431 v 16,050 never & 64,924 cessation	
Outcome	Gene	Locus	SNPs	Effect Size	SNPs	Effect Size	SNPs	Effect Size
Consumption Level	CHRNA6/ B6	8p11					rs647441 2T	0.29 cpd ($p=1.4*10^{-8}$)
		10q25			rs1329650G	beta=0.367 ($5.7*10^{-10}$)		
					rs1028936A	beta=0446 ($1.3*10^{-9}$)		
	CHRNA3/ A5/B4	15q25	rs55853698 *	$1.31*10^{-16}$				
			rs6495308	$3*10^{-5}$				
					rs1051730A **	beta=1.03 (2.8*10-		
					rs1051730A	beta=0.333 (1.0*10		
	CYP2A6/ B6						rs410514 4C	0.39 cpd ($p=2.2*10^{-12}$)
Initiation	BDNF	11			rs6265C	OR 1.06 ($p=1.8*10^{-8}$)		
Cessation	DBH	9			rs3025343 ***	OR 1.12 (3.6*10-8)		
Lung Cancer		8p11					rs647441 2T	1.09 (p=0.04)
		19q13					rs410514 4C	1.12 (p=0.0006)

* two OxGSK SNPs may tag more cpd than rs1051730A
** tags rs16969968G
*** may be linked to DBH upstream variant controlling plasma levels C1021T rs1611115

Table 1.2: Summary of recent genome-wide association studies. Strongest associations are between level of tobacco consumption and markers in the cholinergic receptor gene cluster on chromosome 15. Replicated associations are also observed between the CYP450 gene that metabolizes nicotine and numbers of cigarettes smoked each day and also the dopamine-b hydroxylase gene and quitting. The lowest p-values have been highlighted in bold.

1.5.1 Nicotine metabolism

The major genes responsible for nicotine metabolism are the cytochrome enzymes P450 2A6 (CYP2A6) and P450 2D6 (CYP2D6). It is well documented in the literature that polymorphisms at the CYP2A6 locus associated with reduced enzymatic activity are generally result in smoking fewer cigarettes per day and greater cessation success (Huang et al., 2005; Malaiyandi et al., 2006; Sellers, Tyndale, & Fernandes, 2003).

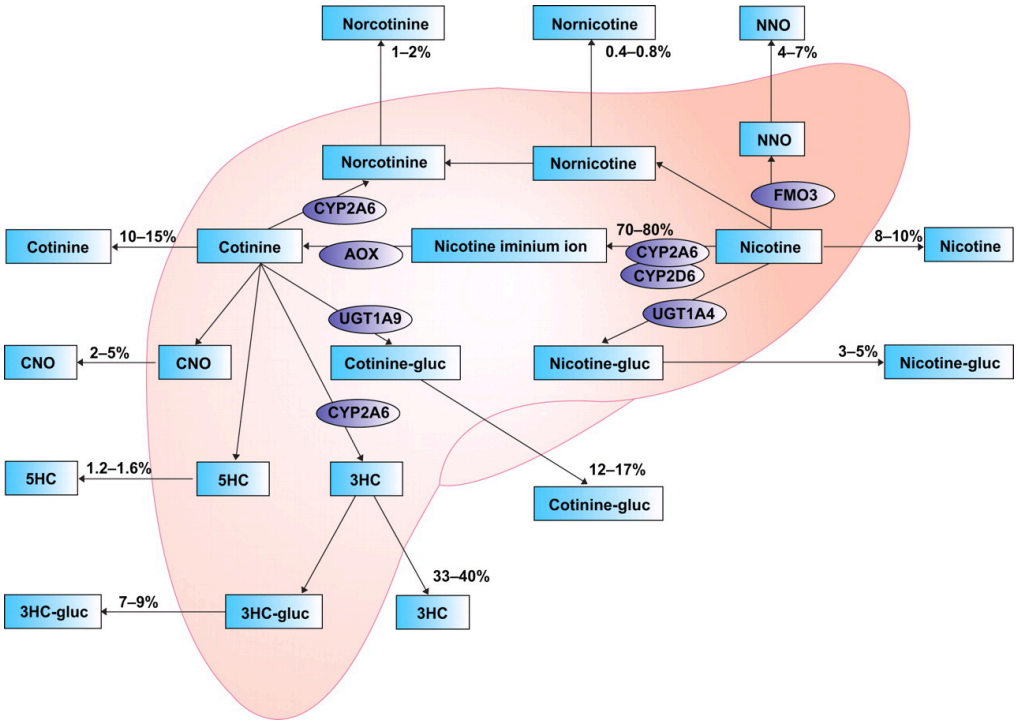


Figure 1.2: Nicotine metabolism in the liver. Mechanisms by which nicotine is metabolised into cotinine in the liver by the cytochrome enzymes P450 2A6 (CYP2A6) and P450 2D6 (CYP2D6).

Altered nicotine metabolism displayed in CYP2A6 variants has also been shown to have an effect on smoking cessation. Individuals with a lower plasma 3'-hydroxycotinine/cotinine ratio (an indicator of CYP2A6 activity) have been shown to be associated with more successful smoking cessation in transdermal nicotine therapy trials (Malaiyandi et al., 2006; Schnoll et al., 2009). This is due to the higher therapeutic doses of nicotine the slow metabolizer sub-group obtain from comparable levels of transdermal nicotine treatment when compared with individuals with normal CYP2A6 activity (Schnoll et al., 2010). It is clear that the effectiveness of a particular nicotine replacement therapy could be predetermined by an individuals CYP2A6 activity. Treatments tailored specifically to an individual's genotype could prove more cost effective and improve chances of successful cessation.

There have been recent studies investigating the duration of nicotine replacement where it was found that smokers receiving an extended transdermal nicotine therapy of 6-months were more than twice as likely to be abstinent at the end of treatment than people on the normal, 8-week treatment (Lerman et al., 2010). However due to the high cost of administering this type of extended therapy, being able to identify biomarkers which can inform the best course of therapy to take on an individual basis, both saving on costs and improving the quality of treatment, would be invaluable.

There has been work to this end, with studies looking into the effect of extended-duration transdermal nicotine therapy on participants displaying higher and lower metabolism phenotypes, characterized by their nicotine metabolite ratio (NMR) (Malaiyandi et al., 2006; Schnoll et al., 2010). Both reduced and normal metabolizers were subjected to extended (6-month) and normal (8-week) replacement therapy. It

was found that smokers with reduced nicotine metabolism benefit more from the 6-month extended therapy than normal metabolizers. This suggests individuals with the reduced CYP2A6 activity genotype would be strong candidates for extended transdermal nicotine therapy and an alternative approach may be needed for people with normal rates of nicotine metabolism (Lerman et al., 2010).

1.5.2 Dopaminergic genes

The mesolimbic pathway in the brain transmits dopamine from the ventral tegmental area to the nucleus accumbens. Since the mesolimbic pathway is associated with feelings of reward and pleasure, this pathway is heavily implicated in most neurobiological theories of addiction (Comings & Blum, 2000). Nicotine activates the dopamine reward pathway through nicotinic acetylcholine receptors (nAChRs) thereby increasing dopamine levels in the nucleus accumbens and generating the sense of 'wellbeing' experienced by smokers (Di Chiara et al., 2004). As a result, variants in genes involved in the dopaminergic pathway may have effects on smoking behavior and response to treatment.

1.5.2.1 Dopaminergic receptors

There are five known subtypes of dopamine receptors: DRD1, DRD5 (members of the DRD1-like family) and the DRD2, DRD3 and DRD4 receptors (D2-like family). Polymorphisms affecting the dopamine D2 receptor (DRD2) gene have been most studied in tobacco dependence and may have an effect on smoking behavior and response to treatment. Polymorphisms in DRD2 may confer reduced dopamine-receptor expression or function, and some studies have demonstrated that individuals with this genotype may have a higher chance of becoming addicted to nicotine (Quaak, van Schayck, Knaapen, & van Schooten, 2009b). There are two main DRD2 polymorphisms that have been extensively studied for their effect on smoking cessation treatments: Taq1A and -141C ins/del. The DRD2 Taq1A polymorphism is located approximately 10 kb downstream of the DRD2 coding sequence in the closely linked ankyrin repeat and kinase domain containing the ANKK1 gene (Neville, Johnstone, & Walton, 2004). Carriers of the A1 allele have a higher quit rate on NRT (Johnstone et al., 2004; Yudkin et al., 2004). Those homozygous for the A2 genotype demonstrate greater response to bupropion treatment and display fewer of the withdrawal symptoms normally associated with the drug (Lerman et al., 2003; Swan et al., 2005). The -141C ins/del variant affects transcription of the DRD2 gene. Individuals with at least one copy of delC have a better response to NRT than those without, and those with the InsC allele respond more favorably to bupropion (Lerman et al., 2006). A study investigating the effects of variants in DRD4 on NRT has also been conducted. The variants were variable number non-tandem repeats (VNTRs) which confer lower receptor activity in those with the long allele, and also a -521C/T polymorphism which confers lower

transcription levels in those with the T allele. Alleles at the DRD4 locus had no effect on cessation in NRT trials (David et al., 2008; Quaak et al., 2009b). Typing people for ANKK1/DRD2 markers could therefore potentially lead to more effective bupropion treatment, and minimize the occurrence of side effects. The benefits of using DRD2 gene variants to guide selection of nicotine replacement or bupropion therapy have not yet been examined in prospective trials.

1.5.2.2 Dopamine metabolism and synthesis

Variants in genes involved in dopamine synthesis also have an effect on response to drug therapy in smoking cessation. Dopamine beta hydroxylase (DBH) is the enzyme responsible for the metabolism of dopamine to noradrenaline. A polymorphism (1368A/G) in the promoter region of the DBH gene confers lower enzyme activity and is more frequent in heavy smokers (McKinney et al., 2000). In one NRT trial, individuals carrying at least one A allele demonstrated significantly higher quit rates (Johnstone et al., 2004). Catechol-O-methyltransferase (COMT) is responsible for degrading dopamine. A functional valine to methionine mutation at position 158 (Val158Met) leads to a reduction in enzymatic activity (Lotta et al., 1995). Individuals homozygous at this locus have an increased likelihood of abstinence in NRT trials (Johnstone et al., 2007; M. R. Munafo, Johnstone, Guo, Murphy, & Aveyard, 2008). The same Val158Met polymorphism has been demonstrated not to influence the efficacy of bupropion treatment (W. H. Berrettini et al., 2007). However, the same study identified two additional COMT polymorphisms (rs165599

and rs737865), which demonstrated a significant association with abstinence (W. H. Berrettini et al., 2007).

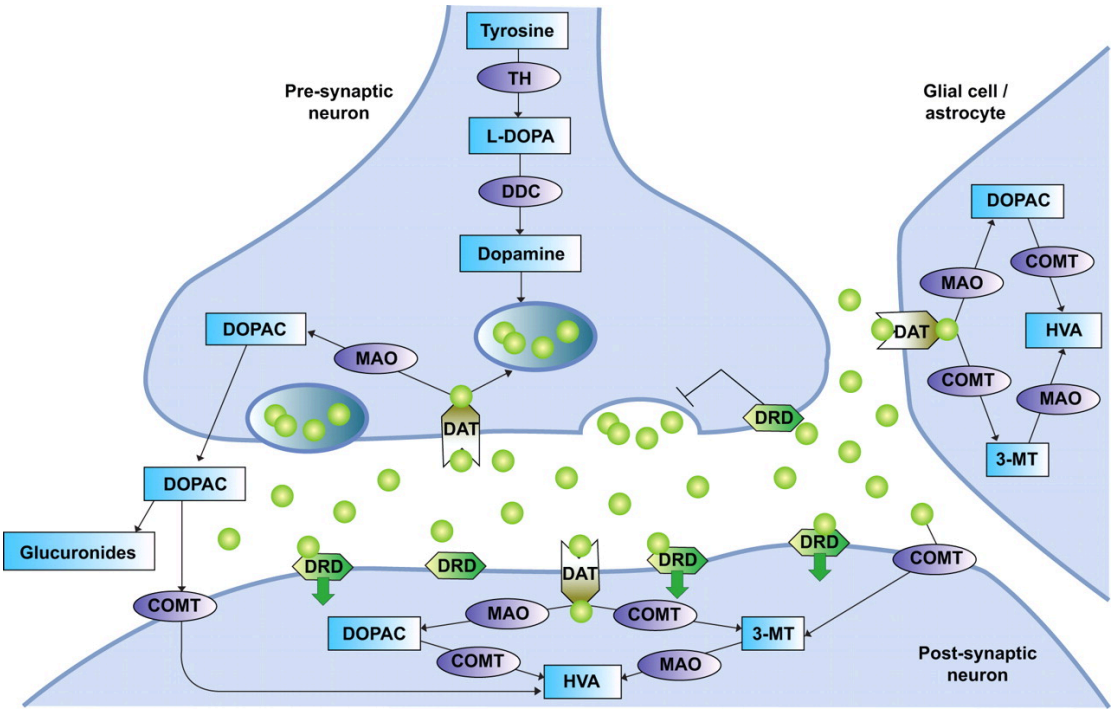


Figure 1.3: Genes involved in the dopamine pathway. TH: tyrosine hydroxylase; L-DOPA: L-3,4-di-hydroxy-phenyllalanine; DDC: 3,4-dihydroxyphenylacetic acid (DOPAC) decarboxylase; MAO: monoamine oxidase; DAT: dopamine transporter; DRD: dopamine receptor; COMT: catechol-O-methyltransferase; HVA: homovanillic acid; 3-MT: 3-methoxytyramine. Circles: dopamine.

1.5.3 Serotonergic and opioid genes

In addition to dopamine, nicotine also causes both serotonin and opioid peptide release as a consequence of stimulating cholinergic receptors. Many symptoms of nicotine withdrawal can be associated with reduced serotonergic neurotransmission

(Quaak et al., 2009b). It therefore appears possible that variation in genes involved in the serotonin pathway could influence individual response to smoking cessation treatment. So far studies have centered on one variant in the serotonin transporter gene (5-HTT) and examined its effect on abstinence in NRT trials. The 5-HTTLPR variant regulates transcription of the serotonin transporter with the short form conferring higher transcriptional activity (David, Munafo, Murphy, Walton, & Johnstone, 2007). As a result, this might be expected to govern the availability of serotonin released by nicotine (Quaak et al., 2009b). A r ecent study has demonstrated individuals with the high-activity variant are more likely to quit in a bupropion trial (Quaak, van Schayck, Knaapen, & van Schooten, 2009a). Nicotine triggers the release of b-endorphin, which targets the μ-opioid receptor (OPRM1), evoking feelings of pleasure. The Asp40 variant of OPRM1 confers increased activity owing to an increased binding affinity for b-endorphin. Studies have demonstrated an association between the Asp40 genotype and increased quit rates when using transdermal nicotine patches (Lerman et al., 2004). Another study has investigated two further genes involved in the opioid pathway, the MOR-interacting proteins b-arrestin 2 (ARBB2) and histidine triad nucleotide binding protein 1 (HINT1). However at the end of NRT treatment, neither HINT1 nor ARRB2 were found to be significantly associated with abstinence (Ray et al., 2007).

1.5.4 Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels of which nicotine is an agonist (Itier & Bertrand, 2001). Neuronal subtypes of nicotinic receptors consist of subunits ranging from a2–a10 and b2–b4. Since nicotine is an agonist for these receptors, which in turn modulate mesolimbic dopamine function, these receptors act as one of the primary mechanisms underlying the development of nicotine dependence (Tapper et al., 2004). Several genome scans indicate that genetic variations in these receptors may influence nicotine dependence (J. Z. Liu et al., 2010; Thorgeirsson et al., 2010; Tobacco & Genetics, 2010). As yet however, only one study has examined two polymorphisms in the a4 subunit coded by the CHRNA4 gene and their effect on abstinence with NRT (Hutchison et al., 2007). Individuals with a polymorphism that affected mRNA stability were more likely to maintain abstinence on NRT nasal spray but not with the transdermal nicotine patch (Hutchison et al., 2007).

1.6 A stratified approach to cessation treatments?

There is a high degree of genetic variability that contributes to individual differences in smoking behavior. Since pharmacological treatments interact with the same neurotransmitter pathways and enzymes involved in nicotine metabolism, it is not surprising that there is also a degree of genetic variability in how individuals respond

to different therapies as the same genes are involved in moderating that response. One possible approach to improving cessation rates could be to target treatment to specific subgroups of smokers. Individuals could be typed at an array of genetic loci related to these differing smoking behaviors, and personalized treatments administered accordingly.

An example where this could be easily implemented would be nicotine metabolism, where biochemical markers (such as cotinine and trans- 3´-hydroxycotinine metabolite ratio) could be used to identify smokers with differing rates of nicotine metabolism, so that an appropriate dose of NRT could be used.

This approach might stratify smokers into different groups according to fundamental biological mechanisms underlying the addictive process. A nosological advance of this kind could lead to more effective use of therapies, reducing side effects and treatment costs whilst at the same time increasing cessation rates.

1.7 Zebrafish as a model organism

Zebrafish have risen markedly in popularity over the last few decades, particularly in the fields of genetics, developmental biology and behavioral neuroscience. Breeding and maintenance of large numbers of zebrafish stocks is both simple and economical, with the ability to house large numbers of animals in a relatively small area. The whole zebrafish genome has been sequenced, with around 70% of human genes found to have at least one zebrafish orthologue (Howe et al., 2013a and Howe et al., 2013b).

The zebrafish also provides researchers with a model amenable to embryonic manipulation and high-throughput screening of genetic mutations and pharmacological agents while also being particularly well suited to such large-scale screens, primarily due to prolific breeding that produces large numbers of rapidly developing offspring (Bang, Yelick, Malicki, & Sewell, 2002; Burns et al., 2005; Gerlai, 2010; Rihel et al., 2010).

1.7 Zebrafish as a model for the study of reward and dependence.

Zebrafish have since the 1950s become established as one of the most widely used comparative model species in developmental genetics, and the genome has now been fully sequenced. More recently, the species has emerged as a valuable model for behavioural neuroscience and addiction biology; the zebrafish encodes an ortholog for nearly every characterised human addiction-related gene including all members of the nicotinic acetylcholine receptor as well as the majority of the dopaminergic, 5-HT-ergic and cholinergic receptor gene families. As a model it allows easy access to a ll developmental stages and imaging of pathological processes as well as automated behavioral assay in adults and larvae (Lange et al., 2013; M. O. Parker, Millington, Combe, & Brennan, 2012). Using a zebrafish model it is possible to knock out genes of interest using a CRISPR/Cas or a TALEN system, and in doing so, investigate whether the gene variants lead to altered connectivity or function in the developing

brain that in turn may influence behaviour. Furthermore, the ability to carry out large-scale screening and mutagenesis strategies makes it unnecessary to know the genes or pathways implicated beforehand.

Recent studies have conclusively demonstrated that zebrafish readily respond to addictive drugs and has led to their growing use in forward genetics screens. Zebrafish provide a useful model system in which to address this question due to the established behavioural assays of drug seeking, compulsive drug taking, and relapse (C. Brennan et al., 2011; L. J. Kily et al., 2008). The zebrafish thus represents an exceptionally powerful platform for efficient whole genome functional assessments of genetic factors that mediate variability in addiction-associated behaviours and the contribution of these factors across specific endophenotypes (Gottesman & Gould, 2003), as well as between drugs of abuse.

Zebrafish have proven to be a useful animal model for studying genetic factors that underlie both complex neurobehavioral phenotypes and drugs of abuse (Bretaud et al., 2007; Clark, Boczek, & Ekker, 2011; Darland & Dowling, 2001; L. J. Kily et al., 2008; Klee, Ebbert, Schneider, Hurt, & Ekker, 2011; Mathur & Guo, 2010; Ninkovic et al., 2006; Stewart et al., 2011). Zebrafish show conditioned place preference responses to cocaine (Darland & Dowling, 2001), amphetamine (Ninkovic & Bally-Cuif, 2006), opiates (Bretaud et al., 2007) ethanol and nicotine (L. J. Kily et al., 2008) and the amphetamine-induced response is modified by pathways known to influence dopamine release in the NAc in other systems (Ninkovic et al., 2006). In addition to this, it has been demonstrated that adult zebrafish develop dependence-related behaviours, such as persistent drug seeking despite adverse stimuli or reinstatement of

drug seeking following periods of abstinence, on prolonged exposure to ethanol or nicotine (C. Brennan et al., 2011).

1.7.1 Conservation of pathways that affect nicotine reward

Despite the topography of the zebrafish brain largely differing from that of mammals, there have been homologous regions associated with addiction and reward identified. There are homologues of mammalian midbrain regions present in the zebrafish. The ventral tegmental area (VTA) is homologous to the posterior tuberal nucleus (PTN) and the nucleus accumbens has homology to the ventral and dorsal telencephalic nuclei (Panula et al., 2010; Rink & Wullimann, 2002b). The dorsal pallium (Dc) has also been described as a likely homologue of the isocortex in in mammals (Mueller, Dong, Berberoglu, & Guo, 2011) (see **figure 4**). Furthermore, there are a number of neurochemical pathways relevant to addiction that show homology to mammals such as the ascending dopaminergic pathways in the midbrain which have been extensively characterized using tyrosine hydroxylase staining (Filippi, Mahler, Schweitzer, & Driever, 2010; Rink & Wullimann, 2002b). There is little evidence of how these systems functionally interact however as much of the evidence pertaining to the cholinergic, DAergic and 5-HTergic neural clusters in the zebrafish brain has been generated from extensive immuno-staining of relevant cell bodies. It is the conservation of the mesolimbic pathway in particular that is vitally important for the experiments covered in this thesis

1.7.2 Mesolimbic reward system in zebrafish.

In mammals the mesolimbic dopaminergic system consists of dopamine neurons that have their cell bodies in the midbrain VTA and send projections to the ventral striatum (NAc), PFC and amygdala (see **figure 1.4**). In zebrafish, dopaminergic neurons are absent in the midbrain; however, experiments have identified a conserved ascending dopaminergic system in zebrafish that is essential for the types of reward responses that will be explored in this thesis. In the zebrafish, the dopamine neurons of the posterior tuberculum of the dorsal hypothalamus, project to the dorsal and ventral (limbic) striatum. These projections are thought to represent the meso-striatal and meso-limbic systems, respectively (Rink & Wullimann, 2002a). The dorsal-medial region of the telencephalon is considered to correspond to the mammalian amygdala (Peitsaro, Kaslin, Anichtchik, & Panula, 2003; Portavella, Vargas, Torres, & Salas, 2002). Evidence in support of the dopamine projection from the posterior tuberculum representing the mammalian mesolimbic projection comes from recent analysis of reward responses in *Too few* mutant fish. *Too few* homozygous mutant fish lack the fez1 transcription factor and lack dopamine and 5HT neurons in the hypothalamus (Levkowitz et al., 2003; Rink & Guo, 2004). Homozygous mutants are indistinguishable from their wild type siblings in terms of size, morphology, anatomy, fertilization, escape, feeding and prey-seeking responses but show a reduced reward response to opiates (Bretaud et al., 2007).

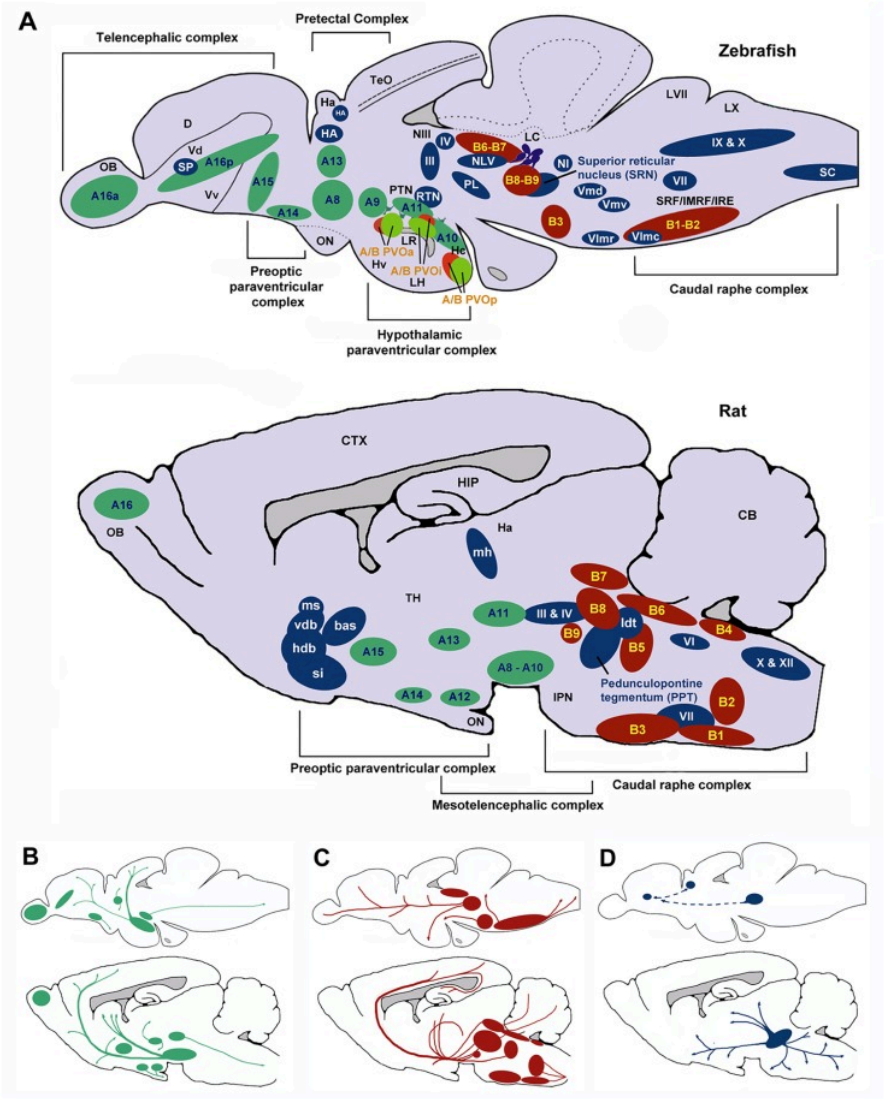


Figure 1.4: Schematic sagittal view comparing dopaminergic (green), serotonergic (red), and cholinergic (blue) neuronal populations in zebrafish (upper) and rat (lower) brains. (A) Cell body distribution (adapted from Manger et al., 2002; Butcher and Woolf, 2003; Mueller et al., 2004; Schweitzer and Driever, 2009; Panula et al., 2010). (B) Schematic drawing illustrating the location of dopaminergic projections in adult zebrafish and rat brains (sagittal view; adapted from Schweitzer and Driever, 2009). (C) Schematic drawing illustrating the location of serotoninergic projections in adult zebrafish (adapted from Gaspar and Lillesaar, 2012) and rat brains (adapted from Di Giovanni et al., 2008; sagittal view). (D) Schematic drawing illustrating the location of cholinergic neuron projections from PPT in adult rats (adapted from (Manger et al., 2002)) and predicted projections from zebrafish SRN to subpallium and habenula.

Further evidence for conservation of neural networks involved in the regulation of reward comes from analysis of the acetylcholinesterase (AchE) mutant zebrafish (*AchE)*. In mammals AchE terminates cholinergic synaptic transmission and AchE inhibitors block cocaine and morphine induced CPP suggesting a critical role of cholinergic systems in the regulation of reinforcement responses to drugs other than nicotine. *AchE* mutant fish have a loss of function mutation in the AchE gene (Behra et al., 2002). Homozygote fish die by 5 days post fertilization but heterozygote fish are morphologically indistinguishable from wild-type siblings. These heterozygote mutants naturally show reduced AchE activity and a reduced reward response to amphetamine as a result (Ninkovic et al., 2006) indicating conservation of the cholinergic regulation of drug-associated reward.

1.8 Genetic tools and mutagenesis approaches

In-breeding of mouse strains have been used to investigate associations between addiction related behaviors and known genetic variants. Reverse genetic approaches in using knockout mice to investigate the role of genes in addiction have also proven fruitful. Although approaches of these types have provided valuable information about the underlying molecular mechanisms of addictive disorders, there are some disadvantages to using these types of methods. Most importantly, traditional approaches require a high degree of knowledge prior to carrying out the experiments, in that the genes you investigate have to be reasonably well known and characterized

before a knock-out mouse can justifiably be generated. When looking at complex neurological disorders this can prove problematic, as it is unlikely such phenotypes are going to be governed by single genes of large effect, rather multiple genes involved in complex pathways with variable penetrance, many of which will be completely novel. As such, significant advances in our understanding of neurological disorders may prove hard to come by if these types of approaches in mice are used exclusively. There is a need for these studies to be complemented with forward-genetic type screens, however this can prove near impossible in mouse models. The relatively long generation gap, small litter sizes and high maintenance costs make it difficult to generate the large numbers needed for forward genetic population screens.

This is where the most exciting possibilities in zebrafish lie; in the ability to perform forward genetic screens for behavioral phenotypes including behaviors associated with complex neurological disorders like addiction. Forward genetic mutagenesis screens in zebrafish have been widely used to identify mutant alleles affecting various behaviors and phenotypes. A typical approach is to use a three-generation mutagenesis screen to identify recessive alleles by screening a family in which 25% of the F3 offspring show a phenotype of interest. This type of classic diploid F2 screen has been carried out (Driever et al., 1996; Haffter, Granato, et al., 1996) with the F3 egg clutches being examined at five stages of development (during the first 6–12 h and on the first, second, third, and fifth days after fertilization) for any signs of abnormal development evident in 25% of the growing embryos (**Figure 1.5**) (Warren & Fishman, 1998). This approach works well for recessive (or dominant) alleles of major effect but is less effective for complex behavioral phenotypes like addiction, as these behaviors are likely to be multi-allelic in nature, rather than being governed by variation at one locus. Nonetheless, forward genetic screens for

behavioral phenotypes have been undertaken including screens for cocaine and amphetamine-induced place preference (Darland & Dowling, 2001; Ninkovic et al., 2006). Each of these screens isolated lines of fish with differential drug seeking behavior, but neither have successfully isolated the causal mutations, possibly due to difficulties in unambiguously identifying the mutant carrier; the performance of control individuals often falls within the range of affected individuals and vice versa (Jain, Wolman, Schmidt, Burgess, & Granato, 2011) making linkage analysis difficult.

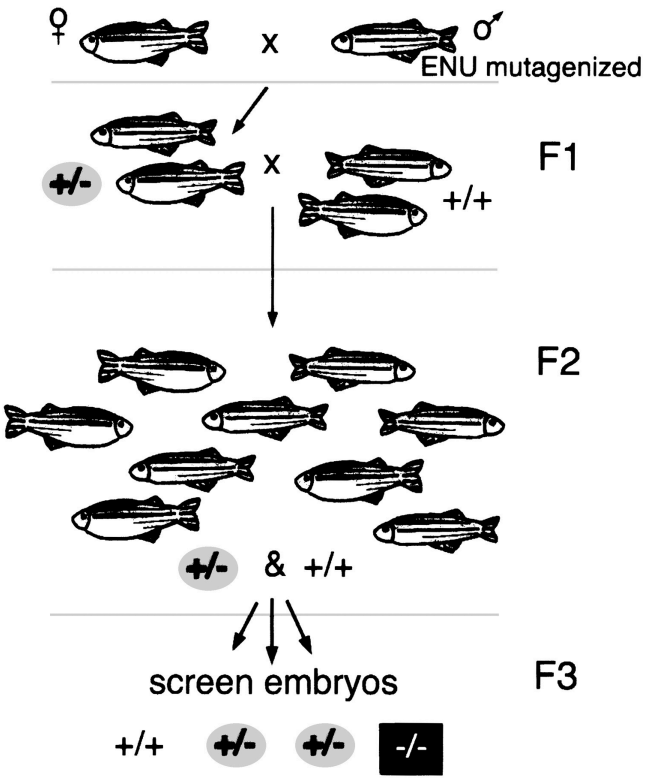


Figure 1.5: Schematic outline of a typical zebrafish F2 mutagenesis screen. G0 male sperm is mutagenized using N-Ethyl-N-nitrosourea (ENU) before outcrossing is performed with wild-type females to produce the F1 generation. Each F1 fish (containing unique mutations) is incrossed with F1 siblings to create the F2 generation.Further in corossing of the F2 generation drive mutations to homozygosity in the F3 embryos where they are screened for a phenotype. (Warren & Fishman, 1998).

Population based breeding and selection, or GFP insertion techniques can be used to address this problem. A good example of this is a study in which a "phenotyping by segregation" approach was implemented, where commonly used breeding and selection strategies were used to map the hypersensitive zebrafish *houdini* mutant (**Figure 1.6**) (Jain et al., 2011). Normally, when carrying out segregation mapping, mutant F2 subjects are pooled based on phenotype, before determining whether a chromosomal region segregates with that phenotype (Michelmore, Paran, & Kesseli, 1991). However, when there is a significant phenotypic overlap between mutant and sibling wildtype populations, often mutants can get misclassified at wildtype and vice versa. This makes it difficult to link genetic variants to the mutant pool. Therefore, to confirm that phenotypic F2 outliers at the larval stage were indeed homozygous mutant individuals, a phenotyping by segregation approach was employed where suspected larval mutants were raised to adulthood, test crossed, and the phenotypic ratios of the F3 larval offspring examined (**Figure 1.4**). Those putative F2 mutant adults producing F3 progeny in the ratios expected for homozygous mutant individuals would then be considered "validated" mutants and used for mapping. Using this method, potential mutants are confirmed both by showing an initial F2 larval phenotype and by producing F3 progeny in expected mendelian phenotypic ratios.

