Development of the Larval Zebrafish as a Genetic Model for the Nicotine Response

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

ANDREW MICHAEL PETZOLD

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

STEPHEN C. EKKER

AUGUST 2010

Acknowledgements

I would like to acknowledge the following people, without whom this work would not exist in its current state.

It is with my gratitude that I humbly thank my supervisor, Dr. Stephen C. Ekker. Without his enthusiasm, inspiration and guidance, my learning and research would not have reached to the heights that it has. Throughout my education, he has provided encouragement, sound advice and plenty of ideas-- I would have been lost without his help.

I am indebted to my committee members: Drs. Mark Thomas, Scott Farhenkrug and Jim Mickleson for providing me with advice and feedback through my time as a graduate student. A special thanks goes to Dr. Mark Thomas for his aid in the development of the nicotine behavioral screening assay, without which my research would not exist.

I would like to thank the many people who have aided me in the process of my research: Aubrey Neilsen for teaching me about the zebrafish and its care; Drs. Darius Balciunas and Sridhar Sivasubbu for their inspired teaching about transposon biology; Dr. Karl Clark for his guidance and advice; Dr. Brent Bill, Stephanie Westcot and Vicki Bedell for their contributions toward an environment of learning and entertainment within the lab and Mark Urban, Kim Skuster and Tammy Greenwood for their aid in technological aspects of my scientific work. Additionally, I would like to thank all others from the Ekker Lab past and present for providing feedback and training.

I am grateful to all others whom have helped me in my educational process including those in academic support at the Mayo Clinic and the University of Minnesota (especially Lisa Hubinger).

Lastly and possibly most importantly, I would like to thank my wife, Logan Petzold; my parents, Don and Aline Petzold; my aunt, Dr. Esther Sternberg; and my friends and relatives. They have provided me support and guidance throughout my schooling.

Dedication

This dissertation is dedicated to all whom have had their lives directly or indirectly changed from the use of nicotine.

Abstract

Tobacco use is predicted to result in over 1 billion deaths worldwide by the end of the

21st century. How genetic variation contributes to the observed differential

predisposition in the human population to drug dependence is unknown. The zebrafish

(*Danio rerio*) is an emerging vertebrate model system for understanding the genetics of

behavior. We developed a nicotine behavioral assay in zebrafish and applied it in a

forward genetic screen using gene-breaking transposon mutagenesis. We used this

method to molecularly characterize *bdav*/ *cct8* and *hbog*/*gabbr1.2* as mutations with

altered nicotine response. Each have a single human ortholog, identifying two points for

potential scientific, diagnostic, and drug development for nicotine biology and cessation

therapeutics. We show this insertional method generates mutant alleles that are

reversible through Cre-mediated recombination, representing a conditional mutation

system for the zebrafish. Additionally, we developed a conditioned place preference

assay for use with larval zebrafish. This assay allows for the perturbation of the

differences in genetic function between the physiological and learned response

representing one of the first associative learning based assays in the larval zebrafish.

The combination of this reporter-tagged insertional mutagen approach and zebrafish

provides a powerful platform for a rich array of questions amenable to genetic-based

scientific inquiry, including the basis of behavior, epigenetics, plasticity, stress,

memory, and learning.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

Section 1: The Importance of Studying Nicotine Addiction

Worldwide, over 5 million deaths attributable to tobacco-related causes occur annually

(See Figure 1 for graphical representation by category of cause of death). By 2030, over

8 million annual deaths worldwide will be attributable to tobacco use, of these deaths,

70% will occur in developing countries [1]. This number will eclipse 1 billion

cumulative deaths by the end of the 21st century [2]. In the United States alone, tobacco

accounts for one in every five deaths and at least 30% of all cancer deaths[3].

Additionally, of those who begin to smoke, nearly half will die prematurely from a

smoking-related disease[3]. The staggering number of people who are affected by

tobacco use, both daily and within their lifetime, has become drastic enough to inspire

the World Health Organization to declare tobacco use as a worldwide epidemic[2].

The realization of the depth of the tobacco epidemic has begun to impact government at

a state and local level. Since the 1990's local governments have began banning

smoking in public areas in an effort to reduce the use of tobacco and its related health

problems. Currently, over 71% of the population of the United States lives in an area in

which smoking is banned in workplaces, restaurants, or bars by law[4]. However only

41% of the population currently lives in an area in which all public areas are smoke-free

[4]. The removal of smoking has reduced the number of air pollutants within public
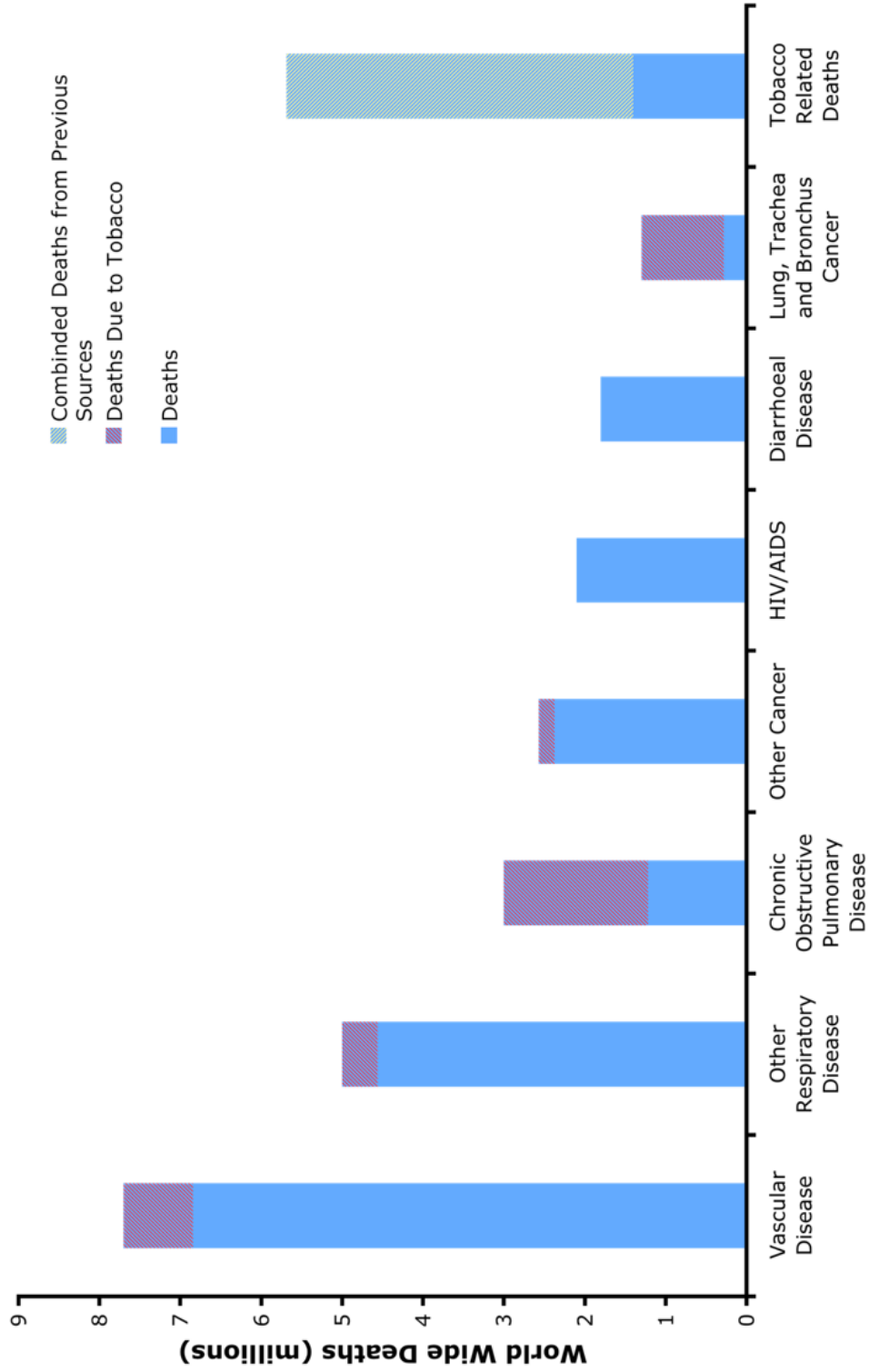
Figure 1. Graphical representation of the percentage of annual world wide deaths due to tobacco according to the World Health Organization MPOWER package.