This method was used Jain et al. to map the *houdini* mutant, which was identified in a screen for genetic factors regulating acoustic startle responsiveness that may be relevant to neuropsychiatric disorders. Using this approach, they were able to map a region of chromosome 5 as being responsible for this altered startle response displayed by the *houdini* mutant.

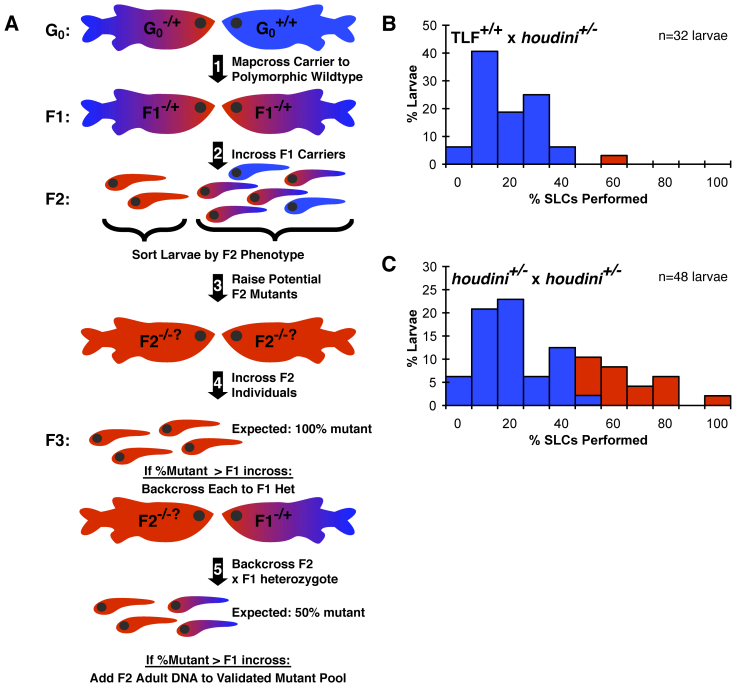


Figure 1.6: The 'phenotyping by segregation' strategy to map the variably penetrant *houdini* mutation. A: F3 phenotypic segregation is used to validate homozygous F2 mutants. F2 larvae at the top 15% of the phenotypic range of the clutch are raised to adulthood as potential mutants, alongside an equal number of siblings (bottom 15% of clutch) as controls. Genomic DNA is then taken from each raised F2 individual before randomly incrosing. Any raised F2 individual that again produced a clutch with a greater frequency of phenotypic outliers than the control F1 heterozygote incross is deemed a "validated" mutant, and is used for segregant mapping. (B-C) Distributions of startle responsiveness to weak sub threshold acoustic stimuli in 5 dpf larval progeny of a *houdini* heterozygote and a wild type (Jain et al., 2011).

This strategy is attractive as it allows for fine mapping of subtle phenotypes that may have variable penetrance in the general population. An alternative approach has been to use fluorescently tagged gene breaking transposons to mutagenize zebrafish (Bill, Petzold, Clark, Schimmenti, & Ekker, 2009). These transposons permit visual sorting of carriers from non-carriers (fluorescent vs. non-fluorescent

larvae) and have the advantage of allowing rapid cloning of the mutagenized gene. This technique has been successfully used to identify two genes involved in the behavioral response of larval fish to nicotine (Petzold et al., 2009).

Application of such breeding and selection-based mutagenesis screening approaches to the adult behavior may lead to the identification of novel genes contributing to complex behavioral phenotypes. It is why such a strategy has been chosen as the main topic of this thesis. By screening mutant populations of fish for nicotine induced CPP, it is hoped novel alleles associated with nicotine reward can be identified. The ultimate aim being to use any alleles identified in this study to inform human-based studies and identify markers, which can be used to stratify people presenting for cessation treatment in the hope of increasing quit rates. Such studies will make a valuable contribution to complement genome wide association studies (Sullivan, 2010) and analyses of copy number variants (Cook and Scherer, 2008) aimed at understanding the genetics of nicotine addiction and psychiatric disease.

Chapter 2

General Methodology

This chapter contains descriptions of general protocols used over the course of this thesis. All general laboratory chemicals were purchased from Sigma-Aldrich unless stated otherwise.

2.1 RNA/DNA isolation and separation methods

2.1.1 Total RNA extraction from zebrafish tissues

This method was deployed at a means of obtaining total RNA stock from zebrafish bodies/heads for generating cDNA stocks. Total RNA from zebrafish head or whole zebrafish tissue was extracted following RNeasy Mini Kit protocol form Qiagen.

- Up to 20 mg of tissue was homogenised in 350 μl of RTL lysis buffer using homogeniser.
- Lysates were cleared by centrifugation at 14000 rpm for 3 minutes.
- Supernatants were then transferred to new tubes and 750 μl of 70% ethanol was added and mixed thoroughly by pipetting.
- 700 μl of sample was added to each RNeasy mini column in a 2 ml collection tube and centrifuged for 15 seconds at 14000 rpm.
- The flow-through was discarded and the column washed by adding 700 μl of buffer RW1.
- The column was centrifuged for 15 seconds at 14000 rpm and the flow-through discarded.
- The column was transferred into a new 2 ml collection tube and 500 μl of buffer RPE was added to wash the membrane.
- The column was centrifuged for 2 minutes at 14000 rpm and the flow-through discarded. This step was repeated without RPE buffer to dry the silica-gel membrane and completely remove all traces of RPE buffer as it can inhibit molecular reaction further down the line.

- To elute RNA, the column was transferred to a new 1.5 ml collection tube and 30 to 50 μl of RNase free water were pipetted directly onto the column membrane and left for 1 minute.
- The column was centrifuged for 1 minute at 1400 rpm and the RNA concentration determined using nanodrop ND 1000 spectrophotometer.

2.1.2 mRNA extraction from embryos using Dynabeads® Magnetic Beads

This method was used for the purpose of obtaining total mRNA from zebrafish embyos for use in quantitative PCR reactions. The optimal amount of tissue for 20ul beads is 1mg. If the tissue is stored in RNA later, remove and put in lysis buffer:

- 2ul PK solution was added to 1mg tissue in 200ul lysis buffer.
- Samples were incubated at 55 degrees ^{0}C until completely digested. This can take 30min or even more. Samples were agitated during incubation period to help digest.
- Once all tissue digested, 40ul of dynabeads was added per 200 μl lysis buffer.
- Digest was rotated for 10min at room temperature to ensure mixing of RNA and beads and to allow binding of mRNA to beads.
- Whilst rotating turn hot block was switched on to 80 degrees. Samples were then transferred to a magnetic rack.
- The magnetic rack was rocked a few times to make sure all beads attracted to magnet. Samples were titrated again to make sure all DNA broken down and all mRNA has opportunity to bind.

- All supernatant was removed including any bubbles taking care not to disturb the pellet.
- Samples were washed with 400ul buffer A (from dynabead kit) – i.e. samples were removed from rack, 400ul of wash buffer added and titrated to wash beads. Samples were then Put back in magnetic rack and the supernatant removed as before.
- Beads were washed with 400ul buffer B. Supernatant was removed making sure all of the buffer B was removed as it inhibits RT reaction.
- 13 μl of Tris-Hcl (from kit, 10mM) was added to re-suspend beads. All 13 μl is used in future reverse transcription reactions
- Samples were placed in heat block at 80 ^{0}C to remove mRNA from beads.
- After 2 min at 80 degrees samples were transferred IMMEDIATELY to rack so that mRNA has no time to recombine with beads.
- Put 7.5ul of RT master mix (prepared as described below) into a PCR tube (thin walled 0.2ml) and add all of the mRNA to it. Then set up cDNA synthesis reaction according to new cDNA synthesis kit. Use a mixture of both oligodT and random primers to prime synthesis.

2.1.3 Quantification of nucleic acids concentration

The concentration of RNA (or DNA) solution was determined using a Nanodrop ND 1000 according the manufacturer's recommendations. RNA or DNA concentration is determined by measuring sample absorbance at 260nm.

2.1.4 Separation of RNA by gel electrophoresis

All RNA/DNA samples were typically run on a 1% (w/v) agarose gel aside from fragments less than 200bp. The agarose gel was prepared by mixing 1g of agarose with 100 ml of 1X TAE buffer in a conical flask. The mixture was heated in a microwave until a homogenous solution. This was then allowed to cool before ethidium bromide (0.5 μg/ml was added. The cooled, but still liquid, agarose was then poured into the casting tray containing a comb for the wells and left to solidify. The gel tray with comb removed was then transferred into the gel tank with 1X TAE buffer. Samples were then added by combing with 6x loading buffer before immediately being loaded into the gel wells. A parallel lane containing 5 μl of DNA markers was then added to determine the size of the obtained fragments. Samples were run a 100 V. The gel was visualized under UV light using a Uvitec illuminometer.

2.1.5 Enzymatic degradation of genomic DNA in RNA samples

To remove DNA contaminants from RNA solutions, samples were subjected to an enzymatic DNA degradation reaction in a micro centrifuge tube containing the following:

- x μl Total RNA (volume = 2 μg RNA)
- 4 μl 5X reaction buffer

- 1 μl DNase I
- 1 μl RNasin
- y μl RNase free water (add up to 20 μl total reaction volume)

DNA digestion was carried out for 30 mins at 37ºC in a water bath. Reaction was stopped by freezing or proceeding to phenol extraction.

2.1.6 Phenol extraction and ethanol precipitation of RNA/DNA

20 μl of RNA/DNA was extracted and precipitated as follows:

- 30 μl of RNase free water and 50 μl of acid phenol was added to the tube and mixed vigorously
- Solution was then centrifuged for 5 minutes at 130000 rpm to separate the two phases
- The top transparent layer containing DNA/RNA was transferred to a new RNase free microcentrifuge tube and concentrated by ethanol precipitation
- 1/10th volume 3M sodium acetate solution was added along with 2.5 volumes of 100% ethanol and triturated thoroughly to mix
- Solution was left at -80 ºC for 1 hour to aid precipitation
- The sample was spun down at 13000 rpm for 10 mins and the pellet washed with 70% ethanol
- The sample was centrifuged for a further 10 mins at 13000 rpm, the ethanol removed and the pellet allowed to air dry.

- The pellet was resuspended in RNase free water.

2.1.7 cDNA synthesis from total RNA or total mRNA from embryos

Reverse transcriptase PCR reaction is set up as follows:

- 4ul 5x buffer
- 2ul dNTP
- 0.8ul primer mix
- 0.4ul RNAsin
- 0.4ul RTase
- 12.4ul RNA

The reaction was carried out in a thermocycler with the following settings:

- 42 degrees for 30 min
- 85 degrees for 5 min
- 4 degrees 5 min.

2.1.8 Polymerase chain reaction (PCR)

All reagents and templates were stored at -20. Primer stocks were stored at 100 μM in water, while working aliquots were stored at 10 μM. The dNTP stocks contained 10 mM each of dATP, dTTP, dCTP, and dGTP and were obtained from Roche. The TAQ polymerase was from New England Biolabs. The reaction mix (for 25μl reaction, for 50 μl reaction double values etc.) contained:

- 2.5μl reaction buffer
- 1μl F+R primers
- 0.5 μl dNTPs
- 1μl template
- 19.875μl ddH20
- 0.125 μl TAQ polymerase

Standard settings on the thermo cycler were as follows:

- Denature at 94° C for 5 min
- Denature at 94° C for 30 sec
- Annealing step at X° C for 30 sec (temp dependant on primer tm)
- Extension 68° C for X min (dependant on template size, 1kb = 1min)
- Repeat steps 2-4 35 times
- 68° C for 10 min

2.1.9 Extraction and purification of DNA from agarose gel

DNA was extracted and purified according to the QIAquick Gel extraction Kit as follows:

- The area of agarose gel containing DNA fragment of interest was excised using a scalpel before being place in a microcentrifuge tube and weighed
- 3 volumes of buffer QG were added per 1 volume of gel
- The tube was incubated for 10 minutes at 50 ° C until the gel slice was dissolved
- 1 volume isopropanol was added to the solution before inverting several times.
- The resulting solution was then added to a QiaQuick spin column in a collection tube and then bound to the column membrane by centrifuging at 13000 rpm.
- The flow through was discarded and 0.5ml of buffer QG was added to remove remaining traces of agarose.
- The column was spun for 1 minute at 13000 rpm.
- To wash the membrane 750 μl of buffer PE was added to the column before centrifuging for 1 minute at 13000 rpm
- The flow through was discarded before centrifuging for a further 1 min to eliminate all traces of buffer PE
- To elute the column was transferred to a 1.5 ml eppendorf and the DNA eluted into 30 μl of distilled water

Concentration of the DNA was determined using the nanodrop as previously described. DNA was stored at -20°C.

2.1.10 Making competent cells

- A 100 ml aliquot of frozen cells was taken from the -80oC and innoculate about 500 ml to 1 L sterile LB broth. Antibiotic was not added, since these cells do not contain a plasmid. Care was taken to work as sterile as possible.
- The cells were grown on a shaker at 37oC until they reached an OD @ 600nm of 0.3 to 0.4 (1 cm path length).
- Cells were then centrifuged in a Sorvall GSA rotor (250 ml centrifuge bottle) at 5,000 RPM for 10 minutes at 4oC. Ice down 100 mM CaCl2 and 100 mM MgCl2 solutions at this point.
- The bacteria pellet was gently resuspended on ice in 1/4 volume of ice cold $MgCl_2$, taking 3 to 5 minutes for this procedure. Centrifuge he cell suspension at 4,000 RPM in the Sorvall GSA rotor for 10 minutes.
- The bacteria pellet was gently resuspended on ice in 1/20 volume of ice cold CaCl2 and then add an additional 9/20 volume of CaCl2. Keep this suspension on ice for at least 20 minutes.
- The cell suspension was centrifuged at 4,000 RPM in the GSA rotor for 10 minutes and the cell pellet resuspended in 1/50 volume of ice cold, sterile 85 mM CaCl2 in 15% glycerol w/v. Dispense in 100 mL aliquots and freeze cells at -80ºC.

2.1.11 Clonong into pGEM®-T Easy

The pGEM®-T and pGEM®-T Easy Vector Systems have been optimized using a 1:1 molar ratio of the Control Insert DNA to the vectors. The pGEM®-T and pGEM®-T Easy Vectors are approximately 3kb and are supplied at 50ng/μl. To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, the following equation was used:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

The steps for ligation of amplified PCR product into the pGEM®-T Easy vector were as follows:

- Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect the contents at the bottom of the tubes.
- Set up ligation reactions as described as follows: 5μl ligation buffer, 1μl pGEM®-T Easy Vector, Xμl PCR product (see above for volume calculation), 1μl T4 DNA ligase, Nuclease-free water up to a final volume of 10μl.
- Incubate the reactions for 1 hour at room temperature (Alternatively, reactions can be incubated overnight at 4°C).

2.1.12 Preparation of LB-Agar plates

- 17.5 g of LB-agar powder was added to 500ml distilled water and then autoclaved
- After autoclaving, agar was left in a water bath at 50 ºC and allowed to equilibrate temperature
- Once agar was 50 ºC, 500 μl of ampicillin was added close to a sterilizing flame
- Agar was then poured into petri dishes with approx. 20-25ml of agar put in each dish and then allowed to dry for 1 hour.
- 50 μl of X-Ga and 100 μl of IPTG solution were poured onto each plate using a spreader fashioned out of a Pasteur pipette and the plates were left to dry for a further 30 mins.

2.1.13 Transformation of competent cells

- Two LB/ampicillin/IPTG/X-Gal plates for each ligation reaction were prepared and equilibrated to room temperature.
- 2 μl of each ligation reaction was transferred to a sterile 1.5ml eppendorf tube on ice
- Frozen competent cells were removed from storage and placed in an ice bucket for 5 mins until thawed. Mix the cells by gently flicking the tube.

- 50μl of competent cells were transferred into each of the tubes containing the ligation reactions. The contents were gently mixed by flicking
- 6. Cells were heat-shocked for 45–50 seconds in a water bath at 42°C. and Immediately returned to ice for 2 minutes.
- 950μl room-temperature LB broth was added to the tubes and Incubated for 1.5 hours at 37°C with shaking at ~150rpm.
- 100μl of each transformation culture was plated LB/ampicillin/IPTG/ X-Gal plates.
- Plates were Incubated overnight (16–24 hours) at 37°C.

Successful cloning of an insert into the pGEM®-T or pGEM®-T Easy Vector interrupts the coding sequence of β-galactosidase; recombinant clones can be identified by color screening on indicator plates. White colonies generally contain inserts; however, inserts may also be present in blue colonies.

2.1.14 Culturing transformed bacterial cells

- Next to a sterilizing Bunsen burner flame, 5 ml of sterile LB broth supplemented with ampicillin (1:100 dilution) was placed in a 25ml falcon tube
- For maxi-prep, 250ml of broth was used in a conical flask

- A colony was picked (white one if blue/white selection) using a sterile pipette tip from agar plate and transferred into tune (tip included)
- Colonies were allowed to grow over night by placing at 37°C with 225 rpm shaking for 16 hours overnight.

2.1.15 Preparation of glycerol stocks

500 μl of transformed bacteria culture was added to a sterile microfuge tube containing 500 μl of sterile glycerol. The solution was mixed thoroughly and the tube stored at -70°C.

2.1.16 Preparation of up to 20 µg of high-copy plasmid

The QIAprep miniprep kit was used following QIAGEN instruction. 5ml of LB broth was inoculated with a single colony or from glycerol stock and incubated overnight using the instructions above. Plasmid was extracted as follows:

- The bacterial cells were harvested by centrifugation at 3000 rpm for 15 minutes at 4°C and the bacterial pellet resuspended in 0.3 ml of buffer P1 (cell suspension solution).

- 0.3 ml of buffer p2 (cell lysis solution) was added and the cell lyse=ate suspension vigorously mixed by inverting the tube several times.
- 0.3 ml of chilled buffer N3 (neutralization solution) was added and the tube contents mixed vigorously by inverting the tube.
- The tube was left to rest on ice for 5 mins and then centrifuged at maximum speed in a microcentrifuge for 10 mins.
- The supernatant containing plasmid DNA was transferred into a QIAprep spin column and centrifuged for 1 minute at 13000 rpm.
- The flow through was discarded and the column wash by adding 0.75 ml of buffer PE before centrifuging again at 13000 rpm
- The column was transferred to a new microcentrifuge tube and the DNA eluted in 20-50 μl of distilled water.

2.1.17 Preparation of up to 100 µg of high-copy plasmid

The plasmid maxi kit was used following the QIAGEN instructions. 250 ml of selective LB broth medium was inoculated with 100 μl from a bacterial glycerol stock and incubated overnight using the instructions above. Plasmid was extracted as follows:

- The bacterial cells were harvested by centrifugation at 3000 rpm for 15 mins at 4°C
- The resulting bacterial pellet was gently resuspended in 4ml of buffer P1.

- 4 ml of buffer P2 (cell lysis solution) was added and the cell lysate suspension vigorously mixed by inverting the tube several times and incubating at room temperature for 5 minutes.
- 4 ml of chilled buffer P3 (neutralization solution) was added to the mixture, again vigorously mixed by inversion several times.
- The tube was left to rest on ice for 15 mins before centrifugation at ~20000 x g for 30 mins at 4°C.
- The supernatant containing plasmid DNA was transferred to a new tube and centrifuged again for 15 mins.
- A QIAGEN-tip 100 was equilibrated by applying 4 ml of buffer QBT (equilibration buffer) and allowed to pass through by gravity.
- The QIAGEN tip was then washed twice with 10 ml of buffer QC (wash buffer).
- The DAN was then eluted with 5 ml of buffer QF (elution buffer)
- DNA was precipitated from the solution by adding 3.5 ml of 70 % isopropanol followed by centrifugation at 15000 x g for 30 mins at 4°C
- The DNA pellet was washed with 2 ml of 70% ethanol and the tube centrifuged at 15000 x g for 10 mins at room temperature.
- The DNA pellet was air dried for 5 to 10 mins and resuspended in 100 μl of distilled water.

2.1.18 Restriction digests

A number of restriction digests were used throughout this thesis to linearize vectors for probe synthesis or to verify the presence of an inserted fragment after bacterial transformation. A standard digestion reaction contained the following components:

- x μl (volume corresponding to between 100 – 500 ng)
- 2 μl 10X reaction buffer
- 1 μl restriction enzyme
- y μl distilled water (add until 20 μl total volume)

Each digestion reaction was incubated in a water bath at 37°C for a minimum of 2 hours. If the amount of DNA digested was greater than 5 μg, the total reaction volume was increased to 40 μl and other components were adjusted equivalently.

2.1.19 Genomic DNA extraction

Genomic DNA extraction was carried out according to the DNeasy® Blood & Tissue handbook as follows

- Zebrafish fin-clip was placed in a 1.5ml microcentrifuge tube. 180 μl Buffer ATL was added.
- 20 μl of proteinase K was added. Contents were mixed thoroughly by vortexing and incubated at 56°C until the tissue had completely lysed.

- 200 μl of Buffer AL was added to the sample, and mix thoroughly by vortexing. Then add 200 μl ethanol (96–100%), and mix again thoroughly by vortexing.
- 200 μl of Buffer AL was added to the sample, and mix thoroughly by
- Then 200 μl of ethanol (96–100%) was added and mixed again thoroughly by vortexing
- The mixture from step 3 was pipetted (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube).
- Columns were then centrifuged at 6000 x g (8000 rpm) for 1 min. and the flow-through discarded.
- The DNeasy Mini spin column was placed in a new 2 ml collection tube, and 500 μl Buffer AW1 was added,
- The spin-column was centrifuged for 1 min at 6000 x g (8000 rpm).
- The DNeasy Mini spin column was then placed in a new 2 ml collection tube (provided), and 500 μl Buffer AW2 added.
- The column was then centrifuged for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane..
- The DNeasy Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and eluted by applying 200 μl Buffer AE directly onto the DNeasy membrane and then centrifuging for 1 min at 6000 x g.

2.1.20 PCR Purification of PCR products, 100 bp to 10 kb

Procedure:

- 5 volumes of Buffer PB was added to 1 volume of the PCR sample and mixed.
- If the colour of the mixture was not yellow *10 µl of 3 M sodium acetate, pH 5.0, was added until the mixture turned yellow*.
- A QIAquick spin column was placed in a 2 ml collection tube.
- To bind the DNA, the sample was applied to the QIAquick column and centrifuged for 30–60 s.
- Discard flow-through. Place the QIAquick column back into the same tube.
- To wash, 0.75 ml Buffer PE was added to the QIAquick column and centrifuged for 30–60 s.
- The flow-through was discarded and the QIAquick column placed back in the same tube before centrifuging for an additional 1 min.
- QIAquick column was placed in a clean 1.5 ml microcentrifuge tube.
- DNA waseluted by adding 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min.

2.2 In-situ hybridization

2.2.1 Generation of template for RNA probe synthesis

Template for probe synthesis was created using a plasmid containing the cDNA of interest and M13 primers to amplify up that cDNA using the previously mentioned PCR protocol. The resulting fragment wad then PCR purified and used s the template in the reaction below.

2.2.2 Anti-sense RNA probe synthesis

To generate probes, the following reagents were mixed in the following order at room temperature (all reagents were obtained from Roche):

- 10 μl sterile distilled water
- 4 μl 10x transcription buffer
- 2μl 0.2 M DTT
- 2 μl nucleotide mix
- Linearised DNA (0.5 μg/μl; 1μl)
- Ribonuclease inhibitor (0.5 μl)
- RNA polymerase 0.5 μl

Probes were synthesised as follows:

- The reaction was incubated at 37°C for over 2 hours up to 5 hours.

- A 1% agarose gel was cast while the sample was incubating.
- A 0.5 μl aliquot was taken and run on a 1% agarose/TBE gel to estimate amount of RNA synthesized. The idea being to look for an RNA band about 20-fold more intense than DNA band, indicating around 10 μg probe synthesized.
- After the probe was synthesised the reaction was diluted to 200 μl with water and add 20 μl of 3 M NaOAc (pH 5.5), 1.3 μl of acetic acid (add this in fume hood), and 550 μl of ethanol.
- The solution was left at -80°C for 30 minutes.
- The solution was spun in a microfuge for 10 minutes and the liquid decanted liquid.
- Pellet was washed with 70% ethanol and air dried.
- Pellet was re-dissolved in low TE (10mM Tris, 0.1 mM EDTA, pH 8.0) at 0.1 μg/μl and store at -20°C.
- The probe was titrated when first used - i.e. to test run at 1/100 to 1/500 dilution.

2.2.3 Whole mount in-situ hybridisation (pretreatment of embryos)

Embryos are fixed and stored as follows:

- Embryos were hand-dechorinated in fish water then transfer to eppendorf, remove liquid and fix in 4% paraformaldehyde/PBS at 4°C overnight or 2hour at room temperature.

- Fixed embryos were washed once with PBS, once with methanol, and then store in methanol at -20°C. Embryos can be stored in methanol indefinitely.

Fixed embryos are readied for in situ hybridization as follows:

- Methanol was removed with a pipette ensuring embryos did not dry out.
- Embryos were rehydrated by taking fixed embryos through:
- 5 minutes in 75% methanol/PBT,
- 5 minutes in 50% methanol/PBT,
- 5 minutes in 25% methanol/PBT
- 4x 5 minutes in 0.5 ml PBT

(Tween20 used in all solutions to prevent the embryos sticking together)

- Embryos were treated with 10 μg/ml proteinase K in PBT for 20 minutes (for 24 hour old embryos, add 20 minutes for every additional day in age
- Embryos were washed once with PBT and refixed in 4% paraformeldehyde/PBT for 20 minutes.
- The Embryos were washed for 5 x 5 minutes with PBT.
- Next there was a prehybridisation step for at least 1 hour at 65°C in hybridization mix (~100 μL, just enough to cover the embryos). *Embryos can be stored at -20°C after prehybridising*.
- Hybridization mix was replaced with fresh mix and probe and incubated overnight at 65°C.

2.2.4 Whole ISH (post-hybridization washes and digoxygenin detection)

The following washes were performed at 65°C (have all wash solutions at 65°C too):

- 3x 10 minutes 25% formamide/2x SSC
- 10 minutes 2x SCC
- 3x 20 minutes 0.2 SSC.

The rest of the protocol was carried out at room temperature as follows:

- 4 x 5 min with PBT at room temperature
- Embryps were incubated in [PBT containing 2% sheep serum and 2 mg/ml BSA] or MABlock: [MAB 1x with maleic acid buffer] with gentle agitation for >1 hour at room temperature.
- The above solution was replaced with anti-DIG-AP antibody (1:5000) dilution in PBT containing 2% blood sheep serum and 2 mg/ml BSA and incubate for 2 hours at 4°C (Or 4°C overnight)
- Wash embryos 8x 15 minutes in PBT at room temperature (or you can wash overnight at 4°C)
- 3x 5 minute washes in BCL buffer. This step brings the ph to 9.5.
- Incubated with BCL buffer including 4.5 μl NBT, 3.5 μL BCIP per ml (or BM Purple) and leave on bench top.
- When colour has developed to the desired extent, wash 3x 5 minutes with PBT and refix embryos in 4% paraformaldehyde/PBS for 30 min - 2 hours.

2.3 Quantitative PCR

2.4.1 Collection of embryos to generate cDNA

- The larvae were killed in MS-222 at 28hr, 3dpf and 5dpf before being placed in RNAlater until assay (4ºC).
- Batches of n=5 embryos were pooled and the mRNA isolated using Dynabeads® Oligo$(dT)_{25}$ protocol outlined in the previous section.
- cDNA was generated from total mRNA to generate samples for analysis

2.3.2 Making standard for target genes

Every gene run with the qPCR protocol was run with a standard curve in order to relatively quantitate the Ct values for the samples. The protocol starts with 20µl of cDNA sample generated in the previous steps.

- PCR fragments were amplified using qPCR primers for target genes using standad PCR protocol
- The PCR products were PCR purified, their concentration determined using the nanodrop before being diluted to 10^{11} fragments using the Avogadro constant.

2.3.3 qPCR reaction set-up

The following negative controls were included:

- non-template control (H2O + SYBR Green + primer)
- no primer/no template control (H2O + SYBR Green).

All qPCR reactions were carried out in triplicate. Reactions were set up on ice in each well as follows:

- 2μl of cDNA
- 2μl each of forward and reverse primer
- 10μl SYBR® Green PCR Master mix (Applied Biosystems)
- 6μl water (for a total reaction volume of 20μl)

Reactions were carried out using a MJ Research PTC-200 Thermo Cycler.

2.3.4 Thermocycler settings

The MJ Research PTC-200 Thermo Cycler was set up as follows:

- 1: Thermocycling begins with 95°C for 5 mins
- 2: 55 cycles of amplification at 95°C for 10 sec,
- 60°C for 6 sec,
- 72°C for 6 sec
- 76°C for 1 sec (data acquisition).

2.3.4 Real-time qPCR data analysis

Relative mRNA expression ratios in the qPCR were calculated with respect to reference gene cycle-threshold (Ct) values, and then subjected to a two-way factorial (between-subjects) analysis of variance (ANOVA). Significant main effects and interactions were followed up with pairwise comparisons All test statistics were evaluated with respect to a type-1 error rate of 0.05. All descriptive statistics are reported as estimated marginal means ± SE unless otherwise indicated.

Chapter 3

The conditioned place preference assay

This chapter explores the conditioned place preference (CPP) assay as a means of measuring the rewarding effects of nicotine and other drugs of abuse. Its uses are explored in the literature before being tested on a range of compounds to assay the robustness of the procedure.

3.1 Introduction

The aim of the project is to use a forward genetic screen in zebrafish to find genes affecting nicotine-seeking behavior in humans. It is based on the hypothesis that as zebrafish are vertebrate with conserved neurocircuitry of reward, homologues of genes that affect zebrafish drug seeking will also affect human drug seeking. In order to demonstrate conservation of reward processes and therefore justify the use of zebrafish, the first results chapter of this thesis explores the zebrafish conditioned place preference assay as a means of demonstrating the conservation of reward responses to common drugs of abuse in zebrafish

An organism's likelihood of survival often hinges upon rapidly learning the conditions and the behavioral responses necessary to obtain natural rewards necessary for survival and propagation, as well as the environmental cues that predict them (Bell, Meerts, & Sisk, 2010; Collier, Khan, Caramillo, Mohn, & Echevarria, 2014; Lau, Bretaud, Huang, Lin, & Guo, 2006). Conditioned learning is primarily mediated by the motivational centers of the mesolimbic system, which are also readily activated psychoactive substances (Alderson, Robbins, & Everitt, 2000a, 2000b; Childress et al., 1999; Everitt & Robbins, 2005a, 2005b). Following the consumption of rewarding drugs, it rapidly becomes paired with a place or emotional state, due to integrated signaling to the memory and motivational centers of the limbic system (McLellan, Lewis, O'Brien, & Kleber, 2000). This pavlovian learning process means that exposure to cues associated with a drug may induce cravings and play a large part in the transition from casual drug use to more habitual and compulsive behaviors (Childress et al., 1999 Alderson et al., 2000 and Everitt and Robbins, 2005). The

identification and understanding genetic components that contribute to this particular facet of addictive behavior would be key to facilitating new treatment strategies.

Conditioned place preference (CPP) is a form of pavlovian conditioning used to measure the motivational effects of objects or experiences (Tzschentke, 2007b). When drugs of abuse are applied to this paradigm, it can be used as a measure of their reinforcing properties. Animals are conditioned to associate drug exposure with specific environmental cues and a compound is considered rewarding if subsequent testing in the absence of the drug reveals a preference for the drug-paired cue. As such, it an ideal model for this particular conditioned learning component of addiction.

This procedure is generally comprised of three testing phases that occur on consecutive days. During the first phase the animal is permitted to explore all compartments of the apparatus, and the time spent in each compartment is measured and used as baseline place preference. In the second phase, animals are sequentially restricted to each compartment for a period of time in which they receive either a drug or control treatment. In the final phase, the animal is once again allowed access to all compartments and final place preference is measured. Change in preference is calculated by subtracting the baseline place preference from final phase preference. If a significant change towards the experimental compartment is observed, CPP is established.

Conventionally, rats and mice have been utilized while investigating the reinforcing properties of drugs, primarily due to them sharing a high degree of anatomical and genomic homology with humans (Lieschke & Currie, 2007). Another common model used in rodents for measuring the reinforcing properties of drugs is

the self-administration paradigm. Drug self-administration consists of recording the number of times an animal produces a response (a button/lever press) that results in an intravenous infusion of drug. There are also a number of differences between the models. CPP is able to provide a measure of both rewarding and aversive effects of drugs, while self-administration can only distinguish the latter. CPP utilizes classical conditioning requiring a maximum of 1 week training, whereas the operant conditioning of self-administration requires extensive training, which is not ideal for high throughput screening. Crucially, no surgery is required for CPP while self-administration requires a catheter implantation, a procedure that would seem unfeasible in a zebrafish model. Another notable difference is that while both CPP and self-administration studies are in agreement with regards to the rewarding effects of a lot of drugs, including psychostimulants and opiates, some drugs produce CPP but may not be self administered (LSD, buspirone, and pentylenetetrazole), conversely, others show self-administration but do not induce CPP (pentobarbital and phencyclidine) (Bardo & Bevins, 2000). It is also apparent that the precise mechanisms that mediate drug-induced CPP and self-administration of a drug may differ. For instance, D2 receptor antagonists have minimal effects on the ability of cocaine to produce CPP, whereas self-administration for cocaine can be readily attenuated (Bardo, Valone, & Bevins, 1999).

Rodents clearly provide a powerful model to investigate the reinforcing and rewarding properties of drugs using either the CPP or self-administration however, challenging husbandry, difficult developmental manipulation, and being unsuited to high-throughput screening impairs utility of rodent models. The zebrafish provides an opportunity to overcome these limitations utilizing a CPP paradigm.

An important methodological concern to consider in CPP studies is the whether the conditioning apparatus is biased or unbiased. A biased CPP relies on the apparatus being designed in such a way that subjects consistently display place preference for one compartment prior to conditioning (Tzschentke, 2007a). This is opposed to an unbiased design where animals do not display a significant initial preference for one stimulus before the conditioning phase of the procedure. The two manifestations of the procedure have been investigated in a mouse assay of ethanol place preference in mice (Cunningham, Ferree, & Howard, 2003) where both designs were utilized. Using the biased apparatus, CPP was only observed when ethanol was paired with the non-preferred side. However, when using the unbiased apparatus CPP was observed regardless of which side the ethanol was paired with. It is therefore important to consider apparatus design when evaluating the rewarding effects drugs with the unbiased design being the most predominately used method.

Previous work has shown that widely abused compounds such as cocaine (Darland & Dowling, 2001), amphetamine (Ninkovic et al., 2006), nicotine and ethanol (C. H. Brennan et al., 2011; L. J. M. Kily et al., 2008), morphine (Lau et al., 2006) are rewarding in zebrafish, reliably inducing CPP in a variety of manifestations of the procedure. Nicotine has previously been reported to be reinforcing in zebrafish (C. H. Brennan et al., 2011; L. J. M. Kily et al., 2008) using an un-biased conditioning paradigm and a tank divided into equal zones with distinct spot versus stripe environmental cues. In order to assess the robustness of this assay and the conservation of reward pathways in zebrafish, CPP responses of adult zebrafish to a range of compounds from different classes of commonly abused drugs were determined. The compounds tested were the stimulants nicotine, amphetamine and caffeine, the opiate fentanyl and the general anesthetic phencyclidine (PCP).

3.2 Material and Methods

3.2.1 Test subjects

All zebrafish were bred and reared in our aquarium facility according to standard husbandry protocols outlined in chapter 2 of this thesis. Fish were housed in a 14h:10h light:dark cycle (08:30am–22:30pm). All fish were 3-5 months old at the start of testing. The housing and testing rooms were maintained at ~25–28◦C. Fish were fed 3 times daily with live artemia (twice daily) and flake food (once). Fish were housed in aquarium water consisting of de-ionized water with added salts. All procedures were carried out under the Animals (Scientific Procedures) Act, 1986, and under local ethical guidelines.

3.2.2 Conditioned place preference assay

CPP was carried out in opaque rectangular tanks of dimensions (l x w x h) of 33cm x 16.5cm x 17cm with a central removable plastic divider (Fig 3.1) containing 3L of water. Both ends of the tank have distinct visual cues; one end "spots" has 1.5cm diameter black spots uniformly dispersed on all sides and the other end, "stripes", has 0.6cm wide black and 2.1cm wide white stripes.

The fish to be tested were singly housed in individual tanks on a re-circulating system (Techniplast) for 3 days prior to habituation to the testing apparatus. On the

fourth day individual fish were placed in the testing tank in 3L of system water and allowed to swim freely (without the divider) for 10-20min. Fish were then returned to their single housing for the weekend. On the Monday a baseline preference was read; fish were individually placed into the CPP tanks, without a divider, for a ten-minute period and tracked real-time using an overhead camera connected to a PC running Ethovision software (Noldus, UK). Videos were routinely saved for record and future re-analysis. The baseline preference for each fish was determined by the time spent in either spots or stripes side over the second 5 minute period (min 5-10 of the 10min period). This time period was used to minimise variation in swimming behaviour due to stress responses on initial placement in the tank (fish habituate to a novel environment over a 5 min period (Parker et al, 2012). Entry into a side was defined as the mid point of the fish body over the mid-line of the tank. Any fish that had a baseline preference for one side over 80% was excluded from the study.

Fish that had a baseline preference of less than 80% were pseudo-randomly sorted into 5 groups such that all groups had approximately the same basal preference that was as close to 50% as possible with each group having approx. equal in-group variance. Fish were conditioned to one of the 5 doses (4 drug concentrations plus a vehicle control) on each of three consecutive days. For conditioning, fish were placed individually into the testing tank and restricted to their preferred side (the side where they spent the majority of time during the basal preference test) for 20min. After this time the divider was lifted and the fish allowed (or shepherded) to enter the least preferred side where it was restricted for the following 20min whilst exposed to the test compound: Once the fish had entered the least preferred side the divider was replaced and the drug administered. Drugs were administered in a volume of 50ml system water that was equally divided between the two halves of the tank to minimise

the establishment of concentrations gradients across the tank. Drugs were poured gently into the tank down the divider. Each drug was tested at four concentrations, with a vehicle control group (i.e. fish water), to determine a dose-response curve. After 20min the fish was returned to its housing tank.

After 3 days of conditioning any change in preference was determined using a probe trial; Similarly to the baseline reading, the fish were placed in the testing tank in the absence of divider and filmed over a 10 min period. The time spent in either end of the tank in the second 5 min (min 5-10 of the whole period) was determined. Results were then calculated as a change in preference (proportion of time spent in drug-paired side after conditioning minus proportion of time spent in drug paired side before conditioning). Drugs were considered rewarding if they induced a change in preference at any dose that was significantly greater than vehicle controls.

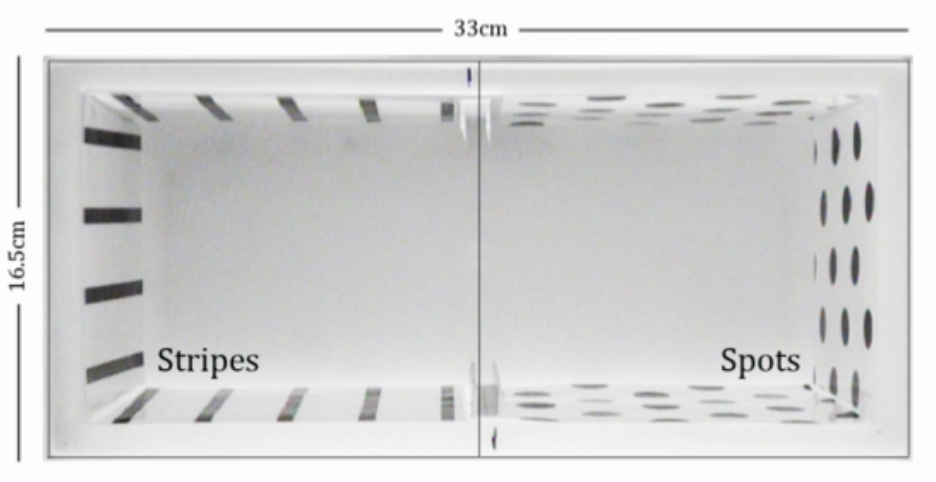


Figure 3.1: CPP tank in zebrafish CPP assay. Overhead view of CPP chamber with removable Plexiglas divider and visual cues on either end of the tank. Tanks are 33x16.5cm in dimension and hold 3 liters of water.

3.2.3 Conditioned place preference data analysis

The data extracted included distance travelled and time spent in the vicinity of the spots and stripes. Data from the first preference test were extracted to determine basal preference for spots or stripes. Fish that fell outside this criterion were excluded from conditioning training. The proportion of time spent in the vicinity of each of the stimuli (i.e., for spots, $Time_{spots}/(Time_{spots}+Time_{stripes})$). Following conditioning, probe trial data (again, including distance travelled and time spent in vicinity of spots and stripes) were extracted, and the time spent in the vicinity of spots and stripes was again calculated as a proportion of total time. The proportion of time spent in the vicinity of the least preferred stimulus during basal (i.e., the subsequently drug-paired cue) was the subtracted from the proportion of time spent by the drug-paired cue during the probe trial to produce a single preference change score for each fish. The change in preference scores was subjected to both linear and polynomial regressions, with dose as the predictor and change in preference as the response variable. To assess the model fit, the model with the highest adjusted (corrected) R^2 value was chosen. The p-values were then compared for each of the regression coefficients to ascertain the best fit of the data. Statistical significance for dose of each drug affecting change in preference was set at $\alpha=0.05$ with the data being analysed in SPSS 21 for Macintosh.

3.2.4 Toxicity testing

Compounds were assessed for effects on pH before use. None of the compounds used caused a significant change in pH at the dilutions used (pH range 7.0-7.5). Prior to CPP analysis, all compounds were assessed for potential toxic effects on the fish: 5 fish were exposed to the proposed highest dose in a volume of 500ml for 20min and observed for signs of toxicity – hyper respiration, difficulty in swimming, subsequent death.

3.2.5 Drugs and doses

Table 3.1 lists the compounds tested, supplier and dose range. Dose ranges were based on previous zebrafish studies or those found to be reinforcing in rodent and with maximum of double the mammalian effective dose. Compounds with no known reinforcing properties were tested at similar ranges to those used in mammals. All compounds were made up as stock solutions in water and stored frozen where applicable. Compounds were diluted from frozen and added in a volume of 50ml of fish water 10 min prior to use.

3.2.6 Nicotine treatment and preparation of brain samples

Fish were placed in 1L tanks containing 5 μM nicotine for a duration of either 5, 10 or 20mins. At each time point, fish were removed and killed using an ice bath, at which point the brain was dissected. Brains were pooled at 3 brains per microfuge tube (2 tubes per time point) and homogenizing in 0.1M HCL before storing at -20 ºC.

3.2.7 Determining nicotine brain concentrations

Samples were analysed by high performance liquid chromatography (HPLC) by Mira Doig at ABS Laboratories, Welwyn Garden City, Hertfordshire UK.

Sample was thoroughly mixed with 1.4 mL of 0.5 mol/L sodium hydroxide. Samples were then transferred to prepacked Extrelut®-3 glass columns (Merck KGaA). The columns had been washed with 15 mL of dichloromethane and left to dry overnight 1 day before analysis.

The analytes were introduced to the MS detector by injecting 20 µL of sample through a HPLC system consisting of an Alliance 2690 separations module (Waters) connected to a µBondapak C18 2 × 300 mm column (particle size, 10 µm; Waters) operated at 30 °C Samples were separated isocratically, using a methanol-acetonitrile-aqueous buffer solvent system (see above) at flow rate 0.5 mL/min. Runtime was 15 min.

3.3 Results

3.3.1 Analysis of baseline

Before continuing on to drug testing, it was important to establish the validity of the exemplars in use. If the zebrafish shows significant preference for one or the other they cannot be justifiably used in the CPP model. The fish had their preference baseline measured, followed by 3 days conditioning to saline, then a 'probe' measurement was taken. The results show the fish (n=20) to have a baseline preference of 0.53 for 'stripes' and a baseline of 0.47 for 'spots' while in the 'probe' trial a preference of 0.46 for 'spots' was observed. There was no significant difference in preference for either exemplar between the two trials (T-test, P=0.59), indicating the conditioning process to have no effect on basal preference.

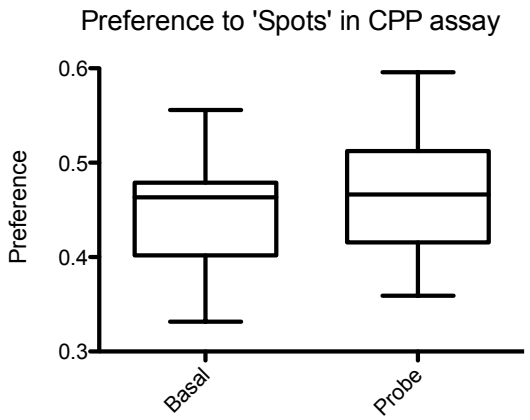


Figure 3.2: Baseline and probe for saline treated fish. Analysis of baseline for n=20 fish showed a preference of 0.53 for 'stripes' and a preference of 0.47 for 'spots' with the 'probe' fish showing a minimal change to 0.46 for 'spots' with there being no significant difference in preference for either exemplars between the two trials (T-test, P=0.59).

3.3.2 CPP results

A total of 5 compounds (including nicotine) were tested at 4 doses in the CPP paradigm and all of the compounds showed statistically significant dose-dependent induction of place preference. The drugs that were tested were the stimulants nicotine (p=0.01), amphetamine (p=0.02), caffeine (p=0.01), the opioid compound fentanyl (p=0.01), and the general anesthetic PCP (p=0.03). Nicotine showed a dose dependent CPP over a range of 0.5 – 10 μM with a maximum occurring at 5 μM. Statistical analysis showed this to be significant when analyzed using both a linear (P=0.01) and polynomial (P=0.01) regressions. Post-hoc t-tests showed only the 5 μM to be significant from control. Amphetamine showed CPP over a range of 2.5 – 15 mg/L with the greatest preference change occurring at a dose of 10 mg/L. This was significant when analyzed with both linear (P=0.01) and a polynomial (P=0.01) regression. The 5 mg/L dose was shown to be significantly different from control post-hoc. Caffeine gave a dose response curve over a range of 5 – 50 mg/L with a maximum change at 10 mg/L. This was significant when analyzed using both a linear (P=0.01) and polynomial (P=0.01) regressions. However there were no individual doses significantly different from control post-hoc. Fentanyl showed dose dependent CPP over a range of 0.004 – 0.16 mg/L with the highest response being at 0.04 mg/L. This was significant when analysed using both a linear (P=0.01) and polynomial (P=0.02) regressions. The 0.016, 0.04, 0.16 mg/L doses were all significantly different from controls. PCP showed a dose dependent response over a range of 0.1 – 1 mg/L with 1 mg/L being the highest.

For example a 0.5 change in place preference would be indicative of a shift from a 50/50 equal preference to a 100/0 preference for the position where a drug is added

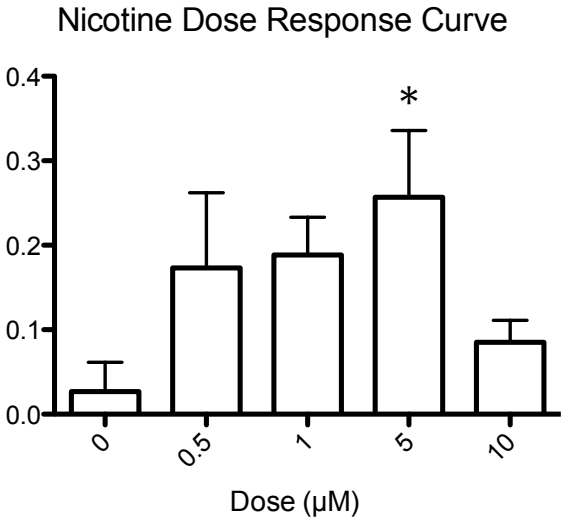


Figure 3.3: Nicotine dose response curve. Nicotine was shown to be highly reinforcing and was significant when analyzed using both a linear (P=0.01) and polynomial (P=0.01) regressions. There was a peak change in preference at a dose of 5μM, which was significant when compared with saline vehicle (p=0.04).

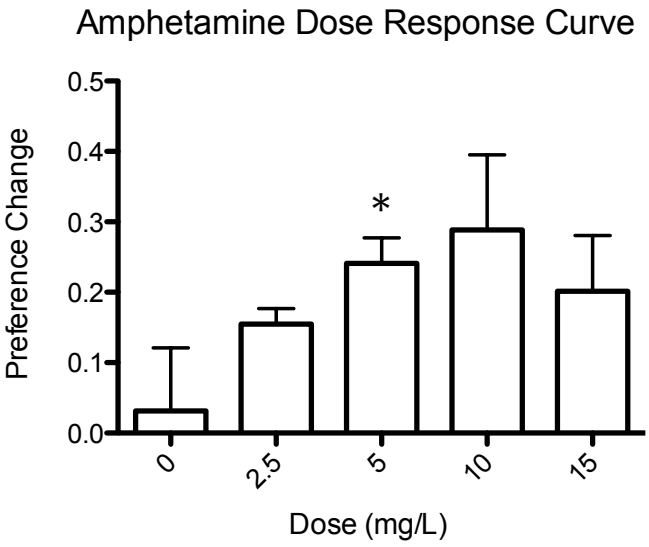


Figure 3.3: Amphetamine dose response curve. Amphetamine was highly reinforcing with the 10 mg/L dose showing the highest CPP. The dose response curve was significant when analyzed with both linear (P=0.01) and a polynomial (P=0.01) regression.

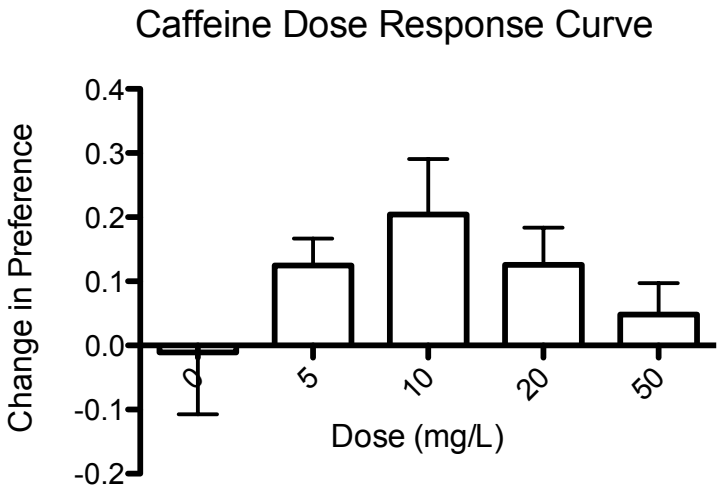


Figure 3.4: Caffeine dose response curve. Caffeine was shown to be highly reinforcing with the 10mg/L dose showing the highest CPP. The caffeine dose response was significant when analysed using both a linear (P=0.01) and polynomial (P=0.01) regressions.

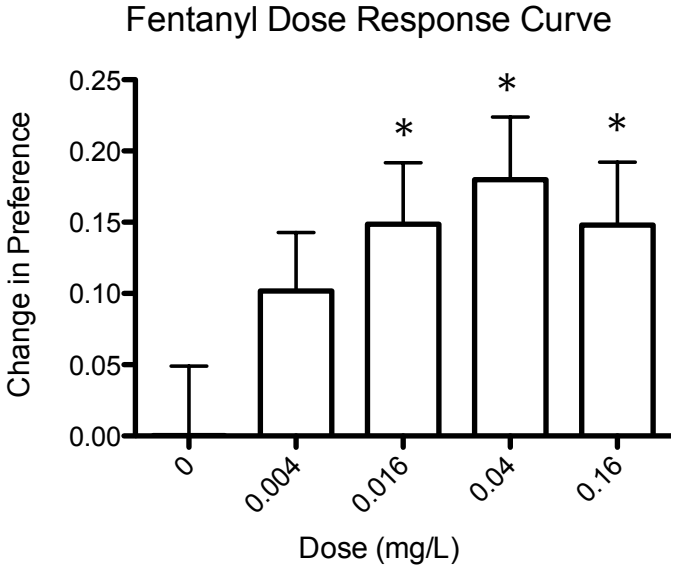


Figure 3.5: Fentanyl dose response curve. Fentanyl was shown to be highly reinforcing with the 0.04 mg/L dose showing the highest CPP. The caffeine dose response was significant when analysed using both a linear (P=0.01) and polynomial (P=0.02) regressions.

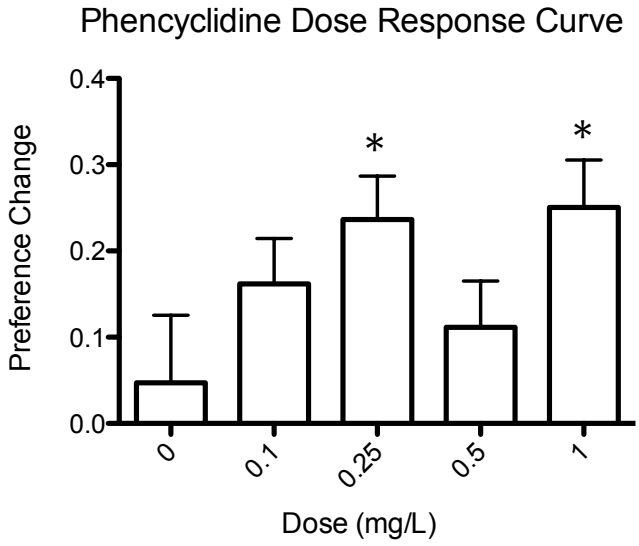


Figure 3.6: Phencyclidine dose response curve. Phencyclidine was shown to be reinforcing with the 1mg/L dose showing the highest CPP. The caffeine dose response was significant when analysed using a linear regression (P=0.01) but not by polynomial (P=0.01).

3.3.3 Nicotine brain concentrations

Fish placed in tanks containing water with 5 uM nicotine showed increasing concentrations of brain nicotine the greater the duration of exposure. Zebrafish assayed after 5 mins nicotine exposure showed a total brain concentration of 0.5 uM. After 10 mins exposure brain concentration rose to 0.77 uM, while after 20 mins exposure brain concentration was at 1.26 uM.

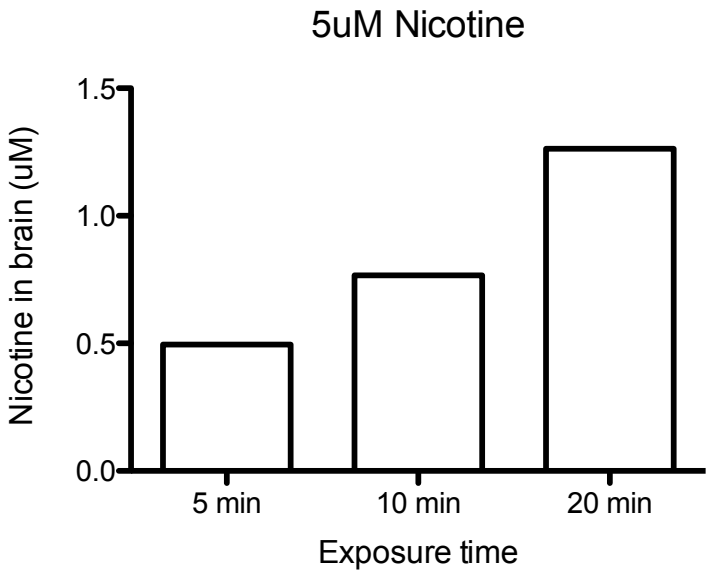


Figure 3.7: Zebrafish nicotine brain concentrations. Graph showing the brain concentration (n=2 for each time point) of nicotine in adult zebrafish brains when submerged in water containing nicotine at 5 uM for 5, 10 and 20 minutes.

3.4 Discussion

3.4.1 Nicotine

The results gave a typical dose response curve, with a peak at 5 μM which was significant when analyzed with both a linear and polynomial model. The only dose significant in post-hoc tests was the 5 μM. As such, 5 μM was selected as the dose to be administered for the mutagenesis screen in the next chapter of this study.

The average blood concentration for a typical smoker (both male and female) varies between 0.004 and 0.72 μM with an average concentration of 0.2 μM (Russell, Jarvis, Iyer, & Feyerabend, 1980). Since the 5 μM present in the water here would be lethal in humans if a corresponding blood concentration was reached (Mayer, 2014) it was important to determine the rate of nicotine uptake in the fish to identify whether the dose being used was translationally relevant. In zebrafish, after 5 mins exposure to 5 μM nicotine brain concentrations were at 0.5 μM, after 20 mins an accumulation of nicotine was observed with a brain concentration of 1.26 μM recorded. This is in concordance with approximate brain concentrations you would find in humans who show similar rates of nicotine accumulation using PET scans and ^{11}C-nicotine loaded cigarettes (Rose et al., 2010).

3.1.2 Caffeine

Caffeine is a stimulant compound belonging to the xanthine class of chemicals naturally found in coffee, tea, and chocolate making caffeine the world's most widely used psychoactive drug. Caffeine's mechanism of action differs from that of most other addictive drugs by being an adenosine receptor A1 and A2A antagonist. Adenosine A1 receptors are presynaptic and reside in many areas of the brain, including the cerebral cortex and hippocampus, where they inhibit the release of dopamine, glutamate, and acetylcholine (Fisone, Borgkvist, & Usiello, 2004). Adenosine A1 and dopamine D1 and receptors also form functionally interacting heteromeric complexes providing another means by which caffeine may affect DA signaling (Svingos, Colago, & Pickel, 1999).

The results showed caffeine to be reinforcing at 10-20 mg/l. Previous research has shown that low doses (1–10 mg/kg) of caffeine are reinforcing for CPP in rats, while high doses (20–30 mg/kg i.p.) are aversive (Bedingfield et al. 1998).

3.1.2 Amphetamine

Amphetamine is a potent psychomotor stimulant that is commonly used in the treatment of attention deficit hyperactivity disorder (ADHD) and narcolepsy. Amphetamine exerts its psychoactive properties by diffusing into dopaminergic cells via the dopamine transporter (DAT), which are found pre-synaptically and is responsible for removing dopamine from the synaptic cleft. Once amphetamine has

diffused into dopaminergic cells, it indirectly causes phosphorylation of DAT. This alters the directionality of DAT, meaning there is no dopamine reuptake but instead there is dopamine release into the presynaptic cell. It also pushes dopamine out of the vesicles in the presynaptic cell, which in turn is released through the reversed DAT. These processes are cumulative and result in increased dopamine levels in the synapse by pushing dopamine out the presynaptic cell and blocking reuptake. At high doses amphetamine has been shown to also inhibit monoamine oxidase-A (MAO-A) reducing dopamine degradation (D. E. Clarke, Miller, & Shore, 1979; Ramsay & Hunter, 2003).

The results for amphetamine and caffeine also validate previous reports that stimulants are reinforcing in zebrafish, as they are often are in humans and rodents (O'Connor et al. 2011). Amphetamine, has been shown in previous CPP studies to be reinforcing in adult zebrafish (Collier & Echevarria 2013), and in rats intravenous injections of amphetamines exhibit a conditioned place preference at a range of 1–3 mg/kg of the drug (Bardo et al., 1999). CPP has also been observed with intracranial injections and intracerebroventricular injections rodents (Gerdjikov & Beninger, 2006; O'Dell, Sussman, Meyer, & Neisewander, 1999).

3.1.3 Fentanyl

Fentanyl is a potent, synthetic opioid analgesic with a rapid onset and short duration of action, mainly due to being a strong agonist at the μ-opioid receptors (MOR). Activation of μ-opioid MORs, which are located predominantly on GABAergic cells

in the VTA (Dilts & Kalivas, 1989; S. W. Johnson & North, 1992; Svingos et al., 1999) selectively hyperpolarizes GABAergic interneurons, thereby disinhibiting DA neurons and increasing dopamine release (Di Chiara & Imperato, 1988; S. W. Johnson & North, 1992). A variety of opiates have been demonstrated to produce CPP in animal models including morphine and heroin (Ashby, Paul, Gardner, Heidbreder, & Hagan, 2003; Cicero, Ennis, Ogden, & Meyer, 2000; L. A. Parker, Corrick, Limebeer, & Kwiatkowska, 2002; Paul, Dewey, Gardner, Brodie, & Ashby, 2001), while fentanyl has been shown to induce CPP at 56 μg/kg in rodents (Suzuki et al., 2005).

Opiates are known to have strong rewarding properties in both humans and rodents. Fentanyl was also shown to be significantly reinforcing in zebrafish. Two independent studies on fentanyl have shown that concentrations of 0.004 and 0.016 mg/kg, in Sprague-Dawley and Long Evan rats respectively, are reinforcing in adult male rats using CPP (Miller & Nation 1997; Vitale et al. 2003), and this is in agreement with the zebrafish results that show reinforcement at between 0.004-0.04 mg/l.

3.1.4 Phencyclidine (PCP)

Phencyclidine, more commonly abbreviated to PCP is a recreational dissociative anesthetic, whose main mechanism of action is as an NMDA receptor antagonist (Giannini, Loiselle, Giannini, & Price, 1985). PCP also shows similar affinity to 5-HT receptors as it does for NMDA, while also showing partial agonism at the D2 receptor (Kapur & Seeman, 2002).

In the zebrafish model, PCP (0.1-1 mg/L) induced place preference. In the literature there are mixed reports of reinforcement for rodents using PCP. PCP dose-dependently produces place aversion at 4 and 8 mg/kg in rats and mice (Noda & Nabeshima 1998; Noda et al. 1998). Place aversion is governed by the same principles at place preference except the testing animal associates the visual cues with a negative stimulus (the drug in this case). However at lower doses (comparable with the doses used in this study) PCP has also been shown to produce reinforcement in rodents when administered at a smaller dose (0.45 mg/kg) with shorter exposure duration (Marglin et al. 1989).

3.1.5 Summary

It was noted during baseline filming that a proportion of fish (~10%) showed large preferences of one of the paired stimuli. Despite habituating subjects to the testing apparatus the week before, when a test subject is added to the testing tank for baseline measurement a freezing response can occur during initial filming. It was for this reason that the last 5 minutes of the total 10 minutes trial was only used as a measure of baseline. This minimized subject loss due to freezing behavior for the most part. Any fish that showed persistent freezing, or an extreme bias for either cue with a basal preference greater than 0.75 was omitted from further testing. Removing these subjects from further analysis was important in minimizing the possibility of habituation responses being misinterpreted as drug-induced CPP.

Overall the results indicate a good degree of correlation between results found in zebrafish CPP and mammalian self-administration and/or CPP assays. Since the assay has been shown to induce robust CPP with multiple drugs, it should be sensitive enough to pick up genetic variations that may affect the sensitivity of zebrafish to rewarding properties of nicotine. As such, it can be implemented in the population mutagenesis strategy discussed in the next chapter.

Chapter 4

A behavioral genetic screen for nicotine reward in zebrafish.

With the parameters of the CPP assay defined and validated, this chapter explores using the assay to screen mutagenized zebrafish for mutations affecting nicotine-induced reward.

4.1 - Introduction

Human genome-wide association studies (GWAS) have identified a number of alleles associated with tobacco use and response to cessation treatment (Brock, Takeda, Brennan, & Walton, 2011; Caporaso et al., 2009; J. Z. Liu et al., 2010; Y. Z. Liu et al., 2009; Siedlinski et al., 2011; Thorgeirsson et al., 2008; Thorgeirsson et al., 2010; Tobacco & Genetics, 2010). The majority of the genes showing high statistical associations with smoking behaviors are those in the cholinergic receptor gene clusters, variants in acetylcholine pathways and those in dopaminergic pathways (Saccone, Saccone, et al., 2009; Saccone, Wang, et al., 2009) (W. Berrettini et al., 2008; Tobacco & Genetics, 2010). However, despite the high concordance rates for smoking, only a fraction of the variation can be explained by candidate genes identified from GWAS analysis (M. Munafo, Clark, Johnstone, Murphy, & Walton, 2004). Although animal models cannot replicate all the complexities of human smoking, they can help with the identification of genetic factors influencing various components of addictive behavior such as reward sensitivity, consumption, persistent drug taking, and relapse. As mentioned in the previous chapter, zebrafish have been show to respond to the rewarding effects of substances by demonstrating CPP to a number of drugs including nicotine. Sensitivity to the rewarding effects of nicotine is one facet of smoking behavior and as such CPP can be used to screen lines of zebrafish for genetic variants that affect that behavior.

By using animal models, it has been possible to track naturally occurring variations in genes using breeding and selection to identify genetic variants that

segregate with behaviours of interest. These studies are obviously limited by both the numbers of important variants that occur naturally and by the severity of the phenotype they induce, meaning a number of important loci are likely to be missed. Mutagenesis is a technique that can introduce 1000s of point mutations into the genome thus dramatically increasing variation and potentially inducing mutations affecting the phenotype of interest.