spaces in these communities by upwards of 90% of pre-smoking-ban levels [5]. This reduction has been accompanied by an increase in the revenue of the smoke-free restaurants areas despite the fear otherwise [6]. The trend towards a smoke-free community, while encouraging does not address the lingering health problems caused by smoking [7]. Cigarette smoke contains over 4000 chemical compounds including: nicotine -- the addictive component of tobacco known carcinogens, particulates, and other harmful chemicals such as: carbon monoxide, formaldehyde, hydrogen cyanide, ammonia and formic acid [8]. The combination of these compounds has many adverse health effects both on the direct user and individuals whom are receiving the smoke second-hand. Current legislation has aided many communities in combating second-hand exposure to tobacco smoke. Although the number of smokers in developed countries is dropping[9], the majority of the annual deaths attributable to tobacco occur in middle to lower income countries [2]. With the staggering number of deaths attributed to tobacco exposure, use, and addiction, the need for an efficient and effective solution is of great importance.

Substantial progress has been made in the last 40 years in reducing the number of smokers in the United States, from 42% in 1965 to close to 20% in 2007 [10]. This has primarily been accomplished through education and reduced initiation of smoking as a whole [11]. Despite the reduction in the prevalence of smoking in the United States and other developed countries, it has been found that fewer than half of smokers achieve long-term abstinence. This is despite over 70% of smokers expressing a want to quit and over one-third of smokers making a serious attempt to quit. Unfortunately, the

majority of these quit attempts do not result in long-term tobacco abstinence [12]. Through meta-analysis of a variety of studies, researchers have determined that with the current state of the field using nicotine replacement therapies (NRT), the most effective current method of cessation is a combination of a biological and psychological intervention [12-14]. These meta-analyses have also shown that novel NRTs have shown greater effectiveness than the nicotine patch and lozenge alone [15]. Despite the effectiveness of intervention, whether biochemical or psychological, the lack of wide-scale use of smoking cessation interventions is largely due to the high cost and small-scale appeal of these interventions [16]. Development of a novel approach to smoking cessation would create opportunities to aid a number of these smokers by providing additional means of cessation.

Genetic influences at each stage of the addiction process have been documented in numerous studies within twins and families of smokers [17, 18]. Parallel human genetic studies have recently identified sequence variations near a nicotine receptor locus associated with a predisposition to lung cancer [19-21]. This association suggests that changes in nicotine response biology can play an important role in the major clinical outcomes of nicotine dependency. Additionally, recent consortiums of anti-smoking groups have banded together to combine efforts on examining the genetic component of smoking at a genome-wide level. These studies have identified a number of single nucleotide-polymorphisms (SNPs) that are associated with the number of cigarettes smoked in a day [22]. These strong associations have been reported to be located on Chromosome 15, in the same region as the CHRNA3-CHRANA5-CHRNA4 and

CHRNB4 loci [22-24]. These loci together help in forming the nicotinic acetylcholine receptor complex (NAChR), which plays a vital role in forming the neurological and physiological receptors within the nicotine addiction pathway [7]. While association to a genetic locus associated with the NAChR complex would seem to be beneficial, and possibly predicted, the results within these studies cannot account for the wide differences in nicotine responses among smokers. Additionally, recent studies comparing smokers and former smokers have identified SNPs within the dopamine β-hydroxylase locus, an enzyme that catalyzes the conversion of dopamine to norepinephrine, creating a greater reward response [23]. This recent finding confirms an earlier study that found variations in expression of dopamine β-hydroxylase correlated with the number of cigarettes smoked in a day [25]. This finding represents a potential novel gene within the nicotine dependence pathway, that can provide a route for future study and drug exploration. Though this research is promising, the potential for additional genes within alternative pathways must be examined to provide a greater success towards smoking cessation [26].

Section 2: Zebrafish as a model organism in behavioral studies in addiction

The relationship between genes and complex behaviors is not straightforward. Behaviors, such as learning and addiction, involve multiple genetic interactions accompanied by environmental influence. Traditionally, rodents, such as the mouse and rat, have been used as models for this research, but each has notable technical limitations when examining and manipulating genetics. Although much of the pioneering behavioral genetics used invertebrates such as the *Drosophila* as a model

organism [27], these animals develop with relatively simplistic neural networks when compared to vertebrates, limiting the complexity of behaviors that can be examined. With its relatively short development time, high fecundity and ease of genetic manipulation, the zebrafish presents itself as an optimal organism for behavioral research[28].

Since its first documented use in science [29], the zebrafish (*Danio rerio*) has found a niche in the study of development, embryology [30] and molecular genetics [31]. Due to the ease of genetic manipulation, the zebrafish quickly became the first vertebrate organism to be used in large-scale mutagenesis screen [32]. This chemical mutagenesis screen, conducted in Boston [33] and Tübingen [34], generated over 4,000 mutations and led to the identification of many genes, most focusing on vertebrate development. Since this pioneering effort there have been additional large-scale screens in the zebrafish using insertional mutagens [35-37], chemical discovery [38, 39], and antisense gene-silencing [40]. These large-scale screening efforts, accompanied with directed mutagenesis [41] and directed gene silencing [42], have increased the presence of the zebrafish as a model organism in the field of behavioral genetics. With millions of people worldwide affected by addiction to a substance, either legal or illicit, the apparent ability of the zebrafish to elucidate the genetic link between addictive behavior and genes is encouraging [28]. Recently, a number of studies have highlighted the use of the zebrafish in addiction research, with some even examining the genetic component of addiction.