Large-scale mutagenesis screens have facilitated forward genetic strategies for identifying the role of genes in behaviour (Hrabe de Angelis et al., 2000; Nolan, Hugill, & Cox, 2002). Nolan et al. reported a large study in which phenotype-driven mutagenesis was used in the identification of novel genes and pathways utilizing a mouse model. Over 26,000 mice were generated and screened, with some 500 new dominant mouse mutants recovered (Nolan et al., 2000). However, the mouse is a relatively expensive and complex model organism to use, and along with relatively long generation gaps and small litter sizes, this makes in vivo genome-wide genetic approaches impractical in many important scientific scenarios (Sivasubbu, Balciunas, Amsterdam, & Ekker, 2007).

In contrast, chemical ethylnitrosurea (ENU) mutagenesis has been used to great effect in zebrafish and has led to the generation of large numbers of fish lines carrying multiple mutations. The external fertilization, high fecundity, rapid development, and production of optically clear embryos, and relatively short generation time allows for high throughput screening of the development and behavior of these mutant lines, offering a significant refinement on mouse models. Recently zebrafish have gained interest as a behavioural model raising the possibility of performing a forward genetic screen in zebrafish for drug seeking behaviour.

Zebrafish are an extremely effective model for investigating genetic factors that contribute to drug seeking behaviour and as such, are increasingly being used to address these questions.

So far, advances in our understanding have relied heavily on hypothesis driven and candidate gene approaches. However, by utilising these large-scale mutagenesis approaches in zebrafish, it is not necessary to know the genes or pathways involved in advance. Such approaches are more likely to lead to novel and unexpected breakthroughs and lead to greater leaps in our understanding of genes and pathways the contribute to disease (Panula et al., 2010). This is particularly important when investigating genetic factors which may contribute to complex neurological disorders like addiction, as due to the complex neural pathways involved there is unlikely to be one or two genes of large effect, but rather a combination of multiple genes of variable penetrance and additive effect. Such factors are unsuited to more traditional reverse genetics experiments, as subtle differences in naturally occurring genes of minor effect will fail to segregate with behaviour due to the subtle phenotypes they confer. However, by introducing a gene breaking mutation by ENU, genes of previously minor effect will have greater penetrance and be selected for in a population screen. This way genes are selected for that would previously be masked in studies of this type.

Mutagenesis studies in zebrafish have been used to good effect in the past. In a special issue of *Development* in 1996, 37 papers presented the results of two large zebrafish mutagenesis screens performed in Tübingen and Boston (Nusslein-Volhard, 2012). Around 1500 mutations in more than 400 new genes were identified, involved in processes such as development and organogenesis. Up to this day, the mutants

provide a rich resource for many laboratories studying embryogenesis, neuronal networks, regeneration and disease. Christiane Nüsslein-Volhard's lab performed a large scale mutagenesis screen which resulted in the identification of approximately 1,000 mutants affecting processes such as early development, organ formation and axonal pathfinding (Brand, Heisenberg, Jiang, et al., 1996; Brand, Heisenberg, Warga, et al., 1996; Haffter, Granato, et al., 1996; Haffter & Nusslein-Volhard, 1996; Haffter, Odenthal, et al., 1996; Hammerschmidt et al., 1996; Heisenberg et al., 1996; Odenthal et al., 1996; van Eeden, Granato, Schach, Brand, Furutani-Seiki, Haffter, Hammerschmidt, Heisenberg, Jiang, Kane, Kelsh, Mullins, Odenthal, Warga, Allende, et al., 1996; van Eeden, Granato, Schach, Brand, Furutani-Seiki, Haffter, Hammerschmidt, Heisenberg, Jiang, Kane, Kelsh, Mullins, Odenthal, Warga, & Nusslein-Volhard, 1996; Whitfield et al., 1996). Wolfgang Driever's group also presented data from a similar ENU mutagenesis strategy to screen F3 embyos from over 2000 families for developmental abnormalities. A total 2383 mutations resulting in abnormal embryonic phenotypes affecting pigmentation, motility, muscle and body shape were identified (Abdelilah et al., 1996; Driever et al., 1996; Malicki, Neuhauss, et al., 1996; Malicki, Schier, et al., 1996; Neuhauss et al., 1996; Pack et al., 1996; Schier et al., 1996; Solnica-Krezel et al., 1996; Stainier et al., 1996; Stemple et al., 1996; Weinstein et al., 1996). More recently, a rapid total coagulation activity (TCA) assay has been used to screen ENU mutants for coagulation defects in individual adult zebrafish (Jagadeeswaran, Gregory, Johnson, & Thankavel, 2000), by screening 1000 lines of these mutant fish, 6 lines were identified with defective clotting.

4.1.1 - Experimental design

The fish used in this screen were obtained through the Welcome Sanger Trust as part of the zebrafish mutation project, an open resource in which they seek to identify, phenotype and distribute a large number of mutant zebrafish lines. The aim of the Sanger project is to identify mutations in the zebrafish using whole exome sequencing, outcrossing followed by re-sequencing until stable lines containing a single mutation in a coding region are generated. Mutants are analysed for morphological and molecular differences and distributed to the community upon request. Through collaboration with the Sanger we obtained a number of these ENU-induced mutant lines at the F2 stage, before the process of outcrossing (**Figure 2.1**). As a result, the fish have thousands of mutations across the genome including functional mutations in between 10 to 15 coding regions. This means that with F1 outcrossing followed by an F2 incross there can conservatively by up to 3.75 mutations for every F3 family generated. Since 30 lines were generated, it can reasonably be estimated that around 112 mutations were included in the first F3 screen. The high numbers of SNPs combined with mutation in known coding regions make these fish ideal candidates for participation in a mutagenesis screen. The F2 siblings can then be inbred to generate F3 fish with these high numbers of SNPs (figure 4.1). The first reason F3 lines were generated, is so that lines could be generated with fish of similar age (within three months). This minimizes any age related effects that may contribute to varied nicotine response. It also means that the

screen will theoretically be able to pick up any recessive phenotypes, which may present themselves.

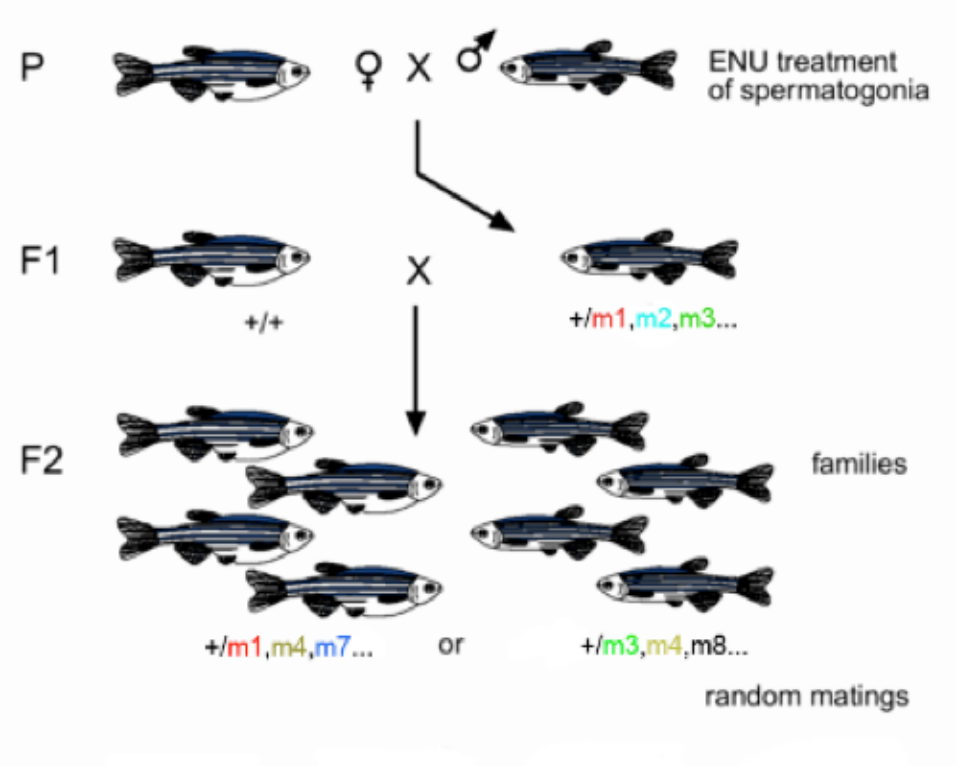


Figure 4.1: Strategy to generate F2 families with multiple gene breaking mutations as outlined on the Sanger website (http://www.sanger.ac.uk/Proj ects/D_rerio/zmp/). After this genotyping and outcrossing will be used to generate lines containing a functional mutation in one gene. For this project the lines were obtained before outcrossing, meaning the fish in the screen have multiple gene-breaking mutations as well as a number of markers in non-coding regions.

The 'Humphrey Bogart' (*HumBog*) line, which has previously displayed decreased locomoter response to nicotine (Petzold et al., 2009), was included in the screen as a positive control. The line contains a gene breaking transposon in the gabbr1.2 gene, which codes for the GABAB receptor, responsible for inhibiting the release of GABA, which is a major inhibitory neurotransmitter in the VTA. A knock out at this locus would essentially increase dopaminergic transmission by reducing GABA mediated inhibition of dopamine neurons.

Using this experimental design, distinct lines can be generated over 4 generations, with screening and selecting fish at the extremes resulting in families that carry mutations affecting nicotine CPP. The screen may pick up dominant or recessive mutations of major effect, which can be verified by screening outlier siblings. The continued selective breeding allows the amalgamation of alleles of lesser effect to generate multiallelic phenotypes. Once these distinct lines are generated the possibility exists in the future to perform highly detailed SNP-mapping procedures to identify SNPs associated with increased and decreased reward seeking behavior.

4.1.2 - Screening for persistent CPP and relapse

While drug-induced reinforcement can be assessed easily using a CPP paradigm, drug addiction is much more complex and multi-faceted encompassing a range of elements including compulsive drug seeking, relapse, loss of control/impulsivity, and continued compulsive drug seeking (Sanchis-Segura & Spanagel, 2006). While it is impossible to model addiction (an exclusively human behavior) in its entirety in a lab environment, specific elements of addictive behavior have been adequately modeled. Therefore it would be ideal to screen the mutant lines for multiple behaviors influencing addiction where possible. Since differing neural pathways control these behaviors, there are going to be different subsets of genes involved.

The primary aims of this study are to show the CPP paradigm to be sufficiently sensitive to detect *Humbog* mutant clustering and use the lines provided by the Sanger Institute and screen them for nicotine reward to identify families that

may house dominant or recessive mutations and to determine if CPP in zebrafish shows significant heritability as in humans. If heritability is established, breeding and selection will be carried out to generate families containing dominant and recessive genotypes influencing behavior. The protocol will also be expanded to allow an additional screening for persistent CPP that lasts following a period of abstinence or in the face of an adverse stimulus. Assaying for CPP in this way acts as a model for both drug reward and drug dependency, an important factor process of addiction, and would allow for selection of mutations encompassing additional components of addictive disorders.

Continued seeking and taking of drugs despite adverse legal, health, economic, and societal consequences is a central hallmark and most debilitating aspect of human drug use disorders (Koob & Volkow, 2010; Tiffany & Conklin, 2000). As such, a pre-clinical model of this compulsive drive for drugs has been modeled in rodents using cocaine and ethanol (Deroche-Gamonet, Belin, & Piazza, 2004; Hopf, Chang, Sparta, Bowers, & Bonci, 2010; Lesscher, van Kerkhof, & Vanderschuren, 2010; Vanderschuren & Everitt, 2004). In rodent models, drug taking is considered 'compulsive' when extended drug-taking history renders drug seeking impervious to adverse stimuli such as signals of punishment (Vanderschuren & Everitt, 2004).

Normally drug-seeking behavior can be readily suppressed by aversive environmental stimuli (Kearns, Weiss, & Panlilio, 2002; Killcross, Robbins, & Everitt, 1997), this is known as conditioned suppression. Heyne and Wolffgramm showed this conditioned suppression to be abolished after long periods of voluntary drug consumption even when the drug was administered with a bitter quinine flavor.

This observation demonstrated persistence in drug consumption regardless of the adverse consequences associated with it.

Kily *et al*. described a zebrafish version of the aforementioned rodent paradigm of persistent drug seeking despite adverse consequences, the model that will be used here. As a continuation of the normal CPP procedure (baseline; 3 conditioning sessions; probe trial) the zebrafish were conditioned for a further 3 week. Subjects were then placed in the testing apparatus and punished when entering the drug paired side, first by restriction (mild punishment) and secondly by removing from the tank by netting (severe punishment). The number of times the subjects persisted with entering the drug-paired side despite negative reinforcement was taken as a measure of compulsive drug seeking period (L. J. Kily et al., 2008).

4.2 - Materials and Methods

4.2.1 – Fish housing

Fish were housed in a 14h:10h light:dark cycle (0830–2230). All fish were 3-5 months old at the start of testing. The housing and testing rooms were maintained at ~25–28◦C. Fish were fed 3 times daily with live artemia (twice daily) and flake food (once). Fish were housed in aquarium water consisting of de-ionized water with added salts. All procedures were carried out under the Animals (Scientific Procedures) Act, 1986, and under local ethical guidelines (Queen Mary University of London).

4.2.2 - Breeding strategy to generate F3 lines for screening

The zebrafish lines included in the experiment were all generated at the Sanger Institute, as part of the Zebrafish Mutation Project. F2 siblings, heterozygous for a number of gene-breaking mutations (generated using the breeding strategy in **figure 4.2**) were obtained and inbred to generate F3 lines as to allow the inclusion of homozygous mutations in to population screen. Each F3 family was created from a different F2 family that has different F1 parents. A total of 100 ENU-induced F3 mutant zebrafish were initially selected for screening. Both male and female individuals were selected as evenly as possible from 30 distinct lines (3-4 fish from each line), each containing between 10-15 known mutations in coding regions and a

large number unknown mutations across the entire genome (based on an expected mutation rate of 1/300 kb in the F_0 founder fish (D. Stemple, personal communication). All fish were removed from their colonies, and housed individually in 29.7 x 15.2 cm tanks 1 week before commencing, and during the behavioral screening. Following the completion of the CPP screen the individuals in the upper and lower 5% of the change in preference distribution curve with the top and low 5% change scores were kept for analysis and further inbreeding, all remaining fish were terminally anesthetized by immersion in MS-222. As a positive control, 5 individuals from the 'Humphrey Bogart' line containing an insertion in intron 6 of the zebrafish *gabbr1.2* gene were included in the screen were. The 'Humphrey Bogart' line has previously displayed a decreased nicotine response profile in a larval motility assay (Petzold et al., 2009).

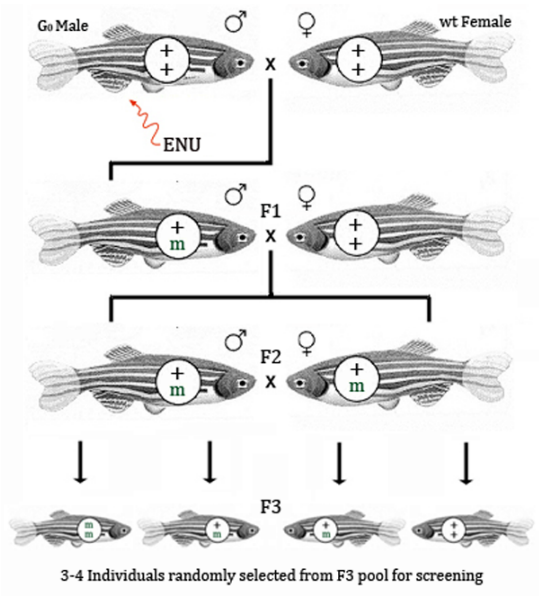


Figure 4.2: Breeding strategy. A general overview of the crosses used to obtain the F3 fish used in the mutagenesis screen. At the Sanger Institute, male sperm was mutagenized and used to fertilize wild-type female oocytes. The resulting F1 were then outcrossed again to generate F2 fish. After generating an F3 in-cross, the fish were then screened for nicotine reward in the CPP paradigm. Both male and female individuals were selected as evenly as possible from 30 distinct lines (3-4 fish from each line), each containing between 10-15 known mutations in coding region.

4.2.3 - Conditioned place preference (5µM Nicotine)

The conditioned place preference procedure was carried out in the same tank as before (W x L x H; 16.5cm x 33cm x 15 cm) and containing 3L water. The visual cues (black spots or black vertical lines) were located on the walls of the tanks (see Figure 3.1). One day prior to the start of the experiment (on Thursday) fish were singly housed. Of the 30 families included in the screen, 4 individuals were selected from each to make a total of 120 fish. The experiment began the following day (Friday), all fish were first placed in the conditioning tanks (including exemplars), with no nicotine added, for a period of 40 mins. CPP procedure was carried out as previously mentioned in chapter 3.

4.2.4 Persistent CPP despite adverse stimuli

The subjects used in the CPP were conditioned for a further 4 weeks, at which point the effect of punishment as well as restriction on the number of returns made to the drug treatment side over a 10 minute period was determined. Zebrafish were placed in the conditioning apparatus, afforded a 5-minute habituation period and then each time the fish entered the side to which they were conditioned to nicotine, the subject was restricted to the non-drug-treatment side for 30 seconds using a white plastic divider. After 30 seconds the divider was removed and the fish allowed free access to the whole tank. The number of returns made over a 10-minute period was determined. An

hour later each fish was returned to the testing apparatus, allowed 5 min to settle and then each time the fish entered the drug treatment side it was removed from the tank to the air for 3 seconds. On return to the tank, the fish was restricted to the non-drug-treatment side for 30 seconds to allow recovery. After this time the divider was removed and the fish allowed free access to the tank. Again the number of returns made over a 10 min period was determined.

Drug seeking despite adverse stimuli was tested by placing individual fish were placed in the testing apparatus, allowed to settle for 5 min and then each time the fish entered its preferred side it was punished by removal from the tank to the air for 3 s. On return to the tank the fish was restricted to its non-preferred side for 30 s to allow recovery. In each case the number of returns to the preferred side over a 10-minute period was determined.

4.2.5 Breeding and selection over 3 generations

Following completion of the first round CPP screen the individuals in the upper (H) and lower (L) 5% of the change in preference distribution curve were kept for analysis and further breeding. All remaining fish were terminally anesthetized by immersion in tricaine solution (MS-222). To generate subjects for the second round screen fish were bred H:H and L:L. 100 progeny from both the high and low crosses were included in the screen (200 in total) with equal representation from each individual cross. A similar strategy was used for the third round screen.

4.2.6 - Statistical analysis

CPP preference scores were calculated as the proportion of time spent in the vicinity of each of the stimuli (i.e., for spots, Time(T)$_{spots}$/ [T_{spots} + $T_{stripes}$]). For the dose-response curve, we fitted the preference scores to a polynomial regression. This allowed us to account for an inverted U-shaped preference curve. Population means between generations were compared using independent 2-sample t-tests, and effect-sizes were ascertained using Cohen's d (Cohen, 1992).

4.3 - Results

4.3.1 - First-generation screen and HumBog mutants

Using the breeding strategy outlined in **Figure 4.1,** 30 lines of F3 zebrafish were generated and 4 fish from each line included in the first generation population. Change in preference (Cp) for the conditioned cue was normally distributed in the first generation (Shapiro-Wilks test $p = 0.83$), with the mean Cp being an increase of 0.11 (**Figure 4.3**). Four fish from a *Humbog* line (Obtained from Stephen C. Ekker, Mayo Clinic, USA) were included in the screen as a positive control. *Humbog* fish have a Gaba-B receptor 1.2 (*gabbr1.2*) knock-out that has previously been shown to alter sensitivity to nicotine (Petzold et al., 2009). The *gabbr1.2* knockouts clustered at one end of the distribution, this demonstrated the assays ability to identify specific genetic lines with altered nicotine response phenotypes.

Figure 4.3: First generation screen (n=100). The mean change in preference of was 0.12. The top five centiles had a change in nicotine preference of 0.6, with the lowest five showing an aversion to nicotine of 0.4. The blue shading on the graph indicates the lowest 5% of fish that were in crossed to generate the 'low responder' lines for the second-generation screen. The pink shaded region of the distribution represents top 5% of responders that were in crossed to generate the 'high responder' lines for the second-generation screen.

4.3.2 – Conditioning for 5 weeks

After two weeks conditioning to 5 μ M nicotine (3 per week, 6 sessions total), the mean preference change was 0.15 compared with 0.12 after 1 week conditioning (**Figure 4.4**), though this change in place preference was not significant. 5 weeks conditioning to 5uM nicotine (3 per week, 15 sessions total). The mean preference change was 0.18 compared with 0.12 after 1 week and 0.15 after 2 weeks conditioning (**Figure 4.5**). Change in CPP was not significant compared with week 2 ($p = 0.21$) though this change in place preference was significant between week 1 and week 5 ($p = 0.0009$). Comparison between different weeks of screening was analyzed using a one-way analysis of variance in SPSS.

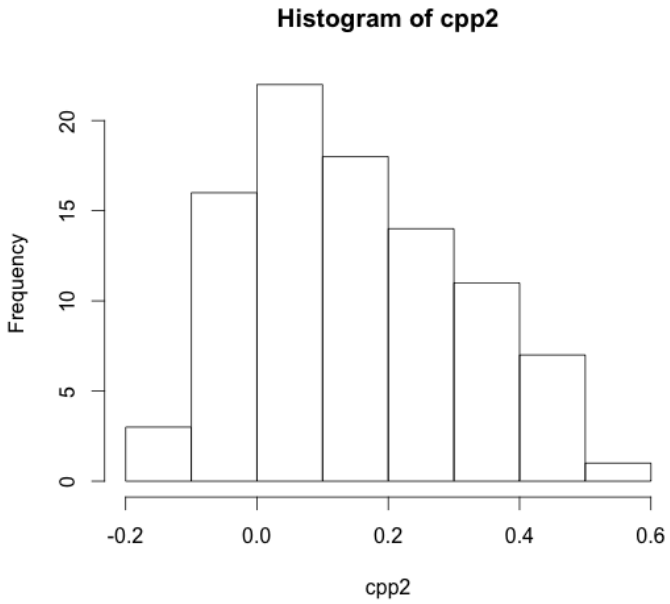


Figure 4.4: Probe of CPP after 2 weeks conditioning. Histogram of F3 ENU population after two weeks conditioning to 5uM nicotine (3 per week, 6 sessions total). The mean preference change was 0.15 compared with 0.12 after 1 week conditioning, though this change in place preference was not significant.

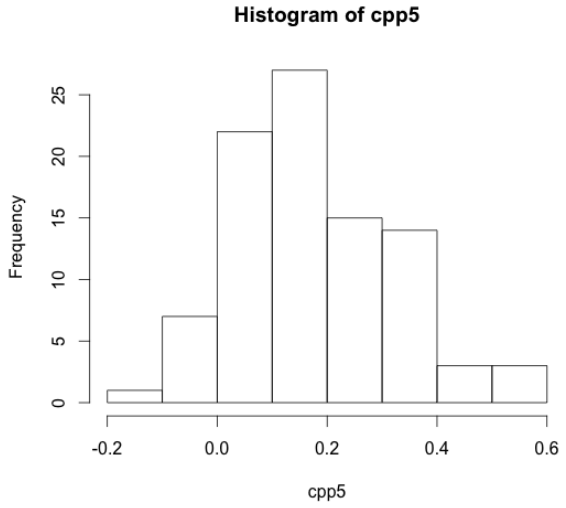


Figure 4.5: Probe of CPP after 5 weeks conditioning. Histogram of first generation ENU population after 5 weeks conditioning to 5uM nicotine (3 per week, 15 sessions total). The mean preference change was 0.18 compared with 0.12 after 1 week and 0.15 after 2 weeks conditioning. Change in CPP was not significant compared with week 2 (p=0.21) though this change in place preference was significant between week 1 and week 5 (p=0.0009).

4.3.3 - Drug seeking despite adverse stimuli

When the subjects were restricted with the divider there was a mean number of 12.29 returns to drug pared side within the 10-minute testing period (**figure 4.6**). The smallest number of returns was 3 with a high of 18. The distribution was negatively skewed. Fish punished by netting showed a mean of 3.49 returns within a 10-minute period (**figure 4.7**). The least number of returns after netting was 1 with the maximum being 7. The distribution was positively skewed which is indicative of how adverse the netting punishment was.

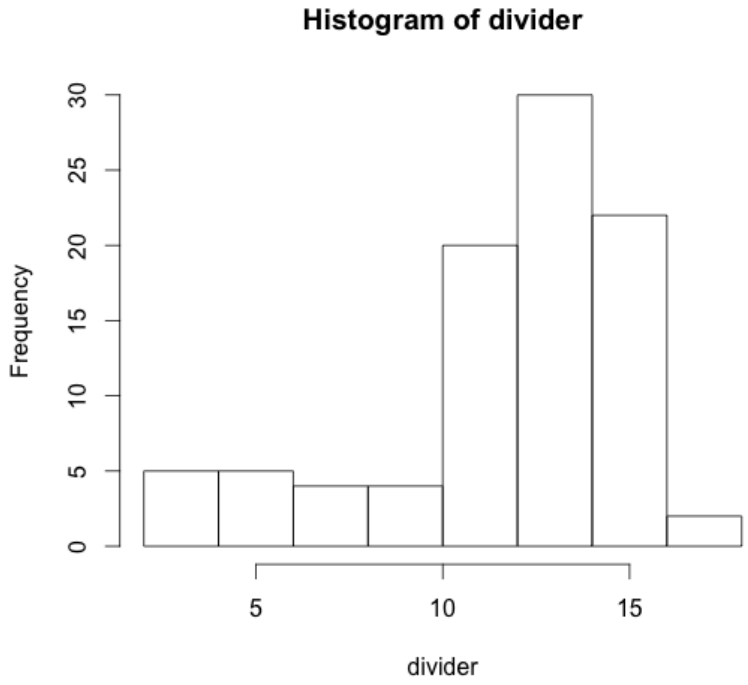


Figure 4.6: Drug seeking despite restriction. The mean number of returns was 12.29. The smallest number of returns was 3 with a high of 18. A total of 94 subjects were included in this assay.

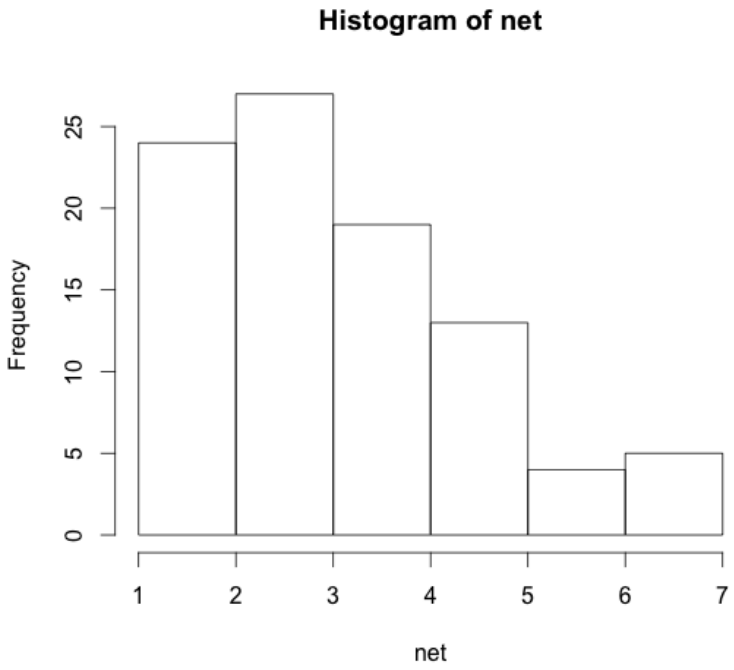


Figure 4.7: Drug seeking despite netting. Punishment by netting showed a mean of 3.49 returns within a 10-minute period. The least number of returns after netting was 1 with the maximum being 7.

4.3.4 - Comparison of netting, restriction and CPP data

The three assays were compared using a Pearson's product-moment coefficient. Netting and restriction were shown to correlate with one another (R^2=0.532; p<0.001). Number of returns when restricted did not correlate with 1 week CPP (R^2=-0.049; p=0.643), 2-week CPP (R^2=0.037; p=0.72) or 5-week CPP (R^2=0.016; p=0.881). Netting did not correlate with 1 week CPP (R^2=0.057; p=0.59), 2-week CPP (R^2=0.093; p=0.376) and 5-week CPP (R^2=0.137; p=0.192). When the mean number of returns for punished (netting) vs. unpunished (restriction) was compared (**Figure 4.8**) there were significantly fewer returns (Students T-Test, $p < 0.0001$) when the fish was netted.

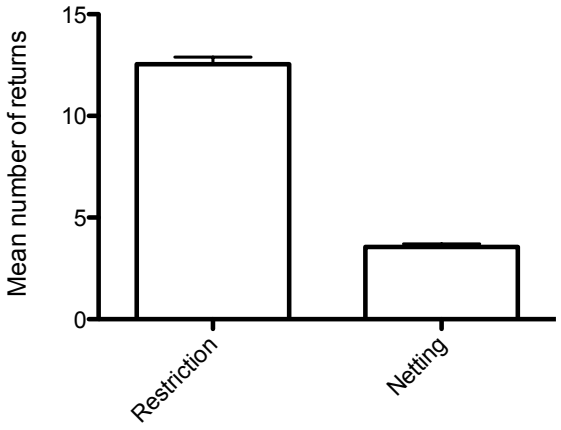


Figure 4.8: Comparison of the number of mean returns to drug-paired side when restricting the zebrafish with a divider and netting. This identies netting is an effective aversive stimulus.

4.3.5 - Second and third-generation screen (F4 and F5)

The second generation showed a shift in mean preference of 0.08 for low responders (Student t-test, p = 0.0002) and 0.07 for high responders (Student t-test, p = 0.001) when compared with the first generation (**Figure 4.9**). The third generation screen gave mean place preference change scores of 0.21 for the high responder lines and 0.01 for the low responder lines (**Figure 4.10**). There was a large effect size (Cohen's d = 1.64) indicating the difference in response to the rewarding effect of nicotine has been genetically maintained. The increase in effect size with each successive generation (d = 0.89 to d = 1.64) indicates that the two populations are becoming more genetically distinct with each generation. These data demonstrate that nicotine-preference is heritable in zebrafish as it is in humans. .

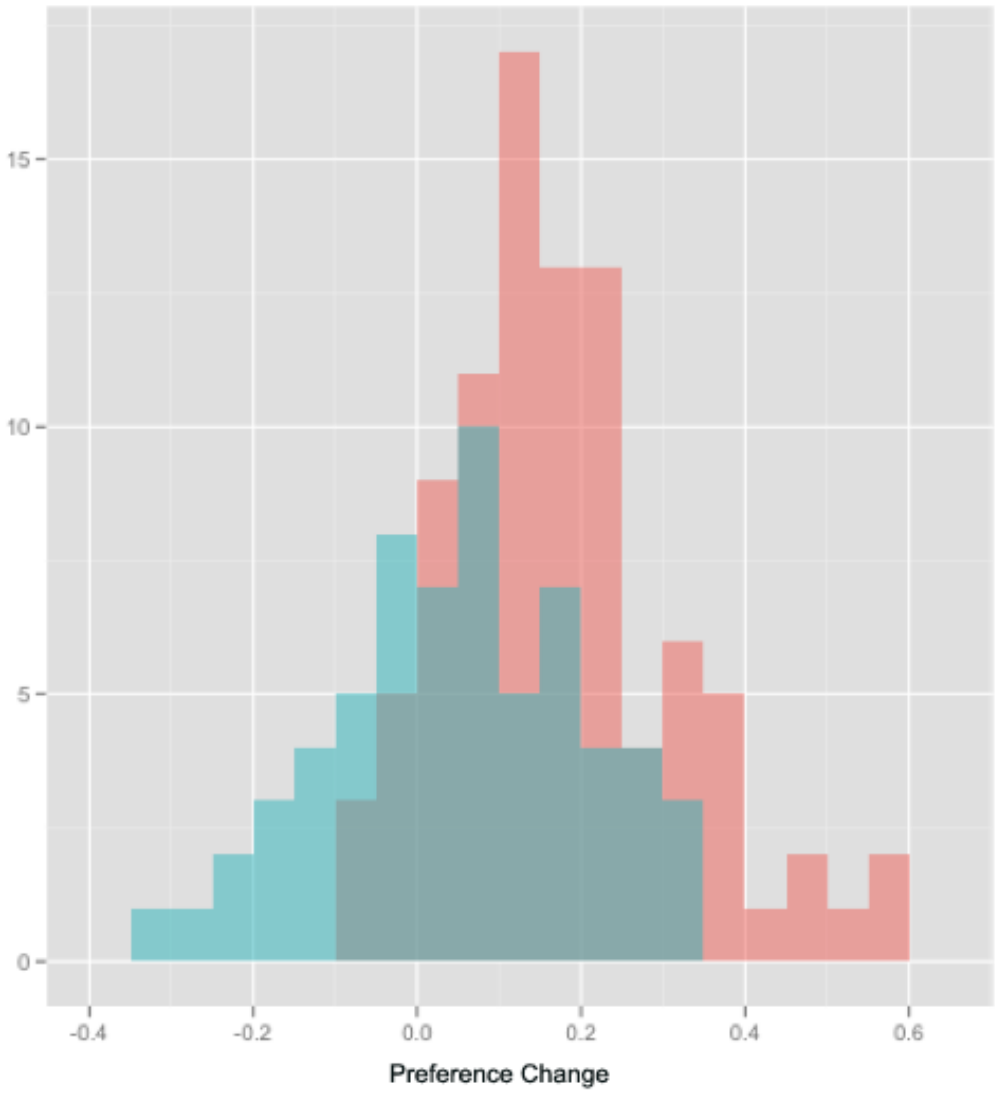


Figure 4.9: F4 Screen (n=184). The mean change score for the high responders was 0.17, while the low responder mean was 0.05. There was a large effect size (D=0.89). There is a statistical shift in nicotine preference for both the high (p=0.03) and low (p=0.01) populations when compared with the first generation screen.

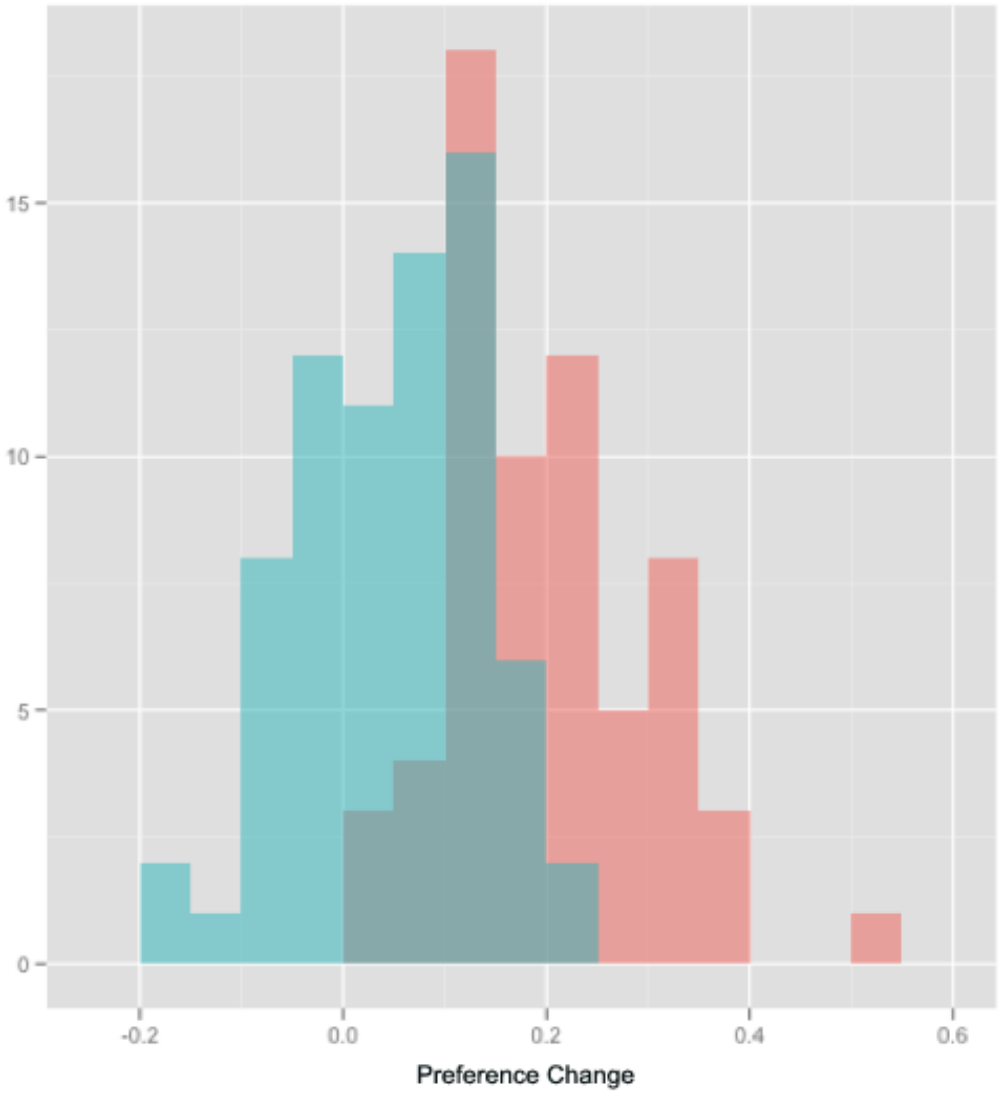


Figure 4.10: F5 screen (n=187). The high responder generation had a mean of 0.21 compared with a population mean of 0.01. There was a larger effect size than the previous generation (D = 1.64).

4.4 - Discussion

The aim of this chapter was to identify lines and individuals showing differential responses to nicotine reward, to determine whether CPP behavior is heritable in zebrafish and generate lines showing differential phenotypes. This was addressed by first running a CPP assays across a large population to see if normal distribution and get evidence that screen was likely to be sensitive enough to detect differences, which was shown by the clustering of *humbog* mutants as well as other lines (discussed in next chapter. The screen was continued for a further 2 generations by inbreeding the outliers at either end of the distribution and screening the offspring for nicotine reward. With each subsequent generation there was a significant shift in mean preference change for both 'high' and 'low' responders indicating the occurrence of a type directional genetic selection.

The second-generation showed a shift in preference in either direction (low responders: $p = 0.03$; high responders: $p = 0.01$) when compared with the previous generation. The mean change score for the high responders was 0.17, while the low responder mean was 0.05. In the case of the third generation screen, there was further divergence from the original population mean with the high responder line showing a change score of 0.21 compared with a preference change of 0.01 in the low responders. There was a large effect size ($d = 1.64$) indicating the difference in response to the rewarding effect of nicotine has been genetically maintained. The increase in effect size from the second generation ($d = 0.89$) indicates the two populations are becoming more genetically distinct with each generation.

The subjects were conditioned for a further 4 weeks after the initial 1-week conditioning period. After 5 weeks there was a marginal but significant shift in place preference from 0.12 to 0.17. After this, the population was tested to see if drug seeking persisted in the face of adverse stimuli, in this instance restriction using a plastic partition and removal from the tank using a net. Both behaviors showed a normal (if skewed) probability distribution, however they failed to correlate with previous CPP data. While you would not expect these behaviors to correlate perfectly as they will be governed by different subsets of neuronal pathways, there was almost no relationship between them whatsoever with R^2 values being close to 0. As a result, it is impossible to say with any confidence that compulsive drug seeking is the behavior being observed in this instance. Another limitation of the procedure is the length of time it takes to assay each fish. It takes 10 minutes in total, though due to the fact only one fish can be assayed at any one time (manual restriction and netting), the 100 fish screen had to be done over multiple days (3 in total). It is for these two reasons that it would not be feasible to screen the fish this for 3 generations.

Nicotine reward in the zebrafish CPP paradigm was established as a heritable characteristic over 3 generations. Now that heritable variation has been displayed and lines with distinct behavioural phenotypes have been established, there is now the potential to use the ENU markers in SNP based genome-association analysis of the families. It will be possible to sequence the parental F3 fish that gave rise to the lines, identify the various ENU markers and use them to identify linked regions associated with the differing nicotine response phenotypes. Once these regions are identified, high density SNP mapping can be used identify linked alleles. The Sanger Institute has in their possession, a 2000 SNP array that can be used now this stage has been

reached (Derek Stemple, personal communication), though this will not occur in time for inclusion in this thesis.

By taking the outliers and incrossing to generate distinct lines, it will then be possible to screen 40 individuals from each family and see whether each 'high' family vary significantly from the average of 'low' families (and vice versa). If there are recessive alleles segregating it is possible to look at all 40 and expect 10 recessive homozygotes (range c 4-16) to be confident of getting two individuals for breeding if there are recessive alleles segregating. By back corossing this identified high lines with low lines it will be possible to identify the alleles that made the extreme individuals have their phenotype by looking at the segregation in backcrosses.

Using this strategy of SNP mapping the lines generated in this screen it will theoretically be possible to identify linked genes of major effect. It also has the power to identify both ENU-induced linked single nucleotide mutations and naturally occurring linked using the principles of mapping by admixture linkage disequilibrium (MALD). The principles of MALD is that any loci in linkage disequilibrium with a locus responsible for vulnerability to nicotine reward will have a greater than expected proportion of markers with ancestry from the high response population. The approach assumes that near a disease causing gene (in this case addiction) there will be enhanced ancestry from the population that has greater risk of getting the disease (high responder lines). An unexpectedly large bump of ancestry in a localized genomic region then suggests that the region may harbor a disease-bearing gene (see **Figure 4.10**).

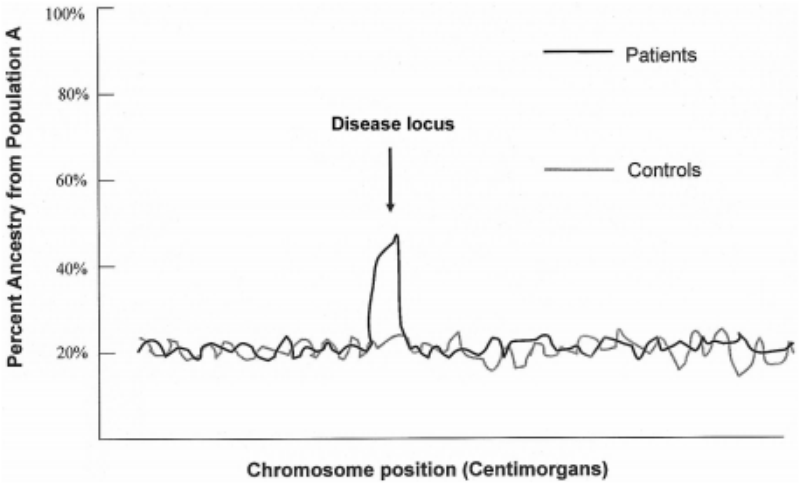


Figure 4.10: Schematic of how a disease locus will appear in an admixture scan. (Taken from ANCESTRYMAP documentation). Around the locus, there should be an unusually high proportion of ancestry from one of the parental populations, because of patients inheriting high-risk alleles from that group.

The linkage mapping of this type can be carried out using ANCESTRYMAP, one of the leading software packages for analysis of this nature. The program uses data from individuals genotyped at a set of markers, where the markers chosen are preferably the ones that differ significantly in frequency between the two ancestral populations. The algorithm calculates a Bayesian-likelihood ratio test to scan for disease association anywhere in the genome. In this calculation, individual ancestry estimates along the genome are averaged across all the individuals to identify genomic regions where there is enhanced ancestry from one of the parental populations, indicating the presence of a disease gene nearby (Patterson et al., 2004).

The results of the first round screen showed that in addition to the humbog clustering, 2 others lines did. The nature of these lines and how they may influence CPP are explored in following chapters.

Chapter 5

Characterization of 2 outlier families and the identification of *slit3* in 'QMhigh' line

The first generation screen is revisited to identify gene-breaking mutations of major effect. Individuals from two of the mutant lines clustered at either end of the distribution indicating the possibility of a dominant mutation being present which may be dictating the phenotype. These two lines are investigated further looking at the known functional mutations in the lines and identifying candidate genes, which may be causal factors in these observed nicotine-seeking phenotypes. This chapter also explores an initial molecular characterization of the QMhigh line to identify possible mechanisms accounting for the observed phenotype.

5.1 Introduction

In the previous chapter, a three-generation selection screen was performed in which zebrafish response to the rewarding effects of nicotine was shown to be heritable. ENU-mutagenised fish were used in the screen, allowing mutations linked to genes affecting the phenotype to be selected for over multiple generations and assimilated using an inbreeding strategy. This facilitates the possibility of using these genetic markers to SNP map regions of the chromosomes affecting the phenotype, using admixture-mapping strategies.

In bred lines with differential nicotine seeking behavior have been established but the SNP mapping is outside the scope of this thesis. However, due to the nature of the lines obtained from the Wellcome Sanger Trust, fish that occur at the extremes of the population distribution curve may be there because they house a dominant mutation of major effect, are homozygous for a recessive mutation of major effect or multiallelic for variants of more minor effect. The fish have thousands of mutations across the genome including 10-20 functional mutations in coding regions. In the first-generation, it was apparent the screen was able to identify mutations affecting nicotine seeking due to clustering of the *gababr2.2* mutant, which has previously shown altered nicotine sensitivity (Petzold et al., 2009). Since 4 individuals from each of the 30 F3 lines generated were included in the screen, any appearance of fish at the extremes suggests family contains a dominant mutation affecting nicotine seeking and warrant further investigation in the siblings of that line.

As previously discussed, the first generation screen gave a normal distribution of phenotypes with a mean change in preference of 0.14. The highest responders had

a 0.6 change in preference, with the lowest responders showing an aversion to nicotine of 0.4 (**Figure 5.1**). The population that was initially screened was made up of 30 families with distinct genetic backgrounds that were generated using the breeding scheme outlined in **Figure 5.1**. Each line contained a possible 10-15 gene breaking SNPs, as well as a large number in non-coding regions.

Of the 30 lines, 1 family clustered towards the right extreme (QMhigh) of the distribution with all 4 fish falling in the top 10 percentile, while another line (QMlow) clustered in the lowest 10 percentile (**Figure 5.1**). This indicates the screen has identified two lines containing a dominant mutation that affects nicotine reward. It can be assumed there is a dominant mutation involved since 4 F3 fish from each line were randomly selected from a pool theoretically containing 25% homozygous mutants, 50% heterozygous mutants and 25% wild type. The probability of each line clustering at either end of the distribution due to a recessive mutation would be low due to all 4 individuals having to be homozygous for that mutation. In the case of the high responders, the chance of pulling 4 homozygous individuals from the original tank of 15 would be just 0.4%. For the low responders the chance would be 0.4%.

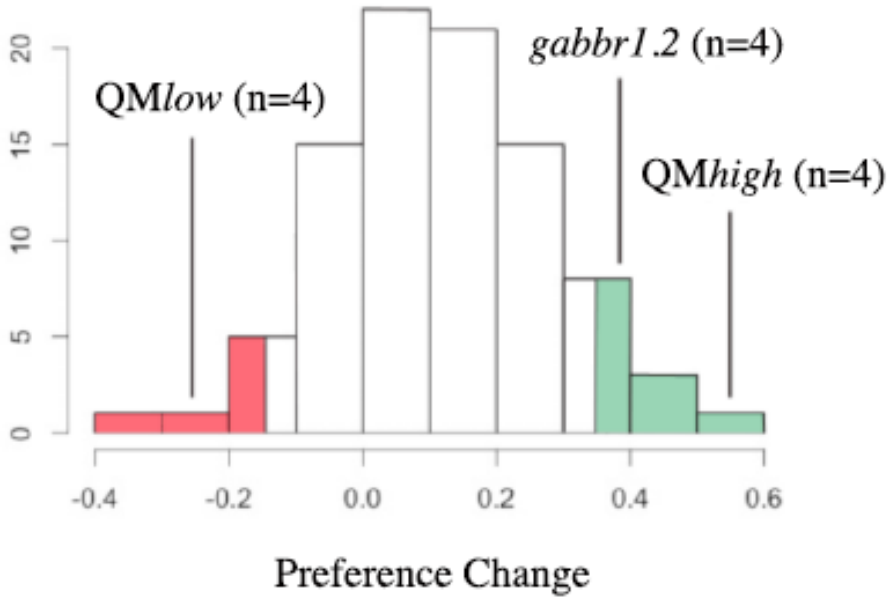


Figure 5.1: First generation outliers. The screen gave a normal distribution of phenotypes with a mean change in preference of 0.14 with the highest responders chowing a 0.6 preference change, with the lowest responders showing an aversion to nicotine of -0.4. The *gabbr1.2* knockouts clustered towards the right side of the distribution. Two mutant lines (named QMhigh & QMlow) clustered toward either end of the distribution in the top and bottom 10 percentiles.

By screening the remaining QMhigh (n=11) and QMlow (n=14) siblings for nicotine induced place preference, it is possible to reafirm the presence of a genetically induced alteration in nicotine seeking through a perseverance of the phenotype in the lines. Exome sequencing (performed at the Sanger) of the parental F1 fish identified a total of 14 possible nonsense mutations that may be contributing to the phenotype in the QMhigh line (**Table 5.1a**) and 12 possible mutations in the QMlow line (**Table 5.1b**). By genotyping the siblings at each of these loci, it may be possible to unravel the identity of specific mutations of causal effect by seeing how they segregate with nicotine seeking behaviour.

A

SNP Name	Description
cacna1ba (Cacna1b)	voltage-dependent N-type calcium channel subunit alpha-1B
vcana (VCAN)	novel protein vertebrate chondroitin sulfate proteoglycan 2
si:ch211-157f15.1 (EVPL)	envoplakin
mobkl2a (MOBKL2A)	mps one binder kinase activator-like 2A
ENSDARG00000068026 (PRKG1)	protein kinase, cGMP-dependent, type I
glis3 (GLIS3)	zinc finger protein GLIS3
si:dkey-220f10.4 (TULP2)	novel tub family member protein
slit3 (SLIT3)	slit homolog 3 protein
dchs1 (DCHS1)	dachsous 1
flad1 (FLAD1)	FAD synthaseMolybdenum cofactor biosynthesis protein-like
si:ch211-199m3.2 (AKD1)	adenylate kinase domain containing 1
si:dkey-4c23.3 (???)	novel protein similar to vitellogenin 1 (Vg1)
magi2 (MAGI2)	membrane associated guanylate kinase,
zgc:101050 (TRIMM55)	hypothetical protein (tripartite motif-containing 55)

B

SNP Name	Description
capn3 (CAPN3)	calpain-3
chrna9 (CHRNA9)	cholinergic receptor, nicotinic, alpha 9
snrnp70 (SNRNP70)	U1 small nuclear ribonucleoprotein 70 kDa
zgc:158677 (SV2B)	synaptic vesicle protein 2B homolog
wu:fa96e12 (AC103686.1)	DNA-dependent protein kinase catalytic subunit
kctd4 (KCTD4)	potassium channel tetramerisation domain containing 4
LOC557854 (SLC19A3)	solute carrier family 19, member 3
tspan3a (TSPAN3)	tetraspanin 3
rapsn (RAPSN)	43 kDa receptor-associated protein of the synapse
si:ch211-132b12.1 (SLC6A11)	hypothetical protein LOC100034467
pkhd1l1 (PKHD1L1)	polycystic kidney and hepatic disease like 1
klf11a (KLF-11)	kruppel-like factor 11a

Table 5.1: Summary of the known possible functional mutations in coding regions of outlier lines QMhigh and QMlow. A: QMhigh line has a possible 14 gene-breaking mutations in the exome. B: QMlow line has a possible 12 gene-breaking mutations in the exome. SNPs were identified at the Sanger Institute through sequencing of the F1 exome. Information regarding SNPs was obtained from Elisabeth Busch, Wellcome Trust Genome Campus, Hinxton, Cambridge.

5.2 Materials and methods

5.2.1 Site-specific polymerase chain reaction (SSPCR)

Four primer pairs were designed to carry out PCR genotyping as previously desribed (Hamajima et al., 2000). For each line, a primer was designed with 3' complementarity to the ENU-SNP with a second primer ~100bp downstream. The second pair had one primer with 3' complementarity to the wild-type base with a second primer ~200bp upstream (**figure 5.2**). The resulting PCR results in one 100bp fragment if homozygous for the mutation, two bands if heterozygous, and one 200bp fragment if homozygous wild-type. A third band is always generated regardless of genotype at around 300bp due to the opposing nature of each primer pair. The 4-primer groups were designed with Tm's as close as possible using the NCBI primer design tool and were ordered from Eurofins, MWG operon.

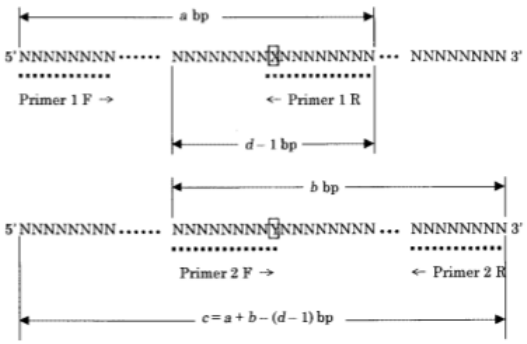


Figure 5.2: Site-specific polymerase chain reaction (SSPCR). Schematic of the logic of polymerase chain reaction with confronting two-pair primers. *a*, size for X allele; *b*, size for Y allele; *c*, size between primers 1 F and 2 R; *d*, sum of the sizes of primers 2 F and 1 R. In this case a fragment of ~200bp was used for wild-type allele and a ~100bp fragment for the mutant allele. This leaves an internal control fragment of ~300bp.

Primer Name	Sequence
cacna1baWT-F	GAG TTC TTA AAG CTG CGC A (19 bp)
cacna1baWT-R	AAA TAG AAG AGT GAC TGG ATT TTT (24 bp)
cacna1baSNP-R	CTT TCG ATC TGC TGT TGT CA (20 bp)
cacna1baSNP-F	TGT TCA TGG ATG TGT CTG C (19 bp)
vcana-WT-F	TTT CCA TCA ACA GAG TCA G (19 bp)
vcana-WT-R	TCT GCT TTG TGG TTC ACT (18 bp)
vcana-SNP-R	GTC CCA TGT TTT GAA CTT G (19 bp)
vcana-SNP-F	TCA GAG AAC ATA GTT GGC (18 bp)
evpl-WT-F	GGT ACT AAA CCA AAA ACA GG (20 bp)
evpl-WT-R	TGC ATA CAT TTT ATT AAC GAT TCA (24 bp)
evpl-SNP-R	TCA ATA TAT TGA ATG TGT ATT CAC AT (26 bp)
evpl-SNP-F	AGC TGT ATG AGC CAT TGA AC (20 bp)
mob3a-WT-F	GAA AAG GCG CTC GCT CGG (18 bp)
mob3a-WT-R	CGT CGT TTT TAA TGG CGT GAC CA (23 bp)
mob3a-SNP-R	GTG TAC AAT GAA GTT GAG GCA AAC TTA T (28 bp)
mob3a-SNP-F	CGA ACA AAA CTG AAA GCT GGG GA (23 bp)
prkg1-WT-F	GCA GTG CAG ATG GTA ATT TCG (21 bp)
prkg1-WT-R	ACA TCA ATG GTG CAG GCA GA (20 bp)
prkg1-SNP-R	TGC TTT TGT CAC TGT GGC CTA (21 bp)
prkg1-SNP-F	CCA GCT TCC TGT CAG AAA ATC (21 bp)
glis3-WT-F	CCC TCT AAC TGT CCC TTC ACA A (22 bp)
glis3-WT-R	GCA GGG TTT CAT GCC TTT TGT TG (23 bp)
glis3-SNP-R	CAA TTA TTC AGC AAA GCT CAT GGA TAT (29 bp)
glis3-SNP-F	CAC AAA GTC ACG CTC CGA TAC (21 bp)
tulp2-WT-F	CCG TCG CTC TGA GCA GAT AA (20 bp)
tulp2-WT-R	TTA AAG ACG CGC GTG GAG AAG (21 bp)
tulp2-SNP-F	CTT TTC ACA GTC ACT GCA ATC AAT AAA G (28 bp)
tulp2-SNP-R	AGG GGG AGG GAA AGA GTC TAA (21 bp)
slit3WT-F	ACG TGT TCT GTC TTC TTT TTT CTA (24 bp)
slit3WT-R	TAA GCC AAG CAA AAG AGC GA (20 bp)
slit3SNP-R	TTG TTG TTG TTG AGA GTG CC (20 bp)
slit3SNP-F	TCT GCT ACA GTG TGT TGT CT (20 bp)
DCHS1_WTF	CTA AAC CTG CTG TAG GGC (18 bp)

DCHS1_WTR	AAT CCT CAG TTC TGT CTC CT (20 bp)
DCHS1SNPR	GAT AAG TGG GGA AAT GTT CA (20 bp)
DCHS1SNPF	TCC CCT TAT TAC TTC TGT AG (20 bp)
FAD1_WTF	TGC TGC AGG CAT TGC CTC (18 bp)
FAD1_WTR	CCG GTG AGT GTA GTC AAA AGC (21 bp)
FAD1_SNPR	TCT TGA CCG CTC TCC TAA TCT A (22 bp)
FAD1_SNPF	GTT CCC AAC AGA CCT TCT CC (20 bp)
si:ch211-WT-F130	TGG ATG GGC ACT GGG CC (17 bp)
si:ch211-WT-R130	GGG CCG CAA GAA CTA GGA TT (20 bp)
si:ch21-SNP-R164	TCC CCT TTT CAG AGC ACA GTC (21 bp)
si:ch21-SNP-F164	TCA GGT GTG TTT TGT CTG CTC T (22 bp)
magi2-WT-F	CTC AGG ACC CCC AGA CAC T (19 bp)
magi2-WT-R	CAT CAC CTT GGC AAC CTT CTT C (22 bp)
magi2-SNP-F	TGG TTC TAG GGT CTC AGC TTG (21 bp)
magi2-SNP-R	CCA TCC TCG CAT GAT TAC ATA CC (23 bp)
trimm55-WT-F	TTG TTT GTT GCA TTG ACT GAC AGT GA (26 bp)
trimm55-WT-R	TCT AGC AAC CCC GAA ATC TGG A (22 bp)
trimm55-SNP-F	GGG TGG TCA GAT ACA CCG TTT (21 bp)
trimm55-SNP-R	GCC TAT CAG GAT TGC AGC AGT (21 bp)
capn3SNP-R	AAG AGA TTC GCA CCC AAC TA (20 bp)
capn3WT-F	AAA ACA GAC GTA AAG AAC GCA (21 bp)
capn3SWT-R	ACG GCG AAA CGT GAA ACA A (19 bp)
capn3SNP-F	GGA GGA TGA TGA AGT GGC (18 bp)
a9WT-F	GTT TTG TGT TTC TTA CAG GAA AAT AT (26 bp)
a9WT-R	TCA GGT GTT GTG CAG TTC TC (20 bp)
a9SNP-R	ATG GTC ATT GTT GCA ATG TAC (21 bp)
a9SNP-F	CCC AGC AAC TGA TTG TGT TA (20 bp)
snrnp70-WT-F	GGG GGT CTT GGT GGC AC (17 bp)
snrnp70-WT-R	CGC GAT CAC TGC AAA ATA AGA CA (23 bp)
snrnp70-SNP-R	TCT GCG CCT CCT CGC CTT A (19 bp)
snrnp70-SNP-F	CGA GGA CCT TGC CTA TCC C (19 bp)
CABZ-SNP-F	GCC TAT CCC ACC ATG CTC AAA T (22 bp)
CABZ-SNP-R	TCA CAC CCT CAC CTT CGA CT (20 bp)

CABZ-WT-R	TGG AAG AGA CAC ACA CCC TG (20 bp)
CABZ-WT-F	CAC CTG CTG CTG CAT TTC CC (20 bp)
wu:fa96e12-WT-F	CTG CTT TAG ATC TTC TAA TCA AAG (24 bp)
wu:fa96e12-WT-R	CCA TAT TTC ACA GCC CTA ACT (21 bp)
wu:fa96e12-SNP-R	TCA TAC AAT ATA TAA ACA AAA GAA CTC AA (29 bp)
wu:fa96e12-SNP-F	GAC ACG TGC ATT GTC GTT TA (20 bp)
kctd4-WT-F	CAG CGA CAA CAC CTT CAT CG (20 bp)
kctd4-WT-R	CAT CAC CGT CTC CAG CTT CA (20 bp)
kctd4-SNP-R	CAG CCG GTT CTT GAC CTT CTA (21 bp)
kctd4-SNP-F	CGA ACC TGC CTT CCT GGA G (19 bp)
SLC19A3-WT-F	CCT TCT AGT AAC TTC ACA GTC TAC (24 bp)
SLC19A3-WT-R	TTA TTT CTT GGC CTG ACT GTT AGC (24 bp)
SLC19A3-SNP-R	GTG CCT CCG CTC CTC CAT T (19 bp)
SLC19A3-SNP-F	GGC TAC AAC CAG ACG GTC AA (20 bp)
tspan3aWT-F	GAG ACC TTT ATC CTG AGG T (19 bp)
tspan3aWT-R	TTA GCC TCC CGT GTT TAT GT (20 bp)
tspan3aSNP-R	TGA CAA AGC AGC TGT TAT TTC TTT (24 bp)
tspan3aSNP-F	CTG GTT TAA GGA GTC CAA G (19 bp)
raspnWT-F	CTG CAG AAG GAA TAT GAT AAG GT (23 bp)
raspnWT-R	TGG GGG CTA ATT TGC AAT AAC T (22 bp)
raspnSNP-R	TTA TCT AAA AGA CAC AAT TAA ATC TTG C (28 bp)
raspnSNP-F	GTC CTC CAT GTG CAT AAT GTC (21 bp)
slc6a11-WT-F	CCA GTT CGA GGT CTC CAT GT (20 bp)
slc6a11-WT-R	GTG GTG CTC TCG ACA CAG AA (20 bp)
slc6a11-SNP-R	CCA ATG TGT GGA TGA AGC AGG (21 bp)
slc6a11-SNP-F	AGC AGA GCA GAG CCG AGT AG (20 bp)
PKH-WT-F	AGG GGG AGG GAA AGA GTC TAA (21 bp)
PKH-WT-R	AGG AAA CTG GCC ATT GTG TAA CTA (24 bp)
PKH-SNP-R	ATG TGA ATG AAT GGC GGT GTG TG (23 bp)
PKH-SNP-F	ATG CGC GTG TCA GAT TTA CCC (21 bp)
???_WTF	ATT AGA CAA CTG TTT CAA GCT G (22 bp)
???_WTR	ACC TGT ATT GCC TGT TCA AGA (21 bp)
???_SNPR	AGT GCA TTT ATA AAA TGT TTC ACC TAT (27 bp)
???-SNP-f	TTT TGA GGT GAG TAG CAG TG (20 bp)

klf11a-WT-F	TCT AAA ACC ACA TCC CAG ACT ATG T (25 bp)
klf11a-WT-R	GCC CTG TGG TTG TTG ATG GA (20 bp)
klf11a-SNP-R	GCT CTG TTG GTC AAG GCT GAT (21 bp)
klf11a-SNP-F	AAA CTT CAG GCA CAG CGG TT (20 bp)

Table 5.2: Primer sequences used for SSPCR. Primers used in the genotyping of the QMhigh and QMlow lines. ??? denotes a gene breaking mutation in a locus with no known human homologue.

5.2.3 Genomic DNA extraction

Zebrafish were anaesthetised using MS-222 in a 1-litre tank of aquarium water. They were then removed from the tank and 4mm^2 of the tail fin removed using a scalpel. The fin-clips were then placed in an eppendorf containing 1ml of 100% ethanol and stored at -20°C until ready to be processed further.

Genomic DNA was extracted from fin-clips by first digesting with proteinase-K for 1 hour at 56°C before extraction using QIAGEN DNeasy® Blood and Tissue Kit. Samples were eluted into distilled water and stored at -20°C until later use.

5.2.4 CPP phenotypic analysis of outlier siblings and sa202

The remaining fish from the QMhigh (n=11) and QMlow (n=14) were screened in the CPP paradigm along with control fish (with and without 5uM nicotine) as described in chapter 3. Fish from the sa202 (n=20) line were obtained from the Sanger and assayed for CPP to nicotine along with controls (with (n=10) and without (n=10) 5uM

nicotine). All lines were fin-clipped and genotyped using SSPCR after CPP. The CPP procedure was carried out as described in chapter 3.

5.2.5 Quantitative PCR of QMhigh embryos

The cDNA from zebrafish embryos was generated as described in chapter 2. All qPCR reactions were carried out in triplicate. Reference genes used were β-actin, ef1α and rpl13α based on previous findings (Tang, Dodd, Lai, McNabb, & Love, 2007). Target genes used were slit3, drd1b, drd2a, drd3, dbh, dat, chrnb2b, chrnb3, chrna2, chrna3, chrna4, chrna5, chrna6 and chrna7 (for primers see **table 5.3**). Absolute quantification was calculated by generating standards for each gene, prepared using the relevant primers to amplify fragments from cDNA. Samples were then PCR purified and diluted to 10^{11} fragments per μl using the Avogadro constant (Gemenetzidis et al., 2010; Teh, Gemenetzidis, Chaplin, Young, & Philpott, 2010). Relative mRNA expression ratios in the qPCR were calculated with respect to reference gene cycle-threshold (Ct) values, and then subjected to a two-way factorial (between-subjects) analysis of variance (ANOVA).