The pioneering work done in regard to addiction in the zebrafish was outlined by Gerlai et al using alcohol as a stimulus [43]. This study described a number of simple assays, developed to examine the reaction of the subject to the addition of alcohol in differing concentrations. Specifically, the study focused upon locomotion, aggression, shoaling behavior, alarm response, place preference, and pigmentation in single adult fish. Aside from demonstrating that the zebrafish reacted in a predictable way to alcohol in a dose-dependent manner, this study also paved the way for other researchers to examine addiction in the zebrafish model system. The first study to focus on the genetics of addiction using the zebrafish examined the perceived reward caused by cocaine in wild-type and mutant zebrafish adults [44]. This study, using conditioned place preference as a model, identified two mutant lines, *dumpy* and *jumpy*, that were differentially affected by cocaine. With this work in place, additional studies examining the genetics of addiction using the adult zebrafish as a model soon followed including those studying alcohol [45], opiates [46], and amphetamine [47]. In general, these studies examined the reward pathway using 3-6 month old adult zebrafish in a conditioned place preference paradigm (CPP). This method uses a tank with distinct visual cues on either side of a tank. Test subjects are exposed to a drug paired to one of these unique environments, creating an association between the visual cue and the effects of the drug. A change in preference for one side of the tank can, in turn, be used as a measurement of the rewarding aspects of the stimulus within the individual. This testing paradigm can successfully be employed in examining a set of genetic mutants at an individual level. These studies used both characterized [45-47]and uncharacterized mutants [48, 49]. Although these studies placed an emphasis on examining the genetics of addiction and

the reward pathway with the zebrafish, the majority of these studies focused upon stimuli that affect a relatively small proportion of the world's population of drug-users, largely neglecting nicotine.

Until recently, nicotine has not been widely associated with addiction research in the zebrafish; however, it has been developed as a resource for the zebrafish research community by pioneering efforts demonstrated on a developmental level from Svoboda et al [50]. This research showed the influence that nicotine has on the developmental secondary spinal motorneurons. The zebrafish was successfully used in a behavioral study examining the effects of nicotine on learning in adult zebrafish by Levin and Chen in 2004. This study provided the initial use of zebrafish in a non-developmental role in nicotine research [51]. Since these pioneering efforts, the zebrafish has been used almost exclusively as an adult in behavioral studies and as a young larvae in studies examining the gross morphological developmental effects of nicotine [51-56]. Despite the research utilizing nicotine with the zebrafish there have been no prior studies focusing on the genetics of the behavioral response to nicotine. Additionally, many of the zebrafish-based genetic studies used individual adult zebrafish. This approach is beneficial on some levels but does not use the naturally shoaling animal [57] to its fullest and lacks the added power of a population-based study [58]. Traditionally, this was due to the method of generating and characterizing mutant zebrafish. These fish were exposed to a mutagen, either N-ethyl-N-nitrosourea (ENU) or gamma irradiation of sperm; making the detection of the mutation difficult and largely only possible through a recessive shelf screen or molecular genetics. With these

studies, the adults were either homozygous viable and needed to be screened

molecularly, or determined through breeding a homozygous lethal clutch, both requiring

a zebrafish of at least 8 weeks in age. To take advantage of the added benefits of

transparency, large offspring populations and rapid development time that is presented

within the larval zebrafish, a novel method of mutagenesis is required.

Section 3: Zebrafish as a tool for genetic manipulation and forward genetic screening

With the introduction of the ENU-based mutagenesis screen performed in Boston and

Tübingen in 1996 [32, 33], the zebrafish has been seen as a viable organism for use in

large-scale forward genetic screening. This reputation has been bolstered with

increased screening methods utilizing targeted-induced local lesions (commonly

referred to as TILLING) [59], insertional mutagens [35, 36] and non-RNA based

antisense technology [40, 60]. These screens demonstrated the usefulness of the

zebrafish for gene discovery, developmental, and genetic research finding over 2,200

mutations in a number of known and unknown genes [61].

The ability of conducting a forward genetic screen for novel behavioral-based loci in

larval zebrafish would require a simple method of mutagenesis that can distinguish

mutants form wild-type siblings and can ultimately be persistent through five days post

fertilization. This requirement for a lasting screen eliminates the use of a transient

molecular knockdown method, such as the morpholino, due to the removal of the

insertion through natural cell regulation[42, 62]. Although a powerful tool for

mutagenesis, the high number of genes affected through ENU mutagenesis and the lack of a tag, either expressional or molecular, within the mutated gene raises the difficulty of determining the gene that is mutated. This is avoided with the use of an insertional mutagen such as those used within the retro-virus insertional screen [35] and Gene-Breaking Transposon Screen [37]. However, the increased difficulty of molecular genetic screening (ie through the use of a Polymerase Chain Reaction) involved to identify mutants in both ENU mutagenesis and retro-viral insertional screen, makes larval screening impractical. This impracticality arises due to the requirement of using individual zebrafish with molecular identification occurring after testing. Individualization also removes the zebrafish from their naturally occurring shoals [57] as well as removing the advantage of using a population to examine variation of behavior within a diverse genetic background [63]. To facilitate these requirements, the Gene-Breaking Transposon (GBT) is beneficial. This insertional mutagen provides for a permanent molecular and expressional fluorescent tag distinguishing the mutant from its wild-type siblings. Additionally, with the use of a combination protein-trap/gene-breaking trap, the GBT technology also allows for an expression-biased screening approach [64].

First outlined in Sivasubbu et al[37], the gene breaking transposon technology has provided the ability to expressionally tag a locus while concurrently mutating the locus. This occurs by harnessing the power of a semi-random insertion event integrating a polyadenylation (poly(A)) trap along with an expression vector[37]. In initial versions of the GBT, the *Sleeping Beauty* transposon was used to introduce a poly(A) trap into

single cell zebrafish embryos at a genome-wide level. This was accomplished by

creating a vector containing minimal *Sleeping Beauty* inverted-terminal repeats flanking

a splice acceptor followed by a polyadenylation signal or a gene-boundary element

(ocean-pout terminator), a promoter, a fluorescent protein and finally a splice donor. In

function, this is designed to hijack the splicing of the gene in which the construct

landed, stop the endogenous transcript with the poly(A)/boundary element, and tag the

fish as a genetic mutant by utilizing a promoter followed by a fluorescent protein. This

fluorescent tag, due to lack of a poly(A), must be stabilized using the endogenous

gene's poly(A) signal, removing the occurrence of a tag within a pseudo-gene or non

gene. Additionally, the continued transcription of the 3-prime end of the product

following the poly(A) signal allows for an easier method of gene-finding using the

known sequence of the fluorescent tag combined with the endogenous transcript using

3-prime RACE and other molecular techniques [65]. Following this initial use within a

gene finding application [37], the GBT underwent a series of transformations to allow

for added functionality and gene transfer. These innovations included switching to the

teleost-derived, *hAT* based *Tol2* and *miniTol2* transposons for increased transposition

and cargo capacity[66], the addition of *LoxP* enzyme recognition sites just inside of the

transposon ends to facilitate reversion [64]and addition of a 5-prime protein trap[64]

through the addition of an AUG-free mRFP immediately following the splice acceptor.