Gene	Primer
β-actin-F	CGA GCT GTC TTC CCA TCC A (19 bp)
β-actin-R	TCA CCA ACG TAG CTG TCT TTC TG (23 bp)
eF1α-F	CTG GAG GCC AGC TCA AAC AT (20 bp)
eF1α-R	ATC AAG AAG AGT AGT ACC GCT AGC ATT A (29 bp)
rpl13α-F	TCT GGA GGA CTG TAA GAG GTA TGC (24 bp)
rpl13α-R	AGA CGC ACA ATC TTG AGA GCA G (22 bp)
chrna2-F	GCG GAA AAC CGG ATA AAA ACA CTC (24 bp)
chrna2-R	AGT TTG TCC TCT GCG TGT GCA T (22 bp)

chrna3-F	TGT ACA TCC GCC GAT TAC CGC T (22 bp)
chrna3-R	TCC GCA GTC GGA GGG CAG TA (20 bp)
chrna4-F	TTA CAA GAG GTT TGG GCG CT (20 bp)
chrna4-R	ACA GAC CAG TAG ATC ATC ACT CC (23 bp)
chrna5-F	GGC TCC CAG GTC GAC ATT (18 bp)
chrna5-R	AAC CCC GGT TAC CAG TGG CCT (21 bp)
chrna6-F	CTT TGG GCC TCT TCC TGC AA (20 bp)
chrna6-R	TCA GAG TCT TGA TGT AGT GAC GG (23 bp)
chrna7-F	ACC GTG TCA CAT TGT TCA TTC TC (23 bp)
chrna7-R	ACA GGT CTC TCC AGT GGG TTA (21 bp)
chrnb2-F	GGC TGC CTG ATG TTG TTC TT (20 bp)
chrnb2-R	TGG TGG CAA CCA GAA GAC ACT T (22 bp)
chrnb3-F	CAG GAG TCA ACC TCC GCT TT (20 bp)
chrnb3-R	TGA ATC TGA ACG CAC TGG CT (20 bp)
chrnb4-F	TGA TCA CAT GAT GGG GAA TGA CG (23 bp)
chrnb4-R	CAC CAC ACA CAC GAT CAC AAA G (22 bp)
drd1-F	TGG TTC CTT TCT GCA ACC CA (20 bp)
drd1-R	AGT GAT GAG TTC GCC CAA CC (20 bp)
drd2-F	TCC ACA AAA TCA GGA AAA GCG T (22 bp)
drd2-R	CAG CCA ATG TAA ACC GGC AA (20 bp)
drd3-F	ATC GAG TTT CGC AGA GCC TT (20 bp)
drd3-R	TCC ACA GTG TCT GAA AGC CG (20 bp)
dat-F	TAT GTG GTC CTG ACC GTG CT (20 bp)
dat-R	CAC ATG TGT AGG CGC AGG AA (20 bp)
nat-F	AGG TGA CAT TGT TTG AGA TGT CTT (24 bp)
nat-R	TGT CTT GGT AGT GTC AAG TTG T (22 bp)
oprm1-F	CCG TAT GTG ACA GGA CGC CA (20 bp)

Table 5.3: Quantitative real-time PCR primer sequences. Reference genes used were β-actin, ef1α and rpl13α.

5.2.6 Statistical analysis

CPP change scores were calculated as previously described. For the rescreen of outlier siblings, lines were compared using an independent two-sample t-test. To determine association between SNPs and phenotype, for each locus, CPP change scores were grouped based on genotype (wildtype vs. +/- and -/-) before comparing means using a t-test. The independent sa202 line, were compared by independent two-sample t-test after genotyping.

5.3 Results

5.3.1 Rescreen of QMhigh and QMlow siblings

The fish used from each family were selected at random from a tank of at least 20 siblings, and as a result of the breeding strategy, only 25% of the family would be expected to be homozygous at any single locus. Therefore clustering of a particular family in the distribution suggests that there is a dominant mutation within the line that influences response to nicotine. To test this hypothesis we screened siblings from the QMhigh and QMlow families for nicotine-induced place preference. QMlow showed a significant reduction (Student t-test, p=0.04) and QMhigh a significant increase (Student t-test, p = 0.01) in nicotine-induced place preference compared to wild type controls consistent with the existence of dominant mutations affecting nicotine preference within the lines (**figure 5.3**).

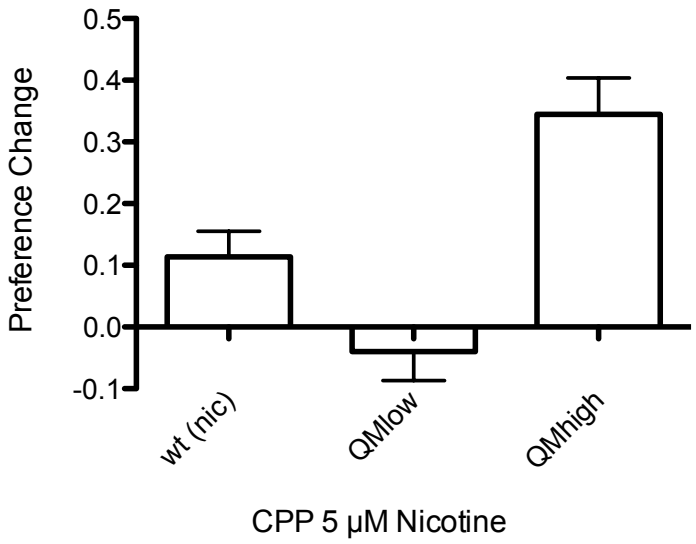


Figure 5.3: Rescreen of siblings. Siblings of QMhigh and QMlow lines were rescreened to verify the genetic component of the phenotype. QMlow (n=14), QMhigh (n=10) and control (n=10) fish were tested for nicotine induced CPP using 5uM nicotine hemisulphate. Phenotypes were similar to those seen from outliers in the population screen; QMlow showed a significant reduction (p=0.04), while QMhigh siblings showed an increase in preference change compared with controls (p = 0.1). There was a significant difference between the QMhigh and QMlow lines (p<0.001).

5.3.2 SSPCR of QMhigh and QMlow line

Exome sequencing of the parental F1 fish used to generate the QMhigh and QMlow families identified 14 gene-breaking splice mutations in the AJBQM1 line and 12 in the QMlow line (**Table 5.1**), plus up to 1000 additional missense mutations. The screen fish and siblings from the QMhigh and QMlow lines were genotyped at each of the known gene-breaking loci and results compared with each individual's place preference change scores (**Table 5.4 and 5.5**). None of the known candidate gene-breaking mutations present in the QMlow line segregated with the behavior

suggesting that the causal mutation may in fact be a missense mutation with linkage to the low responder phenotype. Of the 14 candidate mutations present in the QMhigh line only 1 (**figure 5.4b**) segregated with the 'high responder' phenotype (Students T-Test, $p < 0.001$). This mutation introduces a stop codon at position 176 in the *slit3* coding domain.

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)

GENE	CPP Change Score										p-value
	0.32	0.15	0.07	0	0.01	0.44	0.51	0.6	0.47	0.43	
SLIT3	HET	WT	WT	WT	WT	HET	HET	HET	HET	HET	**7.6592E-05**
CACNE	MUT	WT	WT	WT	HET	WT	WT	WT	WT	HET	0.691
VCAN	MUT	HET	WT	HET	HET	HET	WT	WT	HET	WT	0.259
EVPL	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	-
MOB3a	MUT	MUT	HET	HET	HET	WT	WT	HET	HET	HET	0.236
PRKG1	HET	HET	HET	HET	HET	HET	MUT	MUT	HET	HET	-
GLIS3	WT	HET	HET	WT	HET	WT	WT	HET	HET	WT	0.602
TULP2	WT	HET	WT	MUT	MUT	HET	WT	WT	HET	HET	0.481
DCHS1	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	-
FLAD1	WT	HET	WT	MUT	MUT	WT	HET	HET	HET	WT	0.981
AKD1	WT	HET	WT	MUT	MUT	HET	WT	WT	HET	HET	0.418
MAGI2	HET	WT	HET	HET	WT	WT	MUT	HET	WT	MUT	0.73
TRIMM55	WT	HET	WT	WT	HET	WT	HET	WT	WT	WT	0.51

Table 5.4 PCR genotyping of QMhigh outlier siblings. The siblings were genotyped at each of the 14 candidate loci using SSPCR and results compared with each individuals place preference change scores. *Slit3* (top row) was shown to segregate with high responder phenotype (see figure 5.6).

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

GENE	CPP Change Score													p-vlaue
	0.04	-0.17	-0.17	-0.09	-0.23	-0.21	-0.12	-0.27	-0.28	0.07	-0.07	-0.09	-0.38	-0.18	
tspan3a	HET	HET	HET	WT	WT	WT	WT	HET	HET	HET	HET	WT	HET	WT	0.583
raspn	WT	MUT	MUT	WT	WT	WT	WT	WT	MUT	MUT	MUT	WT	WT	MUT	0.792
a9	HET	HET	WT	HET	WT	WT	HET	HET	WT	HET	HET	HET	WT	WT	0.339
capn3	WT	WT	HET	HET	WT	HET	WT	HET	HET	WT	WT	WT	HET	WT	0.911
klf11a	HET	WT	HET	WT	HET	WT	WT	WT	WT	WT	WT	HET	WT	WT	0.318
kctd4	WT	HET	WT	HET	HET	WT	HET	WT	WT	WT	HET	HET	HET	WT	0.252
slc6a11	HET	HET	HET	WT	WT	HET	WT	HET	HET	WT	WT	WT	HET	WT	0.697
pkhd1l1	WT	HET	WT	WT	HET	MUT	WT	WT	WT	MUT	WT	WT	WT	MUT	0.499
slc19a3	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	-
sv2b	HET	WT	HET	HET	WT	WT	MUT	HET	WT	MUT	MUT	MUT	WT	WT	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	-

Table 5.5: PCR genotyping of QMlow outlier siblings. The siblings were genotyped at each of the 12 candidate loci using SSPCR and results compared with each individuals place preference change scores. No SNP was found to significantly segregate with behavior.

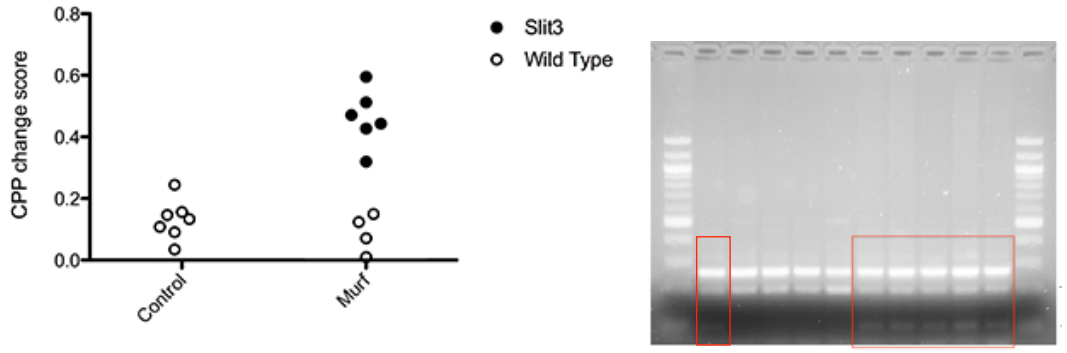


Figure 5.6: Co-segregation of slit3 genotype and CPP phenotype. a: Graph comparing QMhigh with controls. Data points shaded in black were positively genotyped for the *slit3* mutation indicating a clear segregation of phenotype with genotype. b: Site-specific PCR electrophoresis gel. ~100bp band indicates presence of mutant allele.

5.3.3 Phenotype conserved in independent slit3 allele

SSPCR suggests that a mutation in the slit3 gene underlies the high nicotine preference. To confirm this possibility a second independent allele was obtained from the Sanger. The independent slit3 allele sa202 with a nonsense mutation that translates to a protein truncated at amino acid 163 showed a similar high responder phenotype to the QMhigh line when compared with controls. The control fish conditioned to saline showed a preference change of -0.03, with those conditioned to nicotine showing a preference of 0.1 to the drug paired side. The sa202 fish wildtype at the slit3 locus showed a place-preference (0.13) similar to the wildtype fish when conditioned to nicotine. The sa202 fish heterozygous for the slit3 mutation showed a preference change of 0.26, nearly double that of those without a mutation (**Figure 5.5**).

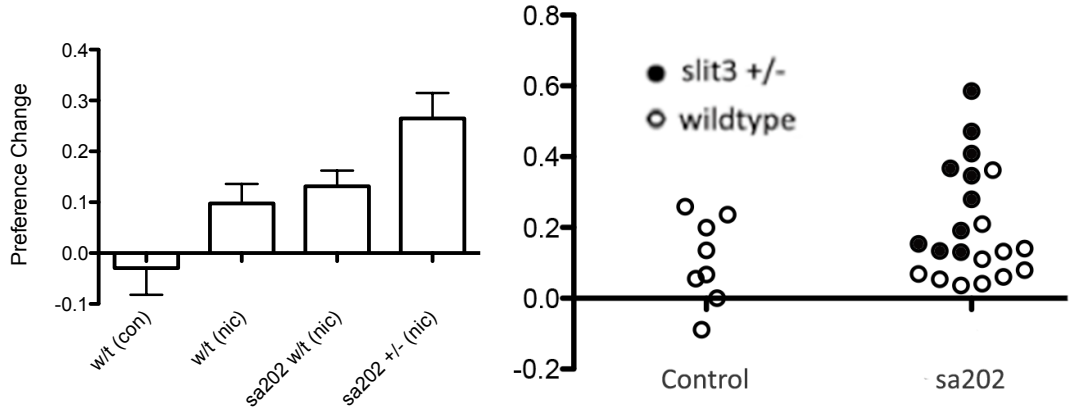


Figure 5.5: CPP of independent slit3 (sa202) line. The high CPP response was conserved in this new mutant when compared with wild-type fish ($p<0.001$). Additionally, after screening, the mutant line was genotyped at the *slit3* locus and those individuals heterozygous (+/-) for the SNP showed significantly higher CPP ($p<0.05$) to nicotine than those homozygous (+/+) wild-type.

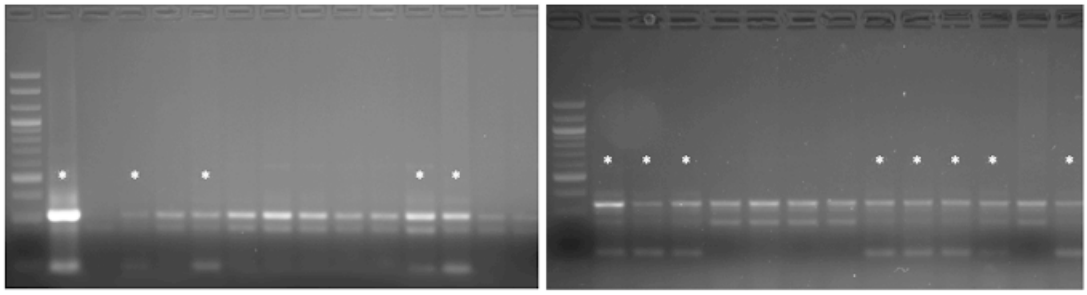


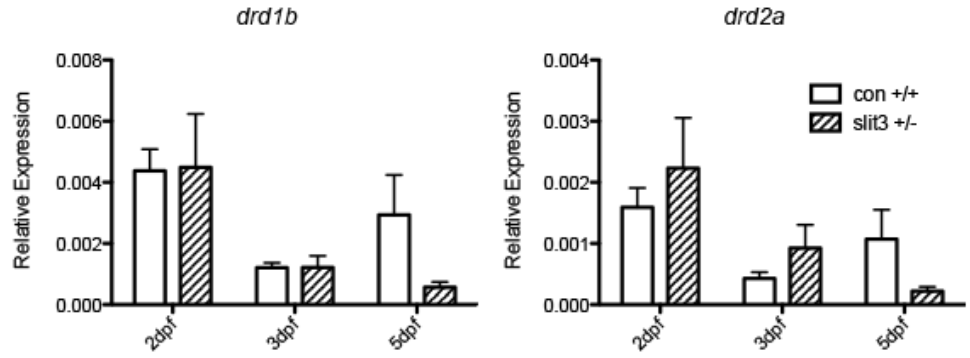
Figure 5.6: sa202 genotyping. Site-specific PCR electrophoresis gel. ~100bp band indicates presence of mutant allele, n=27. 13 subjects were found to be carrying a mutant allele.

5.3.5 qPCR analysis of SLIT3 embryos

Quantitative PCR was carried out on 2, 3 and 5 d.p.f embryos to assay expression levels for cholinergic and dopaminergic related gene expression during development in slit heterozygous incross progeny and in wildtype. No difference was seen when gene expression in progeny from a slit+/+ cross compared to progeny from a *slit3* +/- cross at 2 and 3 dpf. At 5 days differences between the gene expression in wildtype and mutant begin to emerge. There is a down regulation of *drd1b* at day 5 with relative expression levels in the slit3 embryos being around a quarter that seen in the wildtype (**Figure 5.7a**). *Drd2a* also showed a down regulation at day 5 with a slight up-regulation at 3dpf (**Figure 5.7a**). There was also a down regulation of the other dopaminergic genes *drd3* and *slc6a3* at 5dpf (**Figure 5.7b**). Both *chrnb2b* and *chrnb3* (**Figure 5.7d**) showed a noticeably large down regulation in the slit3 mutants, with there being very little expression in both genes when compared with the controls (**Figure 5.7c**). There were no noticeable expression differences between the two groups for *chrna2* and *chrna3* (**Figure 5.7e**) for all three of the time points. There was a down regulation for *chrna4* and *chrna6* (**Figure 5.7f & g**) at 5dpf while there was

no difference in expression levels between the two groups for *chrna7* (**Figure 5.7g**). There was a slight up-regulation in chrna5 in slit +/- in-cross fish (**Figure 5.7f**), which was significant when subjected to two-way factorial (between-subjects) analysis of variance ($p<0.05$). There is no change in slit3 expression across all 3 time points.

A



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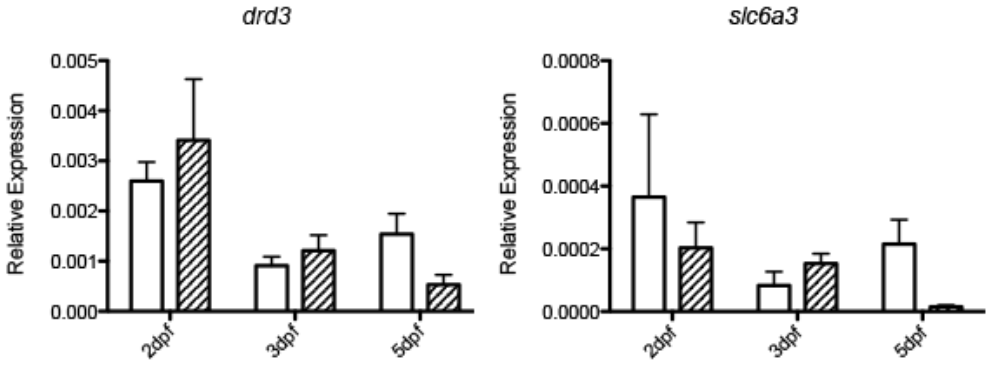

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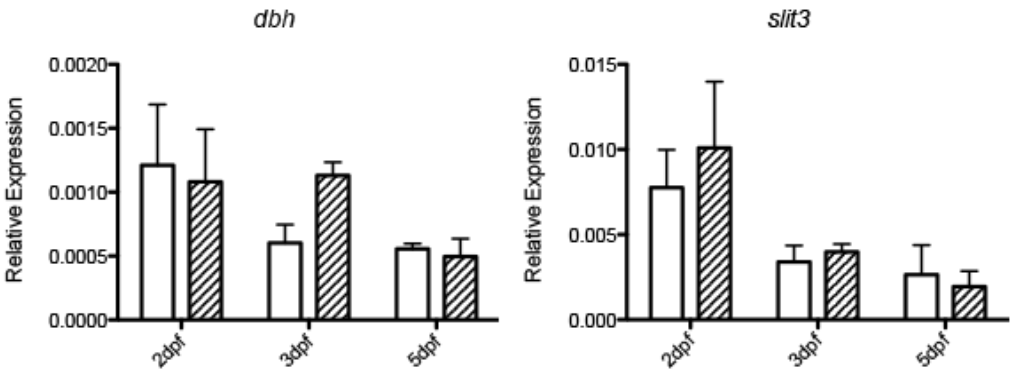
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slit3
Relative Expression
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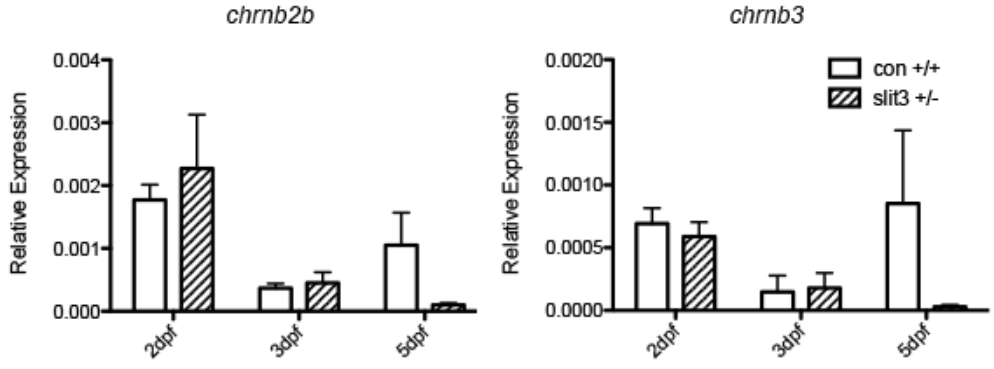
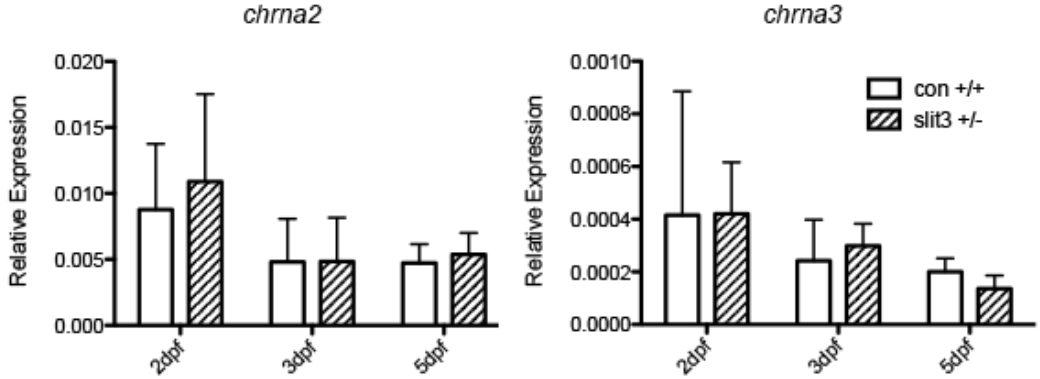
0.0015
0.0010
0.0005
0.0000
0.015
0.010
0.005
0.000
2dpf
3dpf
5dpf

D


chrnb2b
chrnb3
Relative Expression
0.004
0.003
0.002
0.001
0.000
0.0020
0.0015
0.0010
0.0005
0.0000
con +/+
slit3 +/-
2dpf
3dpf
5dpf

E


chrna2
chrna3
Relative Expression
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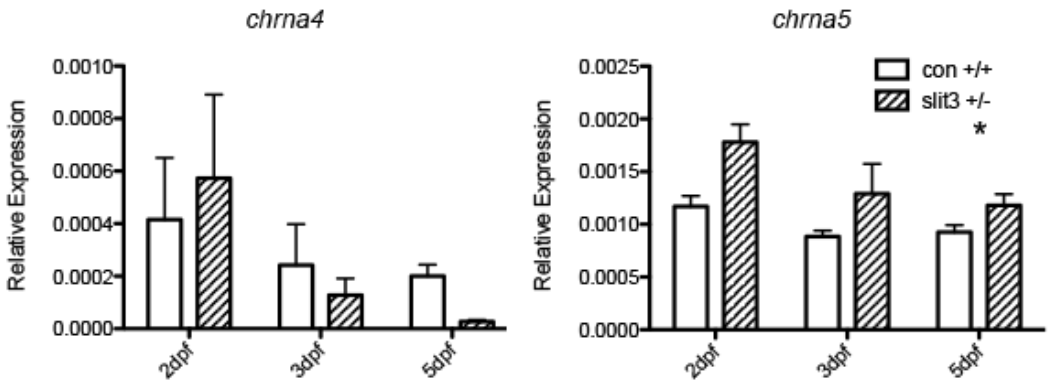
0.015
0.010
0.005
0.000
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
con +/+
slit3 +/-
2dpf
3dpf
5dpf

F



G

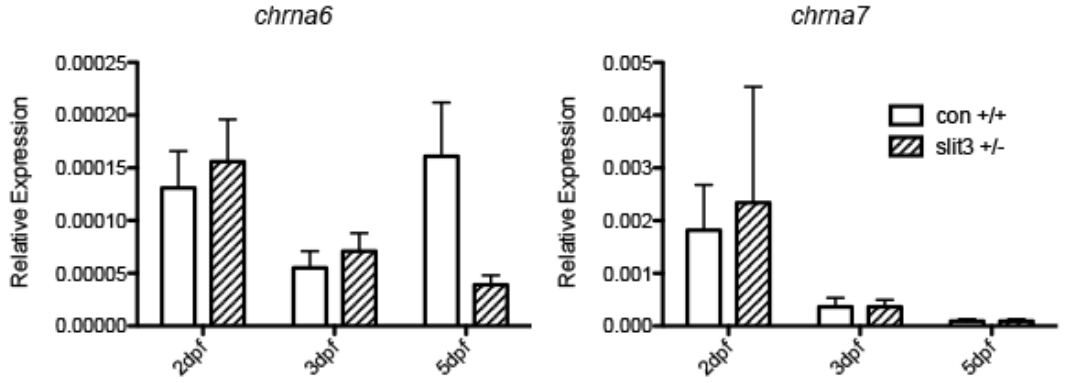


Figure 5.7: Quantitative PCR results. Mean (± SE) mRNA expression ratios of chrnb2b, chrnb3, chrna2, chrna3, chrna4, chrna5, chrna6 and chrna7 genes to β-actin, ef1α and rpl13α of slit3 +/- incross progeny and control (+/+) zebrafish at different periods post-fertilization. Only chrna5 showed significant change on expression levels. Note: * $p < 0.05$.

5.4 Discussion

Of the thirty families included in the screen, one clustered at the right extreme (QMhigh) of the distribution, while another (QMlow) clustered to the left. This clustering on either arm of the distribution curve is a strong indicator of the presence of a genetic component influencing behaviour, in this case to nicotine reinforcement. Due to the lines being obtained from the Sanger, they were exome sequenced at the F1 stage before outcrossing, meaning it was possible to obtain a list of potential mutations in the F3 fish. This led to the possibility of identify specific mutations contributing to the observed phenotype by rescreening the siblings and genotyping at each locus. Exome sequencing of parental fish identified the presence of a 12 possible gene-breaking mutations within the QMhigh line.

The QMlow line was genotyped at all the candidate loci and none of the SNPs were shown to significantly associate with behavior. The SNP showing the closest association with behavior was chrna9 with a p-value of 0.29. When the QMhigh siblings were genotyped for candidate mutations, 1 SNP segregated with the increased nicotine seeking phenotype, suggesting causality. This interaction of the slit3 knock-out with increased CPP phenotype was confirmed in an independent allele

Slit ligands together with Robo receptors form one of the most important ligand-receptor couples among the axonal guidance molecules. Robos were first identified in Drosophila in a screen for genes that regulate the midline crossing of transverse axons connecting the two hemispheres of the brain (Seeger, Tear, Ferres-Marco, & Goodman, 1993). Similarly, Slit was discovered in *Drosophila* as a protein secreted by midline glial cells (Rothberg, Jacobs, Goodman, & Artavanis-Tsakonas,

1990). The Slit/Robo pair not only functions in axon guidance but also in diverse developmental processes including cell migration, axonal branching, axonal targeting or cell differentiation (Chedotal, 2007). There have been studies investigating the role of slit 1, 2 and 3 in the growth and guidance of longitudinal dopaminergic projections in both mice (Bagri et al., 2002; Dugan, Stratton, Riley, Farmer, & Mastick, 2011) and zebrafish (Kastenhuber et al., 2009) in vivo studies.

It has been suggested that during development the combined action of Slit:Robo interactions may initiate a strong repellent signal for midbrain dopaminergic axons with Slit3 expressed in the caudal midbrain being responsible for the repulsive action (Smidt & Burbach, 2007). It is thought that these Slit:Robo repulsive cues in combination with attractive signals from the forebrain, provide important signals that guide the axons to the ventral forebrain. In addition to these effects on dopaminergic axon guidance, Slit proteins have been implicated in 5-HT guidance. In slit1 and slit2 knock-out mice, 5-HT fibers enter the telencephalon normally, but a significant percentage abnormally crossed the midline in the basal telencephalon (Bagri et al., 2002). Although the majority of neural studies to date have focused on slit1 and slit2, since dopaminergic and serotonergic neural systems are intricately linked to reward and addiction and slit3 is expressed in the developing central nervous system, these findings suggest that variants at the slit3 locus may lead to increased drug seeking behavior through a neurodevelopmental role affecting pathfinding and circuit formation.

As well as this axon guidance role, Slit:Robo interactions regulate neurogenesis. In Drosophila, Slit has been shown to modulate neurogenesis by promoting asymmetric terminal division in particular neural lineages (Mehta & Bhat,

2001). In mammals, Slit:Robo signaling modulates the proliferation of central nervous system progenitors (Borrell et al., 2012). As deficits in adult hippocampal neurogenesis are linked to drug seeking behavior (Noonan, Bulin, Fuller, & Eisch, 2010) and slit3 is highly expressed in the adult hippocampus (Marillat et al., 2002), these finding raise the possibility of the slit3 mutations leading to a reduction in hippocampal neurogenesis and increased drug seeking behavior.

This presents two mechanisms by which slit3 variants could affect drug seeking behavior. Molecularly, Slits bind to the Robo Ig1 domains through the concave face of the leucine-rich repeat (LRR) 2 domain of Slit, a domain that is highly conserved among all Slits across species. In the outlier line the gene breaking mutation causes a truncation at amino acid 276 just before the LRR 2 domain, essentially removing the domain responsible for slit3's functional interaction with Robo proteins. The independent allele sa202 has a truncation at amino acid 163, resulting in a similarly truncated protein. This loss of function would in all likelihood impact dopaminergic axon guidance in development or neurogenesis and in doing so, affect the response of adult zebrafish to nicotine reward in the CPP paradigm.

There have been studies looking at slit3 knockout mice and the effect on heart, diaphragm and kidney development (J. Liu et al., 2003; Zhang et al., 2009) but no other phenotypes have been observed in a mouse model. *SLIT3* was previously assumed dispensable for neural development but required for non-neuron–related developmental processes, such as the genesis of the diaphragm and kidney (Zhang et al., 2009). These experiments suggest that there may be more subtle behavioral phenotypes occurring in a *SLIT3* knockout mouse worth investigating and the gene may not be as dispensible for brain development as once thought,

As way of further investigation, quantitative PCR was performed on 2, 3 and 5-d.p.f slit3 (sa202) +/- incross and control embryos comprising of a +/+ wild type in-cross. Quantitative PCR was carried out on dopaminergic and cholinergic genes to test the hypothesis that the slit3 mutation affects development of the reward circuitry. As such, dopaminergic genes *drd1b, drd2a, drd3, dbh* and *dat*; cholinergic genes *chrnb2b, chrnb3, chrna2, chrna3, chrna4, chrna5, chrna6* and *chrna7*; and *slit3* were analysed. There was little difference in expression levels for any genes on 2 and 3 d.p.f, though differences begin to emerge between slit3 +/- and control 5-day old embryos. There was a general trend of down regulation of dopaminergic gene expression seen in *drd1b, drd2a, slc6a6 and drd3*, though these did not reach significance. For the cholinergic genes there was a noticeable relative down regulation of *chrnb2b, chrnb3, chrna4* and *chrna6* when compared with controls embryo, though these also failed to reach significance. There was however a relatively small but significant ($p=0.001$) increase in relative *chrna5* expression in slit3 5-d.p.f embryos, presenting another possible mechanism by which a slit3 knockout zebrafish may show altered nicotine induced CPP.