When in-frame with the endogenous transcript, successful integrations produce a gene-

specific mRFP expression, allowing for further characterization and categorization

based on expression pattern. Additionally, this mRFP, when appended as a fusion

protein, to a signal sequence, would be exported from the cell, collected by the kidney,

and concentrated allowing for further gene discovery (See Figure 2 for a graphical

representation of the combination trap design also, Clark et al. in press). If the

combination trap (named GBT-RP2), lands in frame within an intron of an expressed

gene it will produce: a trace amount of the endogenous gene product, as well as an

mRFP-fusion protein expressed in the location of the endogenous protein as well as the

kidney if secreted, and an independent mutagenesis tag with a continued transcript

fusion that is untranslated (bottom product in Figure 2; for a specific description of the

Gene-Breaking Transposon, see Appendix A). The function of mutation tagging

included within the GBT construct facilitates larval sorting of mutants which is not

possible with other zebrafish mutational methods. This larval based sorting facilitates

the use of GBT mutant lines for forward genetic, dominant screens for behavioral

mutants.

This document aims to demonstrate the utility of the larval zebrafish as a tool to help

elucidate the genetic component of the nicotine response. Because tobacco addiction is

a complex behavior with many discrete processes, we have developed assays for

specific components that comprise the addictive response. These assays take advantage

of the GBT to allow one to conduct a genetic screen for genes required within these

biological steps. A behavioral response assay will be outlined that has been within a

forward genetic screen in conjunction with the GBT to discover new genes that are

involved in the nicotine response pathway. The results from this exemplary screen will

also be examined showing a simple implementation of the assay described as well as

highlighting novel genes involved in the nicotine response pathway. Additional
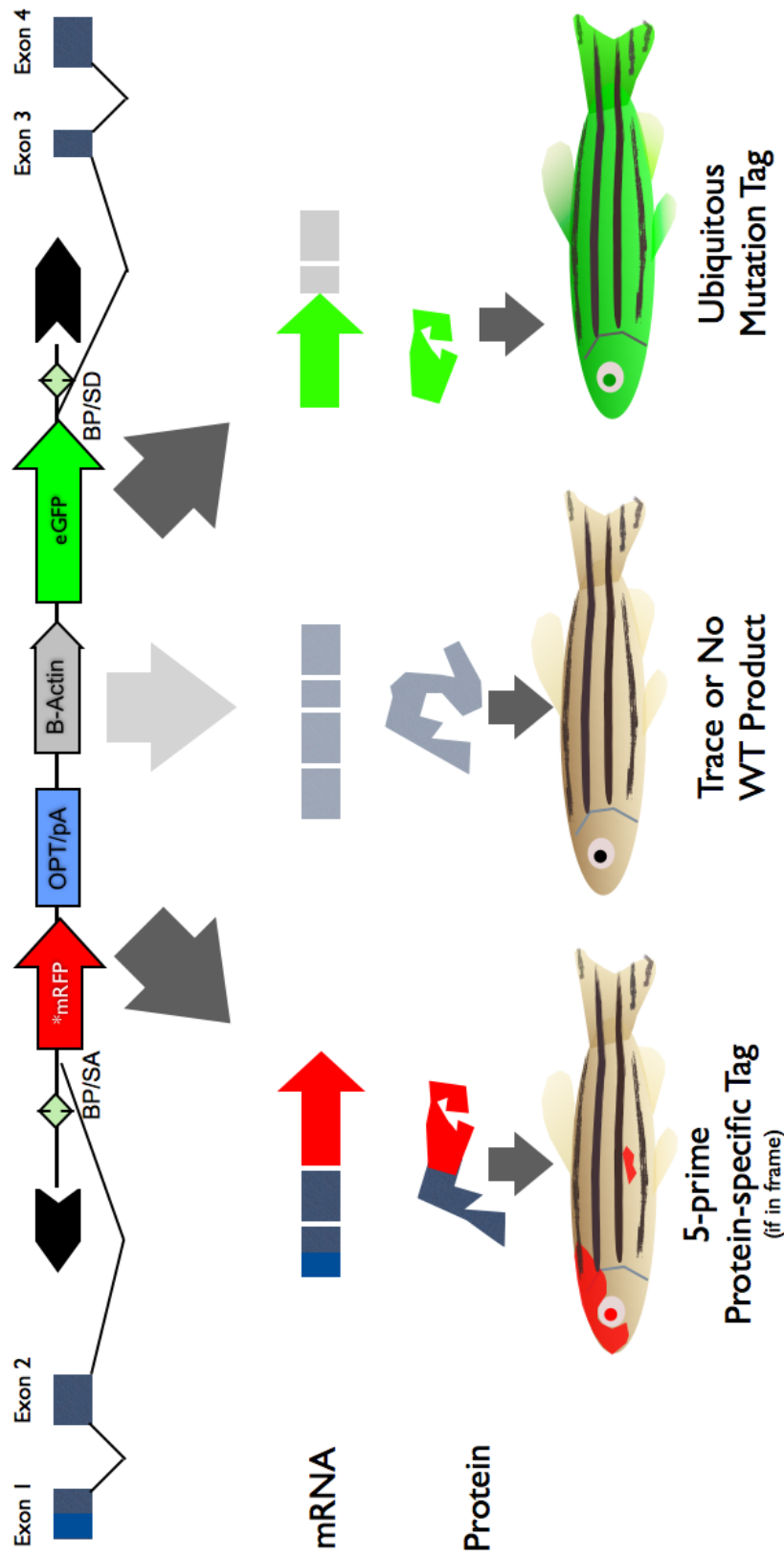
Figure 2. Graphical representation of the mechanism of the Gene-Breaking Transposon. The transposon lands within a transcriptional unit, hijacks the endogenous gene splicing using a branch-point/splice acceptor (BP/SA). If in frame, endogenous splicing creates a truncated protein fused to a transcriptional start site-free monomeric-Red Fluorescent Protein (mRFP) making translation only occur if in the orientation and reading frame of the tagged transcriptional unit. A putative boundary element with full-transcriptional stop follows (OPT/pA). Additionally, a strong promoter (β-Actin) drives an enhanced Green Fluorescent Green Protein (eGFP) expression throughout the fish only if stabilized by an endogenous polyadenylation signal. The remaining gene product is transcribed but not translated. The final product is a truncated, location specific mRFP signal, little or no wild-type product and a ubiquitous eGFP signal. (Clark et al. in preparation)

13

assays will also be described to aid in the further development of the larval zebrafish as

a tool for studying modifiers to the genetic component of the nicotinic response.

Chapter 2

Development and Implementation of a Behavior-Based Assay for Nicotine

Response in Zebrafish

Portions of this chapter have been previously published in Petzold et. al, PNAS, 2009[64].

Section 1: Introduction to Locomotion and Behavioral Sensitization as a Model for the Nicotine Response

Our current understanding of behavior and more specifically the addictive behavior has come from extensive pharmacological model organism research. This research, for example, has led to the identification of the central mechanisms of neural networks and the signaling cascades involved within these pathways [7]. This has largely been accomplished by combining extensive reverse genetic studies within the mouse model system with alternative methods of examining the impact of the drug upon the mammalian model system [67, 68]. This approach has focused upon the examination of the rewarding aspects of nicotine as a drug in attempt to explain the vast behavioral differences in response to nicotine in humans [69].

Numerous studies, in a variety of model organisms from *C. elegans* [70]through rodents [71], have been shown to display an increase in motor stimulant reward following a

repeated exposure to a drug of abuse. This predictable response has been coined

"behavioral sensitization" referring to the change of behavior based state upon the

repeated exposure to a psycho-stimulant drug of abuse [72]. Additionally, this increase

in locomotor activity has been implicated in the development of drug. This link to

addiction provides a potential relevance to continuous self-administration in animals

and drug abuse in human addicts with a possible link to changes in motivation [72].

Although a link appears to exist between sensitization and drug abuse, it is important to

note that the two phenomenon are not equivalent, but can produce predictive

results[73]. It is because of these potential predictive qualities of sensitization that the

mechanism in which behavioral sensitization functions has been greatly studied,

however, a relative paucity of genetic research exists linking these mechanisms to

specific genetic loci. It has been shown that a number of genetic pathways could play a

role within the development of a sensitization to nicotine [74-76]. However, the

correlation between these genetic differences only account for a small sub-section of the

potential genes involved within the nicotine sensitization mechanism and an even

smaller subset of the genes involved within the nicotine addiction pathway as a whole

[68]. This showcases the need for a better exploration of the genes involved in the

mechanism of the nicotine behavioral sensitization pathway.

Section 2: Wild-type response to nicotine in larval zebrafish

The use of nicotine within the confines of the zebrafish model organism is not a novel

concept; for years the teratological affects of nicotine have been examined. These

studies set a precedent for the confines of the behavioral assay that we were proposing.

It was found that nicotine, when given to a larval zebrafish at a dose of 33μM at 22 hours post fertilization (hpf) showed reduced growth, body axis deformities and early death among other developmental deformities [50, 56]. Additionally, these nicotine exposed embryos showed a differential in touch response, becoming effectively paralyzed after 20 hours of exposure (42 hpf) [50]. This reduction in touch response has been attributed to a delay in motorneuron differentiation. These studies highlighted the developmental effects of a chronic exposure to nicotine, they did not examine the specific effects that nicotine has upon behavior, nor did they examine the acute effects of nicotine. Studies that examined these effects focused upon adult zebrafish, specifically of breeding age and older [77]. To elucidate the behavioral effects of nicotine upon the post-hatching, larval zebrafish (54 hpf-adult), we assessed locomotor activity by analogy to prior work with rodent models [72, 78, 79]. Locomotor activity (Figure 3A) was measured using a digital imaging station in a time window where, 30 seconds following a stimulus of water, approximately 15% of a testing population moved (Figure 3A, yellow framed images; yellow lines and bars in all graphs in Figure 3; Description in Chapter 4). Locomotor activity was assessed at different nicotine doses; movement as a function of added nicotine is shown in red bars and graphs in Figure 3. Note the characteristic 'inverted-U' shape of the dose-response curve [80] where higher doses of nicotine result in reduced, rather than increased, movement (Figure 3B). The attenuation in locomotor effects at high doses is known to occur in rodents and depends on the particular complement of nicotinic acetylcholine receptor types activated by these doses [80]. Based on this dose response curve, we selected ten micromolar nicotine for subsequent experiments (box, Figure 1B). Next, we tested
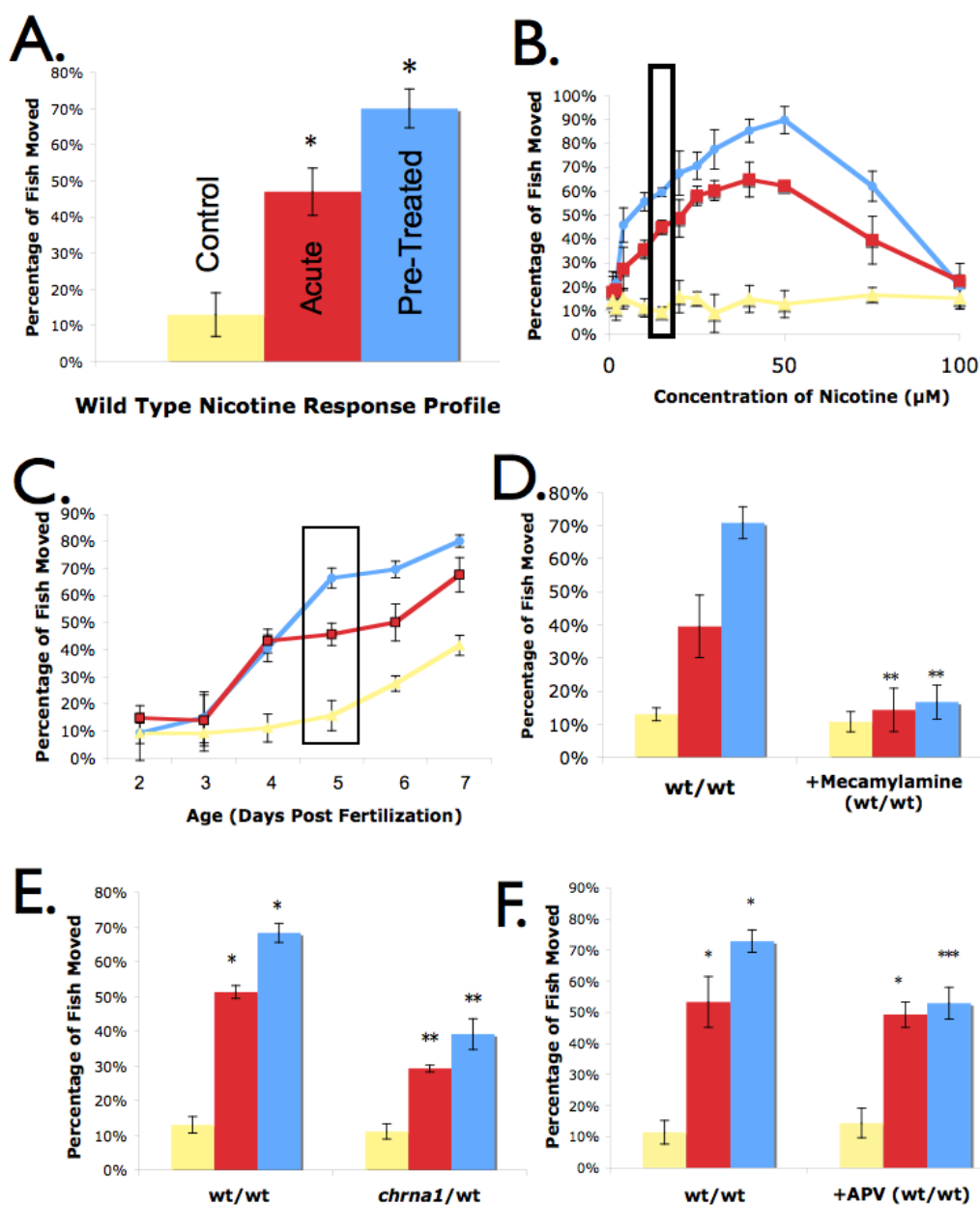
Figure 3. Nicotine response profiling of zebrafish larvae. A. Analysis of the movement of larval zebrafish produces a nicotine response profile of control movement (yellow) acute response to nicotine (red) and the sensitized response to nicotine (blue). All movement rates were determined as the percentage of the total population that changed position within a 1.25 second time window (see Chapter 4 for details). B. Dose-response of 5 day post fertilization (dpf) zebrafish to nicotine. Zebrafish show an increase in movement for doses from 2.5-50μM nicotine. The greatest difference in movement between acute and pretreated fish occurs in doses between 10 and 20μ; the dose used for subsequent work (10μM) is highlighted by a black box. C. Zebrafish respond to nicotine at 4 dpf but not before. Zebrafish can be sensitized to nicotine at 5 dpf and not before (highlighted by black box). D. Mecamylamine, a central nervous system specific nicotinic competitive inhibitor, quenches the overall nicotine response when added before the testing dose of nicotine without affecting the basal locomotion rate in the absence of nicotine. E. *chrna1* heterozygous fish exhibit a reduced nicotine response when compared to their wild-type siblings. F. APV, an NMDAR competitive inhibitor, quenches the development of the sensitization response when added concurrently with initial nicotine dose. *, $P > 0.05$ when comparing to control or acute. **, $P > 0.05$ when comparing to corresponding treatment group. ***, $P > 0.05$ when comparing to control, but not acute. Also $P > 0.05$ when comparing to the corresponding treatment group.