Variations in α5/α3/β4 nicotinic receptors have previously been shown to associate with vulnerability to nicotine addiction in genome-wide association studies, with the α5 subunits showing particularly strong association in independent studies (Bierut, 2010; Exley, McIntosh, Marks, Maskos, & Cragg, 2012; Saccone, Wang, et al., 2009; Thorgeirsson & Stefansson, 2008). Previous studies in rodents have shown CHRNA5 gene deletion to result in decreases in acetylcholine mediated dopamine release (Grady, Salminen, McIntosh, Marks, & Collins, 2010; Salminen et al., 2004). α5 (along with β3) act only as accessory subunits in heteromeric nicotinic receptors forming nAChRs with the stoichiometry $(\alpha4\beta2)_2\alpha5$ and therefore do not take part in

forming acetylcholine-binding sites (Kuryatov, Onksen, & Lindstrom, 2008). α4 and β2 on the other hand, can form binding sites as well as assembling in the accessory position to form nicotinic AchRs with stoichiometries like $(\alpha4\beta2)_2\alpha4$ (Kuryatov, Luo, Cooper, & Lindstrom, 2005; Nelson, Kuryatov, Choi, Zhou, & Lindstrom, 2003). The α4β2 AchRs are the primary subtypes which show a high affinity to nicotine with $(\alpha4\beta2)_2\alpha4$ being the most prevalent in humans (Nelson et al., 2003). Human $(\alpha4\beta2)_2\alpha5$ subtypes have a higher permeability to Ca^{2+} and lower desensitization rates than $(\alpha4\beta2)_2\alpha4$ when expressed in *Xenopus laevis* oocytes using a higher number of free α5 subunits to induce greater formation of this stoichiometry (Tapia, Kuryatov, & Lindstrom, 2007). The up-regulation of *chrna5* in the *slit3* knockout zebrafish may induce greater formation of the $(\alpha4\beta2)_2\alpha5$ nAChR assembly with greater Ca^{2+} permeability and a subsequent increase in the regulation of dopamine transmission. This increased dopamine transmission when nAChRs are activated by nicotine during the conditioning stage of the CPP paradigm, may contribute to the slit3 knockout fish finding nicotine significantly more reinforcing.

There were flaws with these qPCR experiments however. The main problem was that no homozygous mutant was identified. Numerous individuals were genotyped yet no homozygous mutant were ever identified. Slit3 homozygous knockout mice suffered from diaphragmatic hernia. This was caused by a central tendon that remained fused to the liver. In the defective tendon, the collagen fibers did not form tight bundles. Due to the herniation, the orientation of the heart was twisted (J. Liu et al., 2003). Such serious defects would decrease the survivability of homozygous mutants, certainly into adulthood (6-month to 1-year) when the fish were being used for breeding. As such, future experiments will look into genotyping

offspring at various developmental time-points to see if fish are viable up to a certain age. It would also be possible to pool mutants at young age and monitor survival.

The progeny analyzed for qPCR were the result of a heterozygous cross. As such it was decided to pool 5 progeny of a heterozygous in-cross to make sure the target mutation was most likely represented in the sample that was analysed. This is not ideal as the resulting samples analysed will have an undetermined assortment of mutants and wild-type which may have muddied the results. This may explain the lack of significant results despite seeing noticeable trends in down regulation in the dopaminergic genes. The plan to continue these experiments will be to genotype individual empryo's by sequencing genomic DNA from the tail of a heterozygous incross before pooling based on genotype. This would result in decreased variance and help to tease out subtle differences in gene expression.

This series of experiments identified a dominant mutation in one of the outlier lines in the first generation screen. This was shown to replicate in an independent line with a mutation in the same gene. Identification of the slit3 mutation as well as changes in α5 expression in 5-day embryos presents possible mechanisms underlying the altered nicotine seeking phenotype displayed in the QMhigh line. Now a gene has been established in the zebrafish model, the next objective is to address the second goal of this thesis, the translational aspect in which data from these studies is used to inform association studies in humans. This will be explored in the next chapter.

Chapter 6

Analysis of *slit3* SNP panel in human cohort for association with smoking behaviors

With a causal mutation affecting nicotine seeking identified in the zebrafish model, this chapter investigates the human homologue for associations with smoking behaviors in human cohorts with varying smoking status.

6.1 Introduction

In chapter 4, the CPP population screen was explored, during which the heritability of nicotine reinforcement was established over three generations. In so doing, lines with distinct nicotine seeking phenotypes were generated. The first generation screen identified a mutant line, which showed higher nicotine reinforcement than the population mean and clustered at the right of the distribution. The four individuals from this line were selected from an F3 generation containing a mixture of homozygous, heterozygous and wild-type individuals suggesting the line contained a dominant mutation affecting the phenotype. Candidate genes from a list of known possible null mutations (obtained from The Wellcome Sanger Trust) were investigated in this 'QMhigh' line and identified the presence of a sl*it3* mutation that segregated with the observed phenotype. An independent line (sa202) with a nonsense mutation in a similar region of the *slit3* gene was then acquired through the zebrafish mutation project. This line also showed increased nicotine CPP indicating the *slit3* mutation was causal as opposed to other linked alleles which may be co-segregating and causing the observed phenotype.

The aim of this project was to demonstrate that a fish screen in zebrafish could be used to inform human GWAS and find genes/alleles affecting behavior. In order to test that hypothesis and demonstrate the translational relevance of the work in the fish population screen, the *SLIT3* locus was investigated in people. In order to do this, a range of SNP markers covering the *SLIT3* locus were selected, firstly based firstly on previous association with disease in literature, and secondly based on showing linkage disequilibrium (LD) with other SNPs. The SNPs selected for analysis are summarized in **Table 6.1**.

SNP	Phenotype	Literature	MAF
rs11742567	Smoking Cessation, G-allele associated with more increased cessation rates	Uhl et al., 2010	0.39
rs3733975	Schizophrenia-Schizophrenia, gender differentiated in males.	Shi et. al., 2004	0.34
rs2938774	Schizophrenia-Schizophrenia, gender differentiated in females.	Shi et. al., 2004	0.39
rs12521041	Schizophrenia-Schizophrenia, gender differentiated in males.	Shi et. al., 2004	0.31
rs11134527	Cancer, T-allele associated with more increased cancer rates	Zhou et al., 2010	0.33
rs9688032	Parkinsons, GWAS HIT replicated, associated with Vit D levels	Li et al., 2008	0.39
rs17665158	Bipolar disorder.	Glessner et al., 2010	0.22
rs13183458	Alzheimer's disease.	Antonell et. al., 2013	0.24
rs17734503	Obesity (BMI)	Vogel et al., 2009	0.25
rs12654448	Obesity (BMI)	Vogel et al., 2009	0.19
rs4282339	Height.	Lango Allen et. Al. 2010	0.19
rs12515725	GWAS of type II diabetes mellitus	Saxena et. al. 2007	0.5
rs295994	Multiple continuous traits in DGI samples-Height in Type II Diabetes Mellitus Controls;Multiple continuous traits in DGI samples-Waist/height ratio squared;Multiple complex diseases-Type II Diabetes Mellitus, combined control dataset.	Liu et. al., 2002	0.48
rs7728604	HIV-1 disease progression-HIV-1 viral load at set point.	Anand, et. al 2013	0.3
rs10036727	G [Gly] ⇒ S [Ser]	AA Change	0.433
rs297886	High LD with other SNPS	n/a	0.275
rs11749001	High LD with other SNPS	n/a	0.167
rs1345588	High LD with other SNPS	n/a	0.175
rs1559051	High LD with other SNPS	n/a	0.293
rs1421763	High LD with other SNPS	n/a	0.267

Table 6.1: Summary of SNPs used in association analysis. The rs-numbers are provided along with references, phenotype and minor allele frequencies (MAF) where applicable.

The idea is to use these genetic predictors (20 SNPs) to see if they are associated with various smoking outcomes. The impact of SNPs on smoking initiation can be investigated by comparing those who have never smoked with other participants ((Ex

+ Current) v Never). A measure of persistent smoking can be obtained by comparing the genotype of current smokers against the rest (Current v (Ex + Never)). Figures for the number of cirgarettes smoked every day were also recorded and can be compared as a continous variable against genotype.

The SNPs for analysis were initially selected based on biological significance, specifically with regard to their presence in the literature as having previously associated with a disease phenotype in a human GWAS. The rationale for selecting SNPs like this in the first instance, is that a previous association with disease in a GWAS means the SNP is more likely to be a mutation affecting (or acting as a marker for genetic code affecting) expression patterns of *SLIT3*, and thus having an effect on phenotype. The most interesting of these SNPs in the literature was rs11742567, which was shown to associate with smoking cessation success in 925 european-american smokers given 16mg NRT and varying degrees of behavioral support (Uhl, Drgon, Johnson, Walther, et al., 2010). Also of particular interest were 3 SNPs (rs3733975, rs2938774 and rs12521041) associated with schizophrenia susceptibility in the Chinese Han population (Shi et al., 2004). Schizophrenia is a disorder that is partly caused by altered dopamine signaling in the mesolimbic pathway of the brain. It is also a disorder which has historically correlated with smoking, with sufferers showing a high rate of self-medication with nicotine; the therapeutic effect most likely occurring due to dopaminergic modulation through activation of nicotinic acetylcholine receptors (de Leon & Diaz, 2005; Keltner & Grant, 2006). Interestingly rs9688032, was shown to have an association with Parkinson's disease, the degenerative disorder of the central nervous system (Y. Li et al., 2008). The loss of motor function often associated with Parkinson's are a result of dopaminergic cell death in the substantia nigra, which is compelling given the role of SLIT3 in

dopaminergic axonal path finding.

One SNP (rs10036727) was selected due to the polymorphism conferring an amino acid change at protein residue 618 from glycine (G) to serine (S). This is the only missense mutation present in a coding region of the SLIT3 gene and is included due to the possibility of an amino acid change affecting the properties of the translated protein. This in turn has the possibility of having an effect on phenotype, which may influence smoking behaviours.

The final SNPs that make up the list were selected due to being in linkage disequilibrium (LD) with the highest number of other SNPs in the SLIT3 gene. The SNPs in the hapmap input file are then tagged for linkage disequilibrium using the tagger tool incorporated into haploview. Tagger is a tool for the selection and evaluation of tag SNPs from genotype data such as that from the International HapMap Project. Linkage disequilibrium is the occurrence of combinations of alleles in a population more often or less often than would be expected from a random formation of haplotypes from alleles. The amount of linkage disequilibrium depends on comparing the difference between observed allelic frequencies and those expected from a homogenous, randomly distributed model. In this context, the tagger provides a list of alleles which associate with the target SNP at a significantly greater rate than if they were just to segregate randomly. What this allows in terms of the experimental design, is to look for associations between the target SNP and smoking phenotype, as well as those SNPs with which it shows a significant degree of genetic linkage. Correlations among nearby variants can improve the cost-effectiveness of these types of studies, guiding selection of informative 'tag' SNPs and providing information about nearby variants not genotyped (Daly, Rioux, Schaffner, Hudson, & Lander, 2001; Gabriel et al., 2002; G. C. Johnson et al., 2001; Patil et al., 2001). For instance,

rs297886 shows high LD with 17 other SNPs in the slit3 locus and by including it in the panel; it provides greater genetic coverage when looking for association between variants at this locus and smoking outcomes. This increases the likelihood of identifying genetic associations for the four smoking behaviours. The International HapMap Project is a resource that provides empirical genome-wide data to support such analyses

6.2 Methods

6.2.1 Human cohort

Subjects were recruited from a separate cross-sectional study conducted in London, UK, investigating environmental and genetic determinants of vitamin D status in patients with chronic obstructive pulmonary disease (COPD). The participants were therefore derived from three separate cohorts: a group with COPD (ViDiCO; n=272), a group with asthma (ViDiAs; n = 293) and a group containing residents and carers in sheltered accommodation schemes who did not have COPD (ViDiFLU; n=298).

Principal exclusion criteria for the COPD study were smoking history <15 pack-years; age <40 years; and ratio of forced expiratory volume in one second (FEV1) to forced or slow vital capacity (VC) after inhalation of 400 micrograms salbutamol >70%. The cohort comprised of subjects with mild, moderate or severe chronic obstructive pulmonary disease (COPD) treated with the same bi-monthly 3mg vitamin D3 intervention. Inclusion criteria was medical record diagnosis of COPD, emphysema or bronchitis and an age of 40 years or over. Exclusion criteria also included known asthma, sarcoidosis, hyperparathyroidism, terminal illness and malignancy other than non-melanoma skin cancer. The mean age of the cohort was 64.6 (s.d. 8.5), 40% were female and 42% were current smokers.

Asthma patients were selected on the basis of their lack of smoking history, which had to be < 15 pack-years. They were adult patients with asthma treated with inhaled corticosteroids treated with a bi-monthly 3mg vitamin D3 intervention. Inclusion criteria were a medical record diagnosis of asthma (plus corticosteroid

treatment) with an age between 16 and 80 years. The mean age of the participants was 47 (s.d. 14.3), 56% were female while 7% of participants were current smokers.

Principle exclusion criteria for the general population cohort in sheltered accommodation were: age <16 years and diagnosis of COPD or asthma. The cohort was comprised of older adults in sheltered accommodation given 10 mcg vitamin D_3 daily as well as bi-monthly 3mg vitamin D_3 interventions. Participants needed to be residents or carers in sheltered accommodation aged 16 years or over. Exclusion criteria included known asthma, COPD, sarcoidosis, hyperparathyroidism, terminal illness and malignancy. Anyone taking thiazide and calcium supplement, cardiac glycoside, carbamazepine, pentobarbitol, phenytoin, primidone or long-term immunosuppressant therapy was also excluded. The mean age of participants was 66.8 years (s.d. 13.1), comprised of 66% females and 18% were current smokers.

The studies were approved by East London and The City Research Ethics Committee 1 (refs 09/H0703/76 and 09/H0703/112) and written informed consent was obtained from all participants before enrolment.

6.2.2 Ordering TaqMan assays from the Applied Biosystems

TaqMan assay kits were ordered from http://www.appliedbiosystems.com, specifically the TaqMan SNP Genotyping Assays product page. Assays can be found by searching for gene name, SNP ID, Assay ID, Assay type or SNP type. There were

15 SNPs for which Applied Biosciences already had genotyping assays available, these are listed in **table 6.2**.

To design SNP targeting primers for the 5 remaining alleles the sequences shown below in **figure 6.1** were entered into the Custom TaqMan® Assay Design Tool web site in the 5' to 3' direction. The sequences entered are roughly between 500 and 1000 basepairs in length and generated to the following parameters:

- Use only A, C, G, T, and N, except where SNP or indel target sites are marked.
- Convert the IUPAC codes R, Y, M, K, S, W, H, B, V, and D to N, except for marked SNP target sites where you translate the ambiguity code to the appropriate bases.
- Enclose each target site with square brackets [ ].
- **SNP targets:** Enter the base for the first allele followed by a forward slash (/), and then the base for the second allele. For example, convert R to [A/G].

Assays that are designed in regions of a sequence that contain repeats and polymorphisms are likely to produce nonspecific amplification and probe binding. To reduce the likelihood of nonspecific amplification and probe binding, these regions need to be masked (which is accomplished by replacing such regions with an 'N' as mentioned above.

SNP	Taqman Assay
rs11742567	Not Available
rs3733975	C__25753801_10
rs2938774	C___1457640_10
rs12521041	C__31235183_10
rs11134527	C__30743954_10
rs9688032	C___3220724_10
rs17665158	C___3073893_10
rs13183458	C___1457618_10
rs17734503	C__32834038_10
rs12654448	C___3220757_10
rs4282339	C__27909684_10
rs12515725	C___3200020_10
rs295994	C___1030672_10
rs7728604	Not Available
rs10036727	Not Available
rs297886	C___1012759_10
rs11749001	Not Available
rs1345588	Not Available
rs1559051	C___3200034_10
rs1421763	C___7526127_10

Table 6.2: List of 20 SNP rs numbers included in smoking status association study along with the TaqMan assay numbers. SNPs that do not include a TaqMan number in the second column indicate that a pre-designed assay did not currently exist. Those 5 need to be custom designed.

rs11749001

AATGCATTTGGGAAANGCACATCTANACATTTGGGAAGCTGATGTTCACA
TCCTCCGCCTCAAATACCTCTGAGGTAATGAATTCCATAAAATTGACATCT
GCTCTTACAGCACAACTTCCCATTCATGCATTTTTTCCATTAAAAAATATT
TTAGNNCTGTACTAAACCCTGCTAATAAAAAATAAAANTACAATTCCC**[C/**
T]GCTCACAAGTAGCTCATAGCATGGTNGGGAAGGCAGTTAAAGCACNAG
TCTCGGAGTTGGAGTATGGCTGATGCTCAATAATAGTTTGCTGGGGAAGC
TAGAGCTAGAAGAAGTCTTTCTAATAAATAAGACCAGCATAGTCAGGGAG
GGCTTCCTAGAGGTGGAGAGATGAGCAATGAATTTTAAAGAACGAGATTC
AGCCTGTAATCCCAGCACTTTGAAAGGCGCAGAGGGGTGGATACCNTTGA
GGTCGGGAGTTCGAGACCAGGCTGACTAACATGATGAAACCCCGTCTCTA
CTAAAAATACAAAATTAGCTGGGTGTGGTGGCACATGCCTATAATCCCAG
CTACTTGGGAGGTTGAGGCAGGATAATCGCTTGAACCCGGGAGGCGAGG
GTTGCAGTGAGCNAAGATCGCGTCTTTGCACTCCAGCATGGGCAACAAGA
GTGAAACGCCATCTCAAAAACAAAAAAGAAT

rs1345588

TGCTATCTGTCCATCCTGTTTGGGTGGGGAGCTATCTGAGCTGTGCAGAGG
TGGCAGGGGTGGGCCCTGCTGAGGAATGCAGCAAGGGGCTCACCCCACG
ACCCACAGATGCATGACCCTGCCCAAGGGAGGGCTTCTCATCTTACTCAG
CCTCAGCTAGAAGCCAGTGGTGACACATTCNGGGGTGTCTATTCCTATCCT
GAGATAGAGCCACATGGAAATTCAAAAGATGAAATCNCAGAAGCAGTTT
ATCACCCAAGTATAAAGACAGGCATCTGTCAACAAAGGAACATGATCATA
GCTGAGTGTTCCTTGAGTGGCTTTTCCTTCATTGCTCTCAAAGCCTTTTGAG
ATCTCGAAAAGTCTCCAGTGGGCAAGACTTGACATTCTATGTGTGAGACA
CAGATGCAGAATCAGACATGGATACCGTGATTCGCTGCTACATGNCCGGT
GTCTTCAGCTCANNTCTGCTCACTCACACANGAGGTGACCAATCCCAAT**[A
/G]**ATNGGGGATCCTGTCCCAGCCCCCAGTTCTCCAGCAGTGTCTCCTGGCT
CCCCTCTTTAGANCCAGCACCACTCTCATTCTTGCAAGCCACCTGACTCAT
GGCTTTCCCATTCACCTCCTGCCCTGTCCTTGGTACCCCACATTGCTCTGG
ACACCACAGTGCCCTGTATAGCCACTGGTCCCATGGCGGGGATCCTCAGG
CAGAGAGAAGATACTAAAATCAACTCACTTGCAAACAGGCCCTCTTCCTC
ACAAACTGGAAGATGGATCCTAGCCAAATGNNAAAAAAAAANNAAAAGC
TTAAATTTTTAGGTTTTAAAAAAATCAATGTAAGGAAAATTCTCAAATGGT
TGAAAAATATTCAATCTACAGTAGGCCTTGGGGATGTGTATTTTTTTGGCT
AGGGGACCTCCTGAGTTTCTTTAATCACACCAAATAAATGATTCTCTTCTC
CAACTATGGATAAGGCTGGCTCTGATTGGTATATGAGATCACACACATAT

rs7728604

TATTAGGNAGTCCTGGAGCTTGTGGAATGAGTTGCTCTGCCTAAGAGCAT
GNAAGAGAAAAGAATTCCCAACACCCCATTCTGGCACAATAATAGATAGC
CCAGGTGTCAGGCCTACCAGTCAGTTAANCAAATTTGCAAGACCGAGGGG
CTGTCTCCAGTGCTTTCTACTGNTTCTCTGCTTTGATACAGGTTGAGTATCC
CTAATCCAAAAATCCAAAANTCTGAAATGCTCCAAAATCTGAAACTTTCT
GAGCACTGACATGANNTCACAAGTGGAAAATTCCACACCTGACCTCATGT
GACTGATGGATTGCAGTCAAAACTTCATATCATGCACAAAATTATTAAAA
ACTTTGTATAAATTACCTTCAGCCTATGTGTGTGAGGTATATATGAAACAT
AAATGAGTTTCATNNTTAGACTTGGATTCCATTCTCAAGATATCTCATTAT
GTATGTGAAAATATTCCAAACTTTGAAAAATTCAGAACGCTGAAATG**[C/T]**
TTTTGGCCCCAAGCATTTTGGATAAGGAATACTCAACCTTTTAGTTTGTAC
TTGGAATGTTTTTTATACNAAGGTAACCACTAGGAATAGGCAGAGACAAA
GCCATTCATTCAGAGAGAATTCTTTTTTTTNCTCTCCCCCCTCCCCTTTTTT
CTTTTCTTTTTTGGTAAGAGANAAGAGCAGGATGTGNTTCAGTCACAGTTG
AGGGGGAAACTTCCTCCTGTTAGATATTAGGGATAAACTGGTCACGTATA
TTTAGGAAAAATTAGGGTTTTGAAATCCATTCTCACCAGGGACCCTACCTC
TCCCAAACCATTGATTGCACTTCAGTCTAGGTTTCTGAAGTTCCATTAGTC
GAACATTTTTAAGTGTGCTCTCTTCTCTATTGCCATCCTCTTGGGGGAAAA
AAAGGGCATCATAATAAATATCAGTAGCATCATAAGTGATCAAAAATTAA
ATTCACTTTCCAGGTCGTGACTGAGAAGGTCTTATGAATTTTGA

rs10036727

GTAGAGAAGGGGTTTCACCATGTTGGTCAGGCTGGTTTCGAATTCCTCACC
TCNGGTGATCCACCCACCTCAGCCTCCCAAAGTGNTAGGATTACAGGCGT

GAGCCACCATGCCTGGCCTTCCAGCTTGGGTTTTCAAATCACTGTGGACAT
GCTTTCTGTGGCAATTCACTATGCTGTGAGTTCCAGGAAGGAGGGGCNNG
GTGTGTGCAGCCTCAACACCTAGACGATGCCTGCCTGGCACATACAGAAA
GCCTGANGAGTTTTTAAGTGGACAGACAGATAGATGAGCCTGGCTAGAGG
TAGCAGGGAGCTGGGAGGAGAGAGCGCTGCAGAGTGGGANCCCAGAGTC
CGTGGGCAGTGGACCCAGGAGAACTTACATGGTGGACAGGGAGACAAGC
GTGGTGAAGGCCCCAGGGGTGATGGTGGTGATCCGATTGTNATAGAGGGA
CAGCAGTCTCACCGAANTCAGGCCGGCAAAGGTGTCATTACTCACACAGC
[C/T]GATCAAGTTACNTCCTCAGCATCNTACAGGNAGAGGGGTGGGGATG
AGAGAGCACAGGCATGATCTTTTCTGTCCCAAATGGTGCCACGGTGGTGT
GTGTGTGTGTATAGGTGAGATGCTTTTGCCAATTCTAAGCTCAGTCCAGCC
ATGTTGATCAGGTTTGTCCTGTGATAGCAGGGACTGGAGCCCCACTGGGG
CTGTGTGTAAATGTGTATGTACATTATTGTTTGTGCATGCTTTCTTGGAAG
CACATGTCATGCATATGTGTGTATACACACGCATATTGTGTGGCTGCTAGG
GTGTGCACACATATTNCTGGGAAGAGAAGAGAGAATCAAGACGTGGTTGT
AGACATCAGCTTGTATGTATGTGTGTGCTTGTGTGTCTGTGCCTGTTTGTA
TATATGTTTATGTGCATGTTGCACGTGTCTGCATNTATCATTTTTATAAAG
GTCCTTATGCTTATGTTTGTGGGTAAATGTCTGTTTGTGTGCCTCCTTCA

rs11742567

CCTGAGAGGGTACAATAGATTTGGAAAATGCATTCAATCCAGAAAAGGGC
TGTGTGCCTGACAGNGTTTCATCTCCAAGAACAACTTCTCCCCTCCTCCTT
TCTGAATTCTGGCACTCATAGAGATTAGGATAATACAGATAATGAAGCTC
AGGTACAGAAGGAGATACTTNAAGCAACAGCCTCCAGGAGATGTTAGAA
GAGGGTCATTGGCAGTGAGGAAGTTCTGATGATCTTTGGGATAATCTACA
CTGTGATCTGACTCCTTCCAGGCAAATTGTCTAAAAGCTCTGGAAACCCTC
CCAGAGGCTGAAGATGTACAGAAATAAAGTTGAGGGTTGTAGTGAGGTG
AGGAGGTAAGAAAAAGAGAGGAAGTCAGCGGGCTAAGGCACTGCAGTTT
GGGAGAAGTTGAAGCCACTTCCTAGTGTCTACCCCACTGTAACCTTAAAT
GTTCAGCTTGATCATGAAGAAAGGATTCTTGGAACATTGCTCCGGATCTA
C**[C/G]**TAACCCTAACACTCTTTGTGATATGGCACTAGAGTTCCTCTTATGTG
GCCCACTCTGTTCCACNGAGGAAACATGAACATGCCAGGGCAGGGTGGG
GAGGGGCTGTGCCTGGGCTGCGGCTGCCCAGAGGTTGGAGTTTGGCAGGC
AGCACTGCAGCTCAAGGACATGGGACTTGTNTATGTCTGTGCTTTCTTGTA
ACTTTTCAGAAGACCATTAAACAAGTGAAACCTCAATGGTAGAGGTGAGA
TGCTATCCTGGGGCCATCTGCTGATAAAATCAGTATGCTTCAGAAAAGCT
CAAAATTATCTTCCACCAAAGCAGGAAAGCAATTCAGCAAGAGCTCAGCA
CCNTGAGTTGCAAAGCTGGCCTCCAGAAGCCCAGTAACGGATGAAATATA
TAGGAGCTTAAGCCATTAAGCTATGTTAATACTATAGAATGACNTTTCTCA
TCATGACCCCATGCGCTATTTATTGTTCCATTCAAAGCAATTACAGTTATT
G

Figure 6.1: Sequences sent to Applied Biosystems for the design of custom TaqMan SNP genotyping probes. The site for the SNP of interest is donated with two square brackets with the two possible bases. Areas where other polymorphisms and insertions may occur are marked or masked by inserting an N at that location.

6.2.3 Allelic discrimination plate read and analysis

Each DNA sample was diluted with DNase-free water to deliver a final DNA mass in the range of 1 to 20 ng per well. A total of 2.25 μL of genomic DNA was added to each well in a 384-well reaction plate. The master mix was then prepared as to give for each reaction well as follows:

- 2.5 μL TaqMan Universal PCR Master Mix (2X)
- No AmpErase UNG,
- 0.25 μL 20X working stock of SNP Genotyping Assay.

The plates were run on an Applied Biosystems 9800 Fast Thermal Cycler, using the 9700/9600 emulation mode and the following thermal cycling conditions:

- 95°C for ten mintutes
- Denature for 15 seconds at 92°C and an anneal/extention step of 1min at 60°C for a total of 40 cycles.

After the PCR amplification, the plate was read using an Applied Biosystems Real-Time PCR System. The software used the fluorescence measurements made during the plate read to indicate which alleles were in each sample.

6.2.4 Downloading dbSNP files and SNP tagging using haploview

Initially, it is necessary to generate a hapmap style input file for the slit3 gene, which can be done on dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) by searching for

the gene, selecting the whole gene (plus any flanking sequence of interest) and selecting export. This generates an output file of the gene of interest containing all known SNP.

Haploview is based on Paul de Bakker's Tagger. Tagger is a tool for the selection and evaluation of tag SNPs from genotype data such as that from the International HapMap Project. It combines the simplicity of pairwise tagging methods with the efficiency benefits of multimarker haplotype approaches (de Bakker et al., 2005). As output, Tagger produces a list of tag SNPs and corresponding statistical tests to capture all variants of interest, and a summary coverage report of the selected tag SNPs.

SNP	Haploview Tagged SNPs
rs11742567	rs11134544, rs11742567, rs12521361, rs3923474
rs3733975	
rs2938774	
rs12521041	rs11741109, rs11744726, rs11748325, rs2194099, rs898401, rs2054274, rs10475884 rs12521041, rs11746295, rs1864953, rs7705016, rs17553140, rs4868519, rs4867894 rs4867802, rs11750194, rs4868530, rs10056503, rs17553274, rs1870563, rs4868094 rs10516049, rs17553085, rs2277933, rs17732425
rs11134527	rs9784690, rs1368355, rs6860336, rs11134527
rs9688032	
rs17665158	rs1048307, rs11743244, rs17665158, rs13179467, rs2303002, rs12655287 rs11749992, rs1460100, rs6869359, rs10516048, rs1155191, rs11750308 rs17665285
rs13183458	rs13183458
rs17734503	
rs12654448	rs17734605, rs1432898, rs17666992, rs3749674, rs2278386, rs7700954, rs17667033 rs12654448, rs17734593, rs1059160, rs6887076, rs6555846
rs4282339	rs6555846, rs2974438
rs12515725	rs7722860, rs12515725, rs2879149
rs295994	rs295994, rs6863538
rs7728604	rs7728604, rs3797717
rs10036727	rs7706177, rs1549909, rs10036727
rs297886	rs297886, rs297811, rs297873, rs297884, rs297869, rs190935, rs297867, rs297878 rs297822, rs297876, rs10462982, rs297882, rs297819, rs297877, rs7700961 rs297885, rs7701204
rs11749001	rs6873937, rs11742763, rs11744997, rs11745045, rs6881546, rs4867734, rs4868340 rs17637311, rs10516061, rs4868339, rs17637230, rs297868, rs9313447, rs11749001 rs10462980, rs4868338
rs1345588	rs8180402, rs297850, rs745682, rs1345588, rs17735527, rs978902, rs12514330 rs12516235, rs177073, rs11745049, rs17735570, rs10866632, rs10866631, rs172472 rs11738097
rs1559051	rs297820, rs17637212, rs297864, rs297865, rs10058857, rs297823, rs17637323 rs297817, rs297863, rs17556404, rs297815, rs1559051, rs297818, rs10063923
rs1421763	rs9313446, rs1421762, rs297874, rs1421763, rs17667652, rs10516060, rs17667664 rs7704526, rs2112193, rs1006329, rs7732724

Table 6.3: Table of haploview tagging results for each of the target SNPs.

6.2.5 Generation of LD plot in Haploview

The hapmap input file was loaded in haploview, before selecting the target SNPs from this study for inclusion. Once selected, LD plots can be generated by selecting the 'LD view' tab. Haploview calculates several pairwise measures of LD, which it uses to create a graphical representation. The output visualizes the occurrence of haplotype blocks, which are sizable regions over which there is little evidence for historical recombination with only a few common haplotypes occuring.

6.2.6 Statistical analysis

Genetic association analysis was carried out using PLINK v1.07 (Purcell et al., 2007). We combined smokers of European ancestry from the three cohorts. There was a total of 486 smokers, 254 from the COPD cohort, 100 from the asthma cohort and 132 from the general cohort. Of twenty *SLIT3* SNPs genotyped, one – rs13183458 - exhibited departure from Hardy-Weinberg equilibrium, and was excluded from analyses. Linear regression was performed on the average number of cigarettes smoked per day, controlling for age, sex and cohort under an additive genetic model. Binary outcomes were analyzed using logistic regression against the additive genotype model, again controlling for age, sex and cohort. Multiple testing was taken into account using the Benjamini-Hochberg adjustment controlling for a false discovery rate of 10%. For analyses on heavy smokers there were 249 individuals from the COPD cohort, 17 from the asthma cohort and 66 from the general cohort.

6.3 Results

6.3.1 TaqMan and allelic plate read analysis

In total 843 individuals from across the three trials were genotyped successfully at the 20 different loci. When broken down, this included 293 from the ViDiAs trial, 272 from ViDiFlu and 278 from ViDiCO. Allele frequencies from the genotyped cohort (**Table 6.5**) were comparable to values derived from the Ensembl genome browser (**Table 6.4**). As well as showing allele and genotype frequencies for each of the target SNPs, table 6.5 shows Hardy-Weinberg frequencies. All SNPs were found to be in Hardy-Weinberg equilibrium apart from rs13183458 ($X^2 = 4.008$, $p = 0.04$). When the ratios of homozygous and heterozygous genotypes significantly differ from the prediction under HWE assumptions, it can indicate genotyping errors, batch effects, population stratification. Typically departure from HWE is an indicator that a marker should be discarded. As such, rs13183458 was disregarded from further analysis

SNP	Allele 1	Allele 2	1/1 freq	1/2 freq	2/2 freq
rs11742567	C (0.6)	G (0.4)	0.34	0.52	0.14
rs7728604	C (0.61)	T (0.39)	0.39	0.45	0.17
rs1345588	A (0.14)	G (0.86)	0.02	0.24	0.74
rs17734503	A (0.9)	G (0.1)	0.8	0.2	0
rs11134527	A (0.21)	G (0.79)	0.024	0.38	0.6
rs10036727	C (0.5)	T (0.5)	0.28	0.44	0.28
rs3733975	C (0.73)	G (0.27)	0.57	0.33	0.11
rs4282339	A (0.21)	G (0.79)	0.06	0.31	0.64
rs11749001	C (0.87)	T (0.14)	0.75	0.22	0.02
rs12521041	C (0.73)	T (0.27)	0.57	0.33	0.11
rs297886	G (0.73)	T (0.27)	0.53	0.4	0.071
rs295994	C (0.6)	G (0.4)	0.33	0.53	0.14
rs13183458	C (0.78)	T (0.22)	0.64	0.28	0.08
rs2938774	A (0.62)	G (0.38)	0.38	0.49	0.13
rs17665158	C (0.2)	T (0.8)	0.06	0.28	0.66
rs12515725	C (0.62)	G (0.38)	0.4	0.44	0.17
rs1559051	A (0.67)	C (0.33)	0.46	0.42	0.12
rs9688032	A (0.27)	T (0.73)	0.06	0.42	0.52
rs12654448	C (0.9)	T (0.1)	0.8	0.2	0
rs1421763	A (0.25)	G (0.75)	0.07	0.35	0.58

Table 6.4: Allele and genotype frequencies according to data from the Ensembl genome browser.