multiple developmental time-points, demonstrating a functional nicotine response in four day-old but not three day-old zebrafish (Figure 3C).

One additional characteristic of the behavioral response to nicotine is sensitization – an increase in response upon prior exposure to nicotine; the sensitization response is shown in blue lines and bars in Figure 3. As demonstrated in Figure 3A, fish that had been previously exposed to nicotine become sensitized, yielding a marked increase in activity. This level of sensitization is comparable to that noted in rodents [81, 82]. Five and six day-old but not four day-old zebrafish were capable of being sensitized (Figure 3C). This demonstrates that the development of competency for sensitization was delayed compared to the ability to respond to acute nicotine exposure.

To maintain confidence in our assay, we assessed whether this locomotor response is specific for and subject to perturbation of nicotine receptor signaling in zebrafish. First, we examined the effect of mecamylamine, a nicotinic receptor antagonist [83]. Mecamylamine administered to the embryos four hours prior to nicotine treatment blocked locomotor activation but did not change the basal level of movement of the fish (Figure 3D). Second, we took advantage of the pre-existing alpha polypeptide 1 nicotinic cholinergic receptor mutation (*chrna1*; [84]). Compared to their wild-type siblings, *chrna1* heterozygous animals demonstrate reduced total (both acute and sensitized) locomotor activation due to an initial exposure and to repeated exposures (Figure 3E). This altered response could be due to fewer total nicotinic receptors present in heterozygote animals. We cannot exclude the possible contribution of a

neural circuitry change from reduced *chrna1* in these heterozygote animals. The zebrafish thus represents a new vertebrate system for genetic testing of nicotine response.

An emerging theme in the study of the neural substrates for initiation of behavioral sensitization is the role for glutamate receptor subtypes, in particular the NMDA-type receptor (NMDAR). NMDAR inhibition blocks the development of sensitization in rodents [85, 86]. The co-administration of nicotine with the competitive NMDAR antagonist APV prevented the development of nicotine sensitization (Figure 3F). To further examine the validity of this increased locomotor activity as a behavioral based sensitization, rather than a response to noxious or foreign stimuli, a variety of stimuli were administered to the zebrafish pre-exposure to nicotine. These stimuli, including allyl-isothoicyanate and noxious temperatures, produced acute responses upon administration similar to those previously reported [87] (Figure 4A). However, the stimuli did not induce a sensitization to nicotine (Figure 4B). The ability to create a sensitization response to nicotine within the larval zebrafish allows for a robust locomotion based population response to nicotine to be determined, allowing a forward genetic, population-based screen to be performed.

Section 3: Screening GBT fish

To effectively process a large number of mutant zebrafish an efficient screening paradigm needed to be developed. In its development, we used the previous research in zebrafish insertional mutagenesis [37] to design a screening paradigm that also took
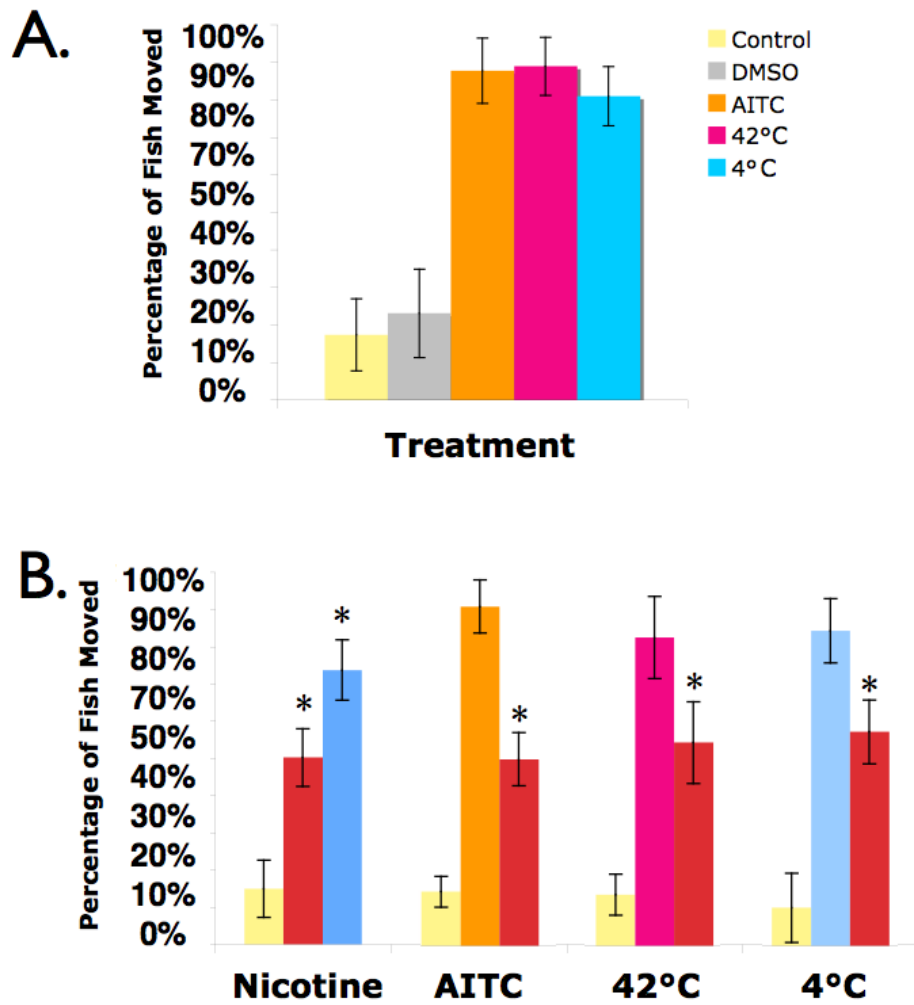
Figure 4. Response of larval zebrafish to noxious stimuli. A. Zebrafish at 5 dpf respond to allyl isothiocyianate (AITC), hot and cold temperatures as previously described [25]. B. A noxious stimulus does not induce an increased locomotor response to a challenging dose of nicotine, however an initial dose of nicotine does.

advantage of the fluorescent tagging supplied by the GBT. After zebrafish mutant

embryos are injected with a cocktail of transposase and GBT-construct, they are raised

and expanded into genetic lines (Figure 3, top half). To facilitate the use of a single

locus and to aid in determining expressed insertional copy number, these fish are out-

crossed to a wild-type fish (Figure 5, F1 line). Because of the Mendelian inheritance of

the transposon, an out-cross of a single insertional line of fish would create a

heterogeneous population of tagged mutant fish and wild-type siblings. These fish are

sorted with aid of the fluorescent-tag. Sorting facilitates examining the difference in

nicotine response of the fluorescent-mutant larvae and their wild-type siblings (Figure

5, F2 line). For each expression tagged candidate mutant locus, we initially examined

two aspects of their behavior. First we eliminated any loci with changes in the basal

movement rate to avoid mutations that overtly altered the mechanics of swimming.

Next we examined the differential nicotine response. This was obtained by directly

comparing the response of the expression-tagged larvae with their wild-type siblings

(blue bars, Figure 6). Once a number of lines are tested, a general background variation

can be determined, allowing for a greater examination of changes in nicotine response.

With the addition of the protein-trap found in the GBT-R14, GBT-R15 and combination

trap vectors (Appendix A), a biased approach is able to be taken, with a preference to

lines expressing in the neural region (Figure 6B).

By examining both the biased and unbiased responses of mutant lines to nicotine, lines