SNP	Allele 1	Allele 2	1/1 Freq	1/2 Freq	2/2 Freq	X^2	P-Value
rs11742567	0.66	0.34	0.43	0.45	0.12	0.026	0.872
rs7728604	0.62	0.38	0.41	0.44	0.16	3.691	0.055
rs1345588	0.14	0.86	0.02	0.24	0.74	0.266	0.606
rs17734503	0.88	0.12	0.79	0.19	0.02	3.711	0.063
rs11134527	0.27	0.73	0.08	0.39	0.53	0.066	0.797
rs10036727	0.48	0.52	0.22	0.53	0.25	2.469	0.116
rs3733975	0.74	0.26	0.55	0.38	0.07	0.026	0.872
rs4282339	0.2	0.8	0.04	0.32	0.64	0.047	0.828
rs11749001	0.85	0.15	0.73	0.25	0.02	0.053	0.818
rs12521041	0.75	0.25	0.56	0.38	0.07	0.094	0.759
rs297886	0.79	0.21	0.63	0.33	0.04	0.122	0.727
rs295994	0.55	0.45	0.31	0.48	0.21	0.515	0.473
rs13183458	0.74	0.26	0.57	0.34	0.09	4.411	0.244
rs2938774	0.57	0.43	0.35	0.46	0.2	1.622	0.208
rs17665158	0.22	0.78	0.07	0.3	0.63	4.008	0.045
rs12515725	0.55	0.45	0.3	0.51	0.2	0.548	0.459
rs1559051	0.7	0.3	0.5	0.41	0.1	0.821	0.365
rs9688032	0.33	0.67	0.12	0.43	0.45	1.169	0.28
rs12654448	0.91	0.09	0.83	0.16	0.01	0.532	0.501
rs1421763	0.22	0.78	0.05	0.35	0.6	0.064	0.8

Table 6.5: Allele and genotype frequencies of each SNP in the cohort of 843 participants in the ViDiAs/CO/Flu trials. Hardy Weiberg frequencies were calculated for each allele and then assessed for whether it deviated from predicted Hardy Weinberg equilibrium.

6.3.2 Statistical analysis

Since there is significant conservation of neural pathways between vertebrate species (M. O. Parker, Brock, Walton, & Brennan, 2013) we looked for associations between 19 single nucleotide polymorphisms (SNPs) in the human *SLIT3* gene and self-reported smoking behavior. Two SNPs, rs12654448 and rs17734503 in high linkage disequilibrium (Figure 6.3) were associated with number of cigarettes smoked per day (p=0.00125 and p=0.00227; Table 6.6a). We repeated the analysis on heavy smokers (>=20 cigarettes per day) to investigate whether effects were related to level of intake. Controlling for age and sex rs12654448 (P=0.008569) and rs17734503 (P=0.01837) were again associated with number of cigarettes smoked as was rs11742567 (P=0.006145, (Table 6.6b). When adjusted for age, sex and cohort, the same SNPs remained significant: rs12654448 (p-value=0.0003397); rs17734503 (p-value=0.0008575) and rs11742567 (p-value=0.004715) (Table 6.6d). rs11742567 was also associated with smoking cessation (Table 6.6d). No other SLIT3 polymorphisms were associated with initiation, persistent smoking or cessation (Supplementary **Tables 6.7a&b**).

A

SNP	P VALUE	β	SE	95% CI
rs12654448	0.001253	-4.241	1.307	(-6.803,-1.68)
rs17734503	0.00227	-3.987	1.299	(-6.534,-1.441)
rs11749001	0.05944	1.972	1.044	(-0.07367,4.018)
rs17665158	0.1308	1.338	0.8837	(-0.3944,3.07)
rs11742567	0.1351	-1.166	0.7786	(-2.691,0.3605)
rs2938774	0.1403	1.101	0.7454	(-0.3599,2.562)
rs11134527	0.2175	1.014	0.8215	(-0.5957,2.625)
rs1345588	0.2401	-1.268	1.078	(-3.38,0.8447)
rs1421763	0.2724	-0.9824	0.8941	(-2.735,0.77)
rs12515725	0.5915	-0.406	0.7561	(-1.888,1.076)
rs297886	0.6198	0.442	0.8903	(-1.303,2.187)
rs10036727	0.6285	-0.3882	0.8018	(-1.96,1.183)
rs4282339	0.669	-0.4335	1.013	(-2.419,1.552)
rs7728604	0.7007	0.2863	0.7444	(-1.173,1.745)
rs295994	0.7135	0.283	0.7702	(-1.227,1.793)
rs12521041	0.9036	-0.1048	0.8652	(-1.801,1.591)
rs3733975	0.9087	-0.09864	0.86	(-1.784,1.587)
rs9688032	0.9478	-0.05017	0.7658	(-1.551,1.451)
rs1559051	0.9611	-0.03998	0.8186	(-1.644,1.564)

B

SNP	P VALUE	β	SE	95% CI
rs12654448	0.00034	-4.83	1.334	(-7.444,-2.216)
rs17734503	0.000858	-4.458	1.325	(-7.055,-1.861)
rs11742567	0.004715	-2.346	0.8245	(-3.962,-0.7301)
rs17665158	0.2363	1.114	0.9393	(-0.7266,2.955)
rs1345588	0.2527	-1.334	1.164	(-3.616,0.9479)
rs7728604	0.3206	0.8273	0.8317	(-0.8027,2.457)
rs11134527	0.3268	-0.8674	0.8833	(-2.599,0.8638)
rs10036727	0.4483	-0.6526	0.8596	(-2.337,1.032)
rs1559051	0.4576	0.6559	0.882	(-1.073,2.384)
rs12515725	0.4875	-0.5652	0.8131	(-2.159,1.029)
rs2938774	0.5281	0.4962	0.7856	(-1.044,2.036)
rs295994	0.6426	0.3776	0.8128	(-1.215,1.971)
rs9688032	0.7698	0.2456	0.8388	(-1.398,1.89)
rs11749001	0.873	0.1765	1.103	(-1.985,2.338)
rs4282339	0.9422	-0.08006	1.103	(-2.241,2.081)
rs297886	0.9613	-0.04786	0.9855	(-1.979,1.884)
rs1421763	0.9775	-0.0271	0.9594	(-1.908,1.853)
rs3733975	0.9817	0.02145	0.9338	(-1.809,1.852)
rs12521041	0.9956	-0.00523	0.9417	(-1.851,1.841)

C

SNP	P VALUE	β	SE	95% CI
rs11742567	0.003909	1.888	0.6441	(0.6258,3.151)
rs2938774	0.01531	-1.655	0.6744	(-2.976,-0.3328)
rs17665158	0.03411	1.62	0.7577	(0.1354,3.106)
rs3733975	0.05848	-1.354	0.7102	(-2.746,0.03772)
rs12521041	0.05848	-1.354	0.7102	(-2.746,0.03772)
rs9688032	0.07968	-1.076	0.61	(-2.272,0.1191)
rs1421763	0.162	-1.074	0.7639	(-2.571,0.4237)
rs11749001	0.2057	1.2	0.9444	(-0.6507,3.051)
rs4282339	0.238	-1.006	0.8492	(-2.67,0.6583)
rs295994	0.2381	-0.7962	0.6722	(-2.114,0.5213)
rs11134527	0.2608	0.7953	0.7046	(-0.5856,2.176)
rs12515725	0.2781	-0.6876	0.6316	(-1.925,0.5503)
rs12654448	0.4095	-1.034	1.251	(-3.486,1.417)
rs17734503	0.4095	-1.034	1.251	(-3.486,1.417)
rs297886	0.489	0.488	0.7036	(-0.891,1.867)
rs1559051	0.5072	0.4552	0.6846	(-0.8866,1.797)
rs7728604	0.654	0.2617	0.5827	(-0.8803,1.404)
rs1345588	0.8691	-0.1497	0.9066	(-1.927,1.627)
rs10036727	0.9403	-0.05146	0.6861	(-1.396,1.293)

D

SNP	OR	SE	L95	U95	P value
rs11742567	1.586	0.1629	1.153	2.183	0.004637
rs11134527	0.6651	0.1646	0.4817	0.9184	0.01324
rs12521041	1.554	0.1783	1.096	2.205	0.01336
rs3733975	1.488	0.1759	1.054	2.101	0.02381
rs2938774	0.7529	0.1484	0.5628	1.007	0.05581
rs17665158	0.7228	0.1724	0.5155	1.013	0.05974
rs17734503	1.616	0.2754	0.9421	2.773	0.08128
rs12654448	1.625	0.279	0.9406	2.808	0.08178
rs1345588	1.417	0.2216	0.918	2.189	0.1155
rs295994	0.7992	0.1544	0.5905	1.082	0.1465
rs297886	1.108	0.1769	0.7837	1.568	0.5606
rs1559051	0.9193	0.163	0.6679	1.265	0.6058
rs1421763	0.9168	0.1759	0.6494	1.294	0.6216
rs7728604	0.9351	0.1493	0.6979	1.253	0.6533
rs9688032	1.066	0.1555	0.786	1.446	0.6804
rs10036727	0.9473	0.1596	0.6928	1.295	0.7344
rs11749001	0.9534	0.2055	0.6373	1.426	0.8165
rs12515725	1.028	0.1508	0.765	1.381	0.8547
rs4282339	0.9839	0.2027	0.6613	1.464	0.9362

Table 1A. Associations of SLIT3 SNPs with self-reported level of tobacco consumption. 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=regression coefficient, which represents the effect of each extra minor allele. SE=standard error of regression coefficient. Benjamini-Hochberg cut-off at q-value 0.1 = 0.01053. **Table 1B. Associations of SLIT3 SNPs with tobacco consumption in heavy smokers (>=20 cigs/day).** Adjusted for age, sex and cohort. (q-value 0.1 = 0.01579). **Table 1C. Associations of SLIT3 SNPs in light smokers (<20 cigs/day).** Adjusted for age, sex and cohort. (q-value 0.1 = 0.00526). **Table 1D. Associations analysis of slit3 SNPs with cessation.** Logistic regression of current smokers vs ever smokers 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. (q value 0.1 = 0.00526). For all panels, associations marked in red remained significant after adjustment for multiple comparisons using a Benjamin-Hochberg procedure to control the false discovery rate at 10%.

A

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

B

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

Supplementary table 5 & 6: Results of association analysis of slit3 SNPs on smoking initiation (5) and Persistent smoking (6). 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.

6.3.3 Linkage disequilibrium & haplotype block analysis

The LD analysis produced the plot seen in figure 6.3, with two main blocks of high LD. Regions are partitioned into segments of strong LD. This display shows lines to indicate transitions from one block to the next with frequency corresponding to the thickness of the line. The blocks of LD were calculated using the default algorithm (Gabriel et al., 2002). The level of LD between pairs of SNPs are represented by colors, with dark red indicating strong LD, through to white indicating little linkage (Barrett, Fry, Maller, & Daly, 2005). The first block encompassed the 3 SNPs rs3733975, rs12521041 and rs17665158. The second comprised of a larger block of SNPs including rs1559051, rs11749001 rs297886 and rs1421763

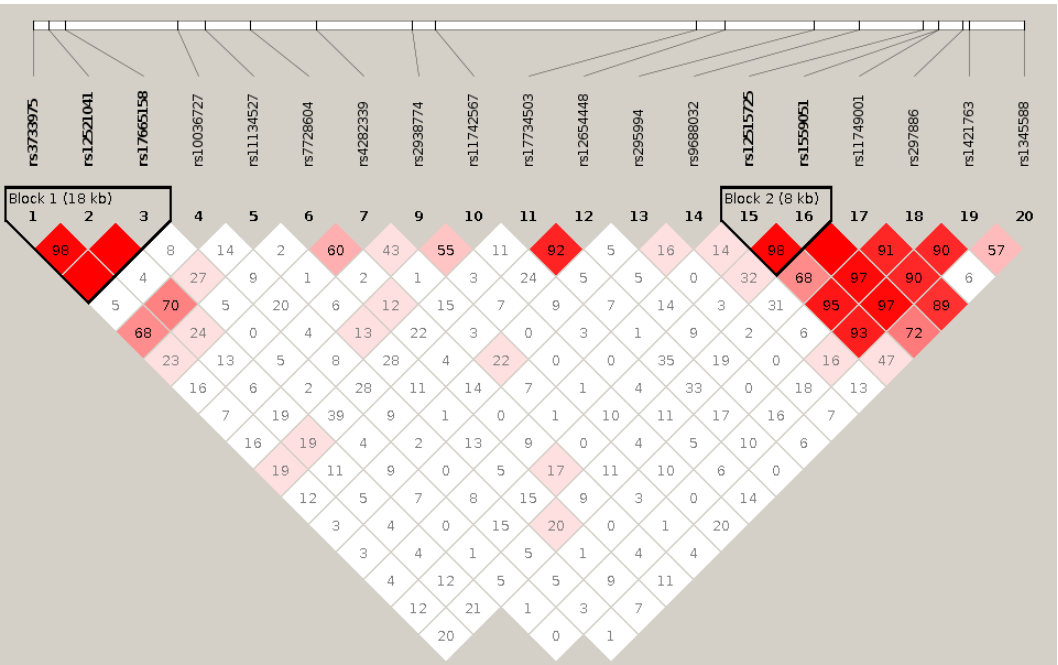


Figure 6.3: Linkage disequilibrium plot constructed by haploview from genotyping data using default LD algorithm. Two LD blocks are shown, the first consisting of 3 SNPs rs3733975, rs12521041 and rs17665158. The second is comprised of a larger block of SNPs including rs1559051, rs11749001 rs297886 and rs1421763.

6.4 Discussion

The analysis presented in this chapter was successful in addressing the second aim of this thesis; namely to use genetic loci identified in the zebrafish behavioural paradigm to both reaffirm previous GWAS studies and inform further clinical studies. The first generation screen of mutants was able to identify a slit3 mutation in a line that clustered to the right arm of the. The gene had previously been identified, along with several others, as having an association with precessation NRT quitting success (Uhl, Drgon, Johnson, Ramoni, et al., 2010). This justified carrying out a *SLIT3* SNP analysis on the COPD and asthma cohorts, chosen due to the participants having a heavy and light smoking histories respectively. In doing so 1 SNP (rs12654448) was found to show significance at the corrected threshold ($p < 0.0027$) with the number of cigarettes smoked per day in the COPD cohort. This SNP also showed an association in the asthma cohort, though this was not significant at the corrected alpha level ($p = 0.04$). Interestingly, 9 SNPs reached significance in this cohort indicating that there was a likely ceiling effect in the COPD cohort masking possible effects. This is also indicative of rs12654448 being of particular importance due to not being affected by this ceiling effect.

The rs12654448 SNP, located in an intronic region of the *SLIT3* gene. This SNP was initially selected for inclusion in the genotyping analysis based on previously appearing in GWAS literature as a polymorphisms that was associated with a human disease phenotype. This SNP had previously showed association with

increase body-mass index in two studies (Y. J. Liu et al., 2008; Zhao et al., 2007) though this was not found to be replicable in a third study (Vogel et al., 2009).

Variations in the *SLIT3* gene may affect BMI due to alterations in dopamine signaling, which plays an important role in appetite control (Hardman, Herbert, Brunstrom, Munafo, & Rogers, 2012). Hormones from the gut, pancreas and fat stores influence dopamine signaling as a means of detecting nutritional status and regulating feeding behaviour (Clifton, 2000; Wise, 2006). It therefore seems plausible that abnormalities in the dopaminergic system contribute to over-eating and obesity with inappropriate cues to eat becoming as compelling as drug addictions.

The *SLIT3* SNPs rs12654448 and rs17734503 (in LD with rs12654448 and significant in general population cohort) and were reported to be associated with increased BMI with odds-ratios and confidence intervals of 1.150 (0.814 – 1.626) and 1.126 (0.791 – 1.602) respectively. In this smoking study, the effect of rs17734503 (4.5 fewer cigs/day) and rs12654448 (2.8 fewer cigs/day) was to reduce the average number of cigarettes smoked per day for those with at least 1 copy of the minor allele. As such, these two polymorphisms in the SLIT3 gene have been shown in this study and previous studies to be a modifier of the reward-motivated characteristics, smoking and obesity.

These two SNPs are located in intron 32 and in LD with one another while also being in LD with a number of other SNPs. The marker rs12654448 was tagged as being in LD with the following SNPs: rs17734605, rs1432898, rs17666992, rs3749674, rs2278386, rs7700954, rs17667033, rs17734593, rs1059160, rs6887076 and rs6555846. All of these SNPs are present in intron 32 of the *SLIT3* gene. There were no SNPs in LD with rs17734503, however it is also present in intron 32 of the

SLIT3 gene. This raises the possibility of the SNPs being linked to variations in the 32nd intron that effect translational or transcriptional processes, which in turn alters phenotype. The intron itself is roughly 318kb in length, which is very large and accounts for roughly 50% of the entire *SLIT3* gene. These findings reaffirm a role for *SLIT3* variation in effecting smoking behavior as well as identifying a number of new SNPs that could potentially act as markers when informing smoking cessation strategies.

There were three common SNPs between the asthma and general population cohorts. The first SNP was rs7728604, previously implicated in Parkinson's disease in GWAS studies (Fung et al., 2006; Simon-Sanchez et al., 2007). The SNP rs17665158 falls within block 1 of the LD analysis while rs12515725 occurs in block 2. These two regions of the *SLIT3* gene may be good targets for further haplotype analysis.

There are also limitations to an analysis of this type. Primarily, the sample is of modest size from the perspective of a genetic association study and this results in the power being quite modest also. This lack of power led to a few promising associations being lost when the new significance threshold was calculated. These associations may have proven to have reached significance with a more adequate sample size. Also, since this was not a smoking trial in the first instance, smoking histories are not as detailed as they could be. Future work like this could really benefit from having a dedicated library of genomic DNA from a larger cohort of people with differing smoking histories. If more detailed information was available about smoking habits (social smoker, number of quit attempts etc.) there might be greater resolutions for picking up gene-behaviour interactions.

Chapter 7

General Discussion

A summary of the work covered in this thesis as well as ways in which the work can be explored further.

7.1 Thesis Summary

The aim of this thesis from the outset was to firstly identify polymorphisms affecting nicotine reward by screening mutagenized fish using the conditioned place preference paradigm. The second aim was to use any information garnered from the model to inform a human study and determine whether genetic variations in human orthologues confer any variations in smoking behavior. Using a forward genetic screen two loss of function mutations in the *slit3* gene that caused increased nicotine place preference in zebrafish were identified. The translational relevance was identified using focused SNP analysis in one cohort of current or previous heavy smokers, one of current or previous lighter smokers, and one of the general population. This is the first report of a novel human functional polymorphism, identified using a forward genetic screen of adult zebrafish to uncover loci affecting a complex human behavioral trait. Taken together, these results provide preliminary evidence for a role for *SLIT3* in regulating smoking behaviour, and may be a useful target when designing tailored treatments to aid permanent smoking cessation in patients.

There are a number of ways in which the work can be continued, both in terms of SNP analysis on the families generated in the three-generation screen and in molecularly characterizing the *slit3* mutants.

7.1.1 The CPP assay

The first results chapter of this thesis explores the zebrafish conditioned place preference assay as a means of measuring drug-induced reward. It was decided that a 'biased' CPP would be used in which the animal is allowed to explore the apparatus during the baseline, and the exemplar they least prefer is the one that the drug is administered in and the one they most prefer is where they receive saline. This allowed it to certain that in the future population screen, fish were going to be selected that showed a shift in preference from one compartment to the other when conditioned to nicotine. To minimise habituation responses being misinterpreted as drug induced CPP, any fish showing a preference exceeding 75% for either compartment was removed from the analysis. The assay was tested using different classes of drugs that included stimulants, opioids and general anaesthetics. The drugs that were tested were (p=0.02), caffeine (p=0.01), nicotine (p=0.01), the opioid compound fentanyl (p=0.01), and PCP (p=0.03. The aim was to assess the validity of CPP in fish as a means of assessing the reinforcing properties of compounds, particularly nicotine. All five of the compounds produced a reinforcing effect comparable with other animal models. This high degree of correlation between results found in zebrafish CPP and mammalian self-administration and/or CPP assays instilled confidence that the developed protocol was robust and sensitive enough to pick up genetic variations that may effect a zebrafish response to the reinforcing properties of nicotine.

7.1.2 Mutagenesis screen

With the paradigm protocol established, progression to the 3-generation mutagenesis screen was possible. The subjects included in the experiment were all generated at the Sanger Institute, as part of the zebrafish mutation project. Founder line males were chemically mutagenized, introducing a SNP roughly every 300kb in their germline stem cells. The resulting progeny were then exome sequenced and outcrossed to generate the F2 lines. The resulting fish contained a number of artificially generated markers including known gene breaking mutations in coding regions. The first generation screen gave a normal distribution with mean change in preference of 0.14. The top five centiles had a 0.6 change in nicotine preference, with the lowest five showing an aversion to nicotine (- 0.4). Four fish from a Humbog line (Obtained from Stephen C. Ekker) were included in the screen as a positive control. Humbog fish have a Gaba-B receptor 1.2 (*gabbr1.2*) knock-out that has previously been shown to alter sensitivity to nicotine (Petzold et al., 2009). The *gabbr1.2* knockouts clustered at one end of the distribution, this demonstrated the assays ability to identify specific lines. The second generation showed a shift in preference for both low (mean = 0.17, $p = 0.03$) and high responders (mean = 0.05, $p = 0.01$) when compared with the previous generation, there was a large effect size when comparing the two groups (Cohen's $d = 0.89$). The third generation screen showed the high responder line to have a mean of 0.21 compared with a population mean of 0.01 in the low responders. There was a large effect size (Cohen's $d = 1.64$) indicating the difference in response to the rewarding effect of nicotine has been genetically maintained. The increase in effect size from the last generation indicates the two populations are becoming more genetically distinct with each generation. In the screened set of F3 fish a unique

strong or weak responding fish with siblings in the "average region" responding range could be homozygous mutant. Genotyping and establishing for which gene breaking mutations for which an outlying fish is homozygous might identify candidate recessive modifiers.

7.1.3 Identification of dominant mutation in first generation screen

Due to the nature of lines obtained from the Wellcome Sanger Trust, there remained the possibility of identifying knockout mutations of major effect that alter phenotype. The first generation screen identified a mutant line, which showed higher nicotine reinforcement than the population mean and clustered at the right of the distribution, as well as a line that clustered to the left arm of the distribution. The four individuals from this line were selected from an F3 generation containing a mixture of homozygous, heterozygous and wild type individuals suggesting the line contained a dominant mutation affecting the phenotype. Candidate genes from a list of known possible null mutations (obtained from The Wellcome Sanger Trust) were investigated in the QMhigh and QMlow lines. While no SNPs were shown to segregate in the QMlow line, in the QMhigh line a *slit3* mutation segregated with the observed phenotype. An independent line with a nonsense mutation in a similar region of the *slit3* gene was then acquired through the zebrafish mutation project and the same phenotype was observed indicating the slit3 mutation was causal as opposed to other linked artifacts in the lines genetic background. Quantitative PCR on embryos from a +/- incross of this *slit3* line showed there to be an up-regulation of nAChR alpha 5, presenting multiple mechanism by which a *slit3* knock-out may have and

affect on nicotine mediated reward processes. This chapter provided powerful evidence for mutagenesis screens of this type to be a useful translational model for investigating the genetic basis of human psychiatric disorders.

7.1.4 Identification of SNPs affecting smoking rate

As zebrafish are vertebrates with significant conservation of neural pathways thought to be important for drug-mediated reward (M. O. Parker et al., 2013) it was hypothesized that findings in fish could be used to predict loci in humans influencing smoking behavior. This hypothesis was tested on two cohorts of patients. The first cohort comprised of heavy smokers, with a diagnosis of chronic obstructive pulmonary disease (COPD) (n=272), and having amassed ≥15 pack-years. The second cohort comprised of lighter smokers (<15 pack years), with a diagnosis of asthma (n = 293). The dependent variables were initiation (only for Cohort 2), persistent smoking (current vs ex) and the number of cigarettes smoked per day (cigs/day). One SNP (rs12654448), was found to be strongly related to number of cigarettes smoked per day in the COPD cohort. No other smoking-related behaviors tested were predicted by any of the SNP. In the asthma cohort, 10 SNP significantly predicted cigs/day. We next validated the association of variants at the *SLIT3* locus with smoking behavior in one further cohort from the general UK population (without asthma or COPD; n=298). Four common SNPs between the asthma and general population cohorts were found, including rs7728604, rs7129099, rs17665158 and rs12515725. These were consistent in terms of the direction of effect between the cohorts. The SNP from the COPD cohort, rs12654448, significantly predicted smoking rate in the general

population, but not in the asthma cohort. However, in the asthma cohort, the SNP was significant at the uncorrected alpha level ($p = 0.04$). There was no SNP that predicted other smoking-related factors (persistent smoking and quitting).

7.2 Future directions

This screen is shown to be capable of identifying subtle nicotine seeking behavioral phenotypes as well as showing nicotine reinforcement to be a heritable trait in zebrafish. A gene underlying nicotine reinforcement was identified using this method and furthermore, was used to inform a human study of smoking behaviors. This presents powerful evidence for mutagenesis screens of this type to be a useful translational model for investigating the genetic basis of human psychiatric disorders. Ultimately, more precise information about genetic influences on the ability to quit smoking from these and previous data sets will aid in constructing predictive genetic quit success scores that could be implemented in a personalized approach to smoking cessation treatment. On top of this, there are numerous ways in which this body of work can be continued both as a means of identifying more genomic regions associated with nicotine addiction as well as characterising the mechanisms by which *slit3* affects nicotine mediated reward processes.

7.2.1 Admixture mapping of high and low populations

The first generation population screen was successful in identifying 1 gene of major effect, which also showed an association with smoking behavior in a human cohort. The selection screen continued for a further 2 generations, allowing the possibility of using additional genetic markers located in the genome to identify genomic regions being selected for in the context of the CPP paradigm.

Having demonstrated heritable variation, key loci can subsequently be identified using SNP based genome-association analysis of the families. As the mutation rate in the founder population is approximately 1 every 300 base pairs, the fish included in the screen carry a large number of unknown ENU-induced single nucleotide mutations (SNMs). The zebrafish genome is approximately 1.4Gb, so with a mutation rate of 1/300kb the founder fish will have had roughly 5000 mutations across the genome. Assuming even distribution of ENU-induced markers and an average chromosomal length of 100cM and 1 recombination event per chromosome, 95% of the chromosomes in the F3 generation will have had at least 1 recombination event and contain regions of ENU-mutagenised fragments with an average of more than 20 ENU SNMs per fragment. As the founder fish were outcrossed to the mapping line with a high density of naturally occurring SNP markers, SNMs and outcross SNPs can then be used to identify genetic regions linked to specific phenotypes using the principles of disequilibrium mapping.

Now that the lines of distinct (high and low) phenotypes have been established the parental F3 fish (that were fin-clipped before the first generation screen) can be sequenced to identify ENU markers that can be used for genome association analysis of the families. Once linked regions are identified, high-density SNP mapping and sequencing can be used to fine map any linked alleles. There exists a 200,000 SNP array at the Sanger Institute that is available for fine mapping once this stage is reached. Using this strategy it will be possible to identify linked genes of major effect as well as more context specific alleles of reduced effect. It will also be possible to identify both ENU induced SNM as well as linked naturally occurring SNPs.

In reality though, this inbreeding strategy needs to be discussed more critically in terms of whether it will be a viable strategy going forward. Much of the positive shift might have been created by inclusion of *slit3* heterozygotes. As such, it is likely that any other remaining genetic contributors will be relatively minor.

It may be more sensible to focus on the identification of strong dominant modifiers. The data that is already present from the low responders may have interesting candidate genes that could be identified by correlating missense mutaions with behavior. Information on these missense genetic varients should be available from the Wellcome Sanger Trust. It is also worth noting that it is unlikely it will be possible to continue inbreeding as due to the indeterminate way sex is determined in fish you are bound to run into genetic bottlenecks, with all male or female skewed stocks.

It would perhaps make more sense to screen further lines using the robust CPP assay designed here to determine loci with major effect on nicotine reward. Knock-outs of major effect in fish provide a good candidate to investigate in humans. It seems unlikely that a total knock-out in a zebrafish that conveys a relatively minor effect will prove to show significant associations of self-reported nicotine intake rates in humans. As such, trying to tease out these small associations through repeated selective breeding seems a waste of resources as well as time in terms of impact and results.

7.2.2 Molecular characterization of slit3 zebrafish embryos

There still remains further work that can be done to unravel mechanisms by which the slit3 may affect drug seeking. The qPCR results presented one possible mechanism in the up-regulation of chrna5, which may result in a greater number of presynaptic $(\alpha4\beta2)_2\alpha5$ assemblies which show lower rates of desensitisation to nicotine as well as a higher permeability to Ca^{2+} than the typically more prevalent $(\alpha4\beta2)_2\alpha4$. This could hypothetically leads to greater dopaminergic transmission. Alternatively, a nonsense mutation in the *slit3* locus may lead to increased drug seeking behavior through a neurodevelopmental role affecting path-finding and circuit formation. A number of molecular experiments could help shed like on a mechanism underlying the change in nicotine seeking in fish and smoking behavior in humans.

Fundamental to understanding the role of variations at the slit3 locus in affecting nicotine reward is to fully understand where the slit3 transcript is expressed in the brain. Slit3 is possibly involved in the repulsive action of the caudal midbrain that initiates the turning of growing fibres towards the rostral brain (Gates, Coupe, Torres, Fricker-Gates, & Dunnett, 2004; Holmes, Jones, & Greenfield, 1995); so you would expect to see a degree of expression in the midbrain.

It will also be possible to look at dopaminergic genes like *drd2* and *slc6a3* and compare expression levels in wildtype and *slit3* knock-outs. While there was no significant change in expression levels shown when using qPCR analysis, more subtle differences that may be present that may be visualized using staining. For example, small changes in *drd2* expression in regions such as the posterior tuberal nucleus (PTN) or the ventral and dorsal telencephalic nuclei (Vv and Vd) (Panula et al., 2010)

would impact upon zebrafish behavior in a CPP assay. Small changes in specific areas like the PTN may be masked when carrying out qPCR on whole empbryos. Also, due to pooling of embryo's from heterozygous in crosses, effects can be masked due to varying phenotypes within a pooled sample. Staining techniques as well as individual geneotyping of embryo's before carrying out RNA-Seq may provide a clearer picture as to what is occurring transcriptionally.

Similarly, while there was a significant increase in *chrna5* mRNA expression in embryos derived from a *slit3* +/- incross, it is difficult to determine what this change in expression means in relation to the phenotype without knowning how expression changes spatially. If the increase in chrna5 expression occurs predominantly in the PTN, it would give credence to the hypothesis that presynaptic $(\alpha4\beta2)_2\alpha5$ assemblies on dopaminergic cells is giving rise to an increase in nicotine mediated reward.

The effect of a *slit3* knockout on axon pathfinding can be investigated using antibody staining. Using tyrosine hydroxylase (TH) staining it would be possible to label dopaminergic cells bodies as well as their projections in the midbrain. In slit3 knockout zebrafish, it may be possible to visualize mesodiencephalic dopaminergic axons aberrantly crossing the midline due to lesser prevalence of repulsive cues from the midline (Kawano et al., 2003; Marillat et al., 2002). A combination of all these techniques and experiments would provide a clearer picture of the mechanisms behind this altered nicotine seeking, as well as an insight into why variations at the *SLIT3* locus affect smoking behavior in humans.

7.2.3 Closing summary

The aim of this study was to use forward genetic screening in zebrafish to identify loci affecting human smoking behavior. Two loss of function mutations were identified in the *slit3* gene that caused increased nicotine place preference in zebrafish. The translational relevance was confirmed using focused SNP analysis in one cohort of current or previous heavy smokers, one of current or previous lighter smokers, and one of the general population. This is the first report of novel human functional polymorphisms, identified using a forward genetic screen of adult zebrafish to uncover loci affecting a complex human behavioral trait. Taken together, these results provide preliminary evidence for a role for *SLIT3* in regulating smoking behavior, and may be a useful target when designing tailored treatments to aid permanent smoking cessation in patients.

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