that significantly differed from the general background variation (Figure 6; stars) could
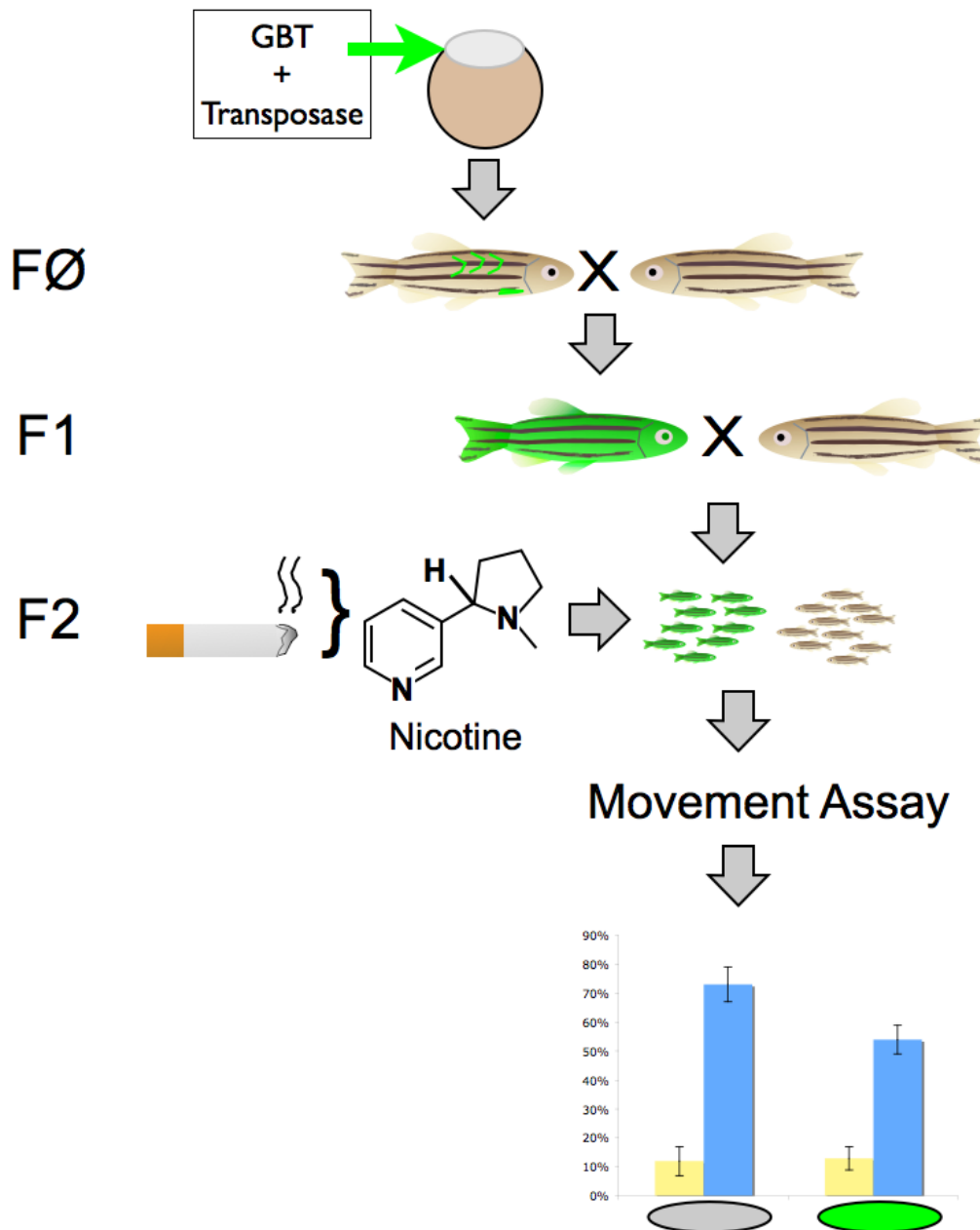
Figure 5. Screening paradigm for behaviorally-based mutants using the gene-breaking transposon. Standard transposase-mediated transgenesis protocols [26] generate a pool of mosaic F0 founder animals. These are outcrossed to generate stable transgenic F1 animals that are then outcrossed to generate mixed clutches of wild-type and mutant siblings. Mutant animals are segregated from their genetically wild-type siblings using fluorescent tags in these GBTs (GFP fluorescence is diagrammed as noted in the GBT-P9, PX and RP2 screens; for the biased GBT-R14, R15 and RP2 screens, red fluorescence was used to sort). Nicotine response profile is obtained on each subpopulation (fluorescently tagged vs. wild-type). Nicotine was administered at the standard dosage of 10 μM.
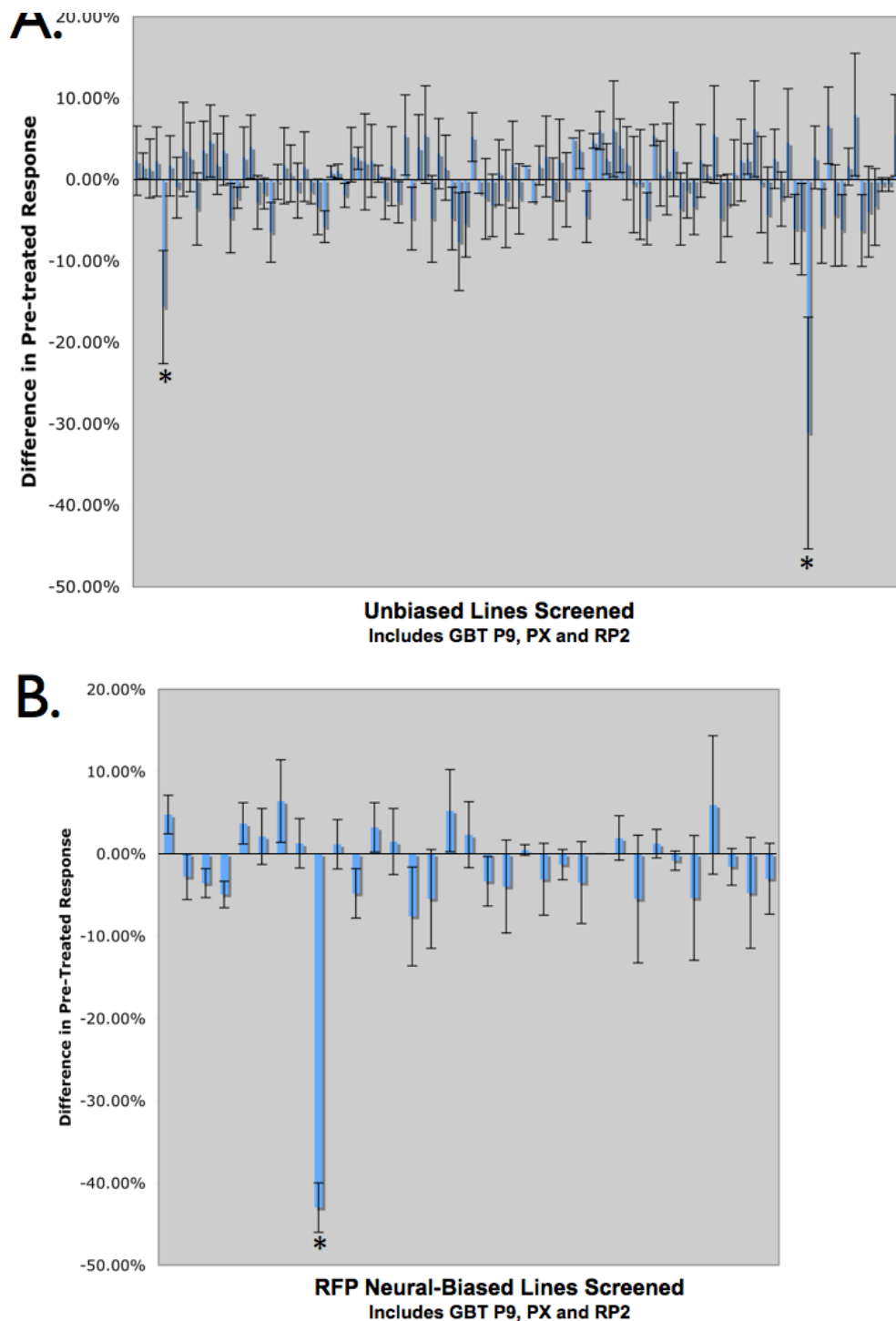
Figure 6. Differential nicotine response profile between wild-type and fluorescently tagged siblings. Response bars are derived by subtracting the pretreated response of fluorescently tagged GBT-mutants from the pretreated response of their wild-type siblings. This analysis identified three nicotine response mutants (*, $P << 0.05$), bette davis (*bdav*) and (* $P << .05$) yul brynner (*yulb*) through an un-biased screen representing an estimated 235 screened loci (A) and (*, $P << 0.05$) humphrey bogart (*hbog*) through a biased mRFP based screen of an estimated 58 screened loci (B).

be easily tested in more depth to determine the full nicotine response range as well as

the full nicotine response profile. This nicotine response profile includes the

background level of movement (signified by a yellow bar), the acute response to

nicotine (signified by a red bar) and the sensitized response to nicotine (signified by a

blue bar; wild type response shown in Figure 3A). Comparing the full nicotine response

profile of the fluorescent-tagged mutant with that of their wild-type sibling allows an

inquiry into the category of the response (Figure 7: Types of response). Possible

mutants could show either increases or decreases over the normal nicotine response

(Figure 7; Category I) within either of the three treatment categories. A change in the

basal level of movement (Figure 7; Categories II and III), would show changes in all

three categories due to the changed liveliness of the larvae as a whole. Changes in

overall nicotine response will produce a linear shift of both the acute and sensitized

levels (Figure 7; Categories IV and V). A change in sensitization level alone would be

seen as a single change in only the sensitized fish, not the acute or control (Figure 7;

Categories VI and VII). From this point, we can also prioritize any mutants found based

upon their nicotine response profile, placing mutants that show a difference in nicotine

response but not in overall movement at a higher priority than movement based

mutants. This distinction also elucidates the need for a consistent measure of basal

movement rate of the zebrafish and the need for a basal movement rate higher than 0%.

In analyzing the mutant lines highlighted by the screening process, three were

determined to have differences in nicotine response profile. These mutant lines were

named for celebrities who suffered due to tobacco-related cancers: *bette davis (bdav)*,

humphrey bogart (*hbog*) and *yul brynner* (*yulb*).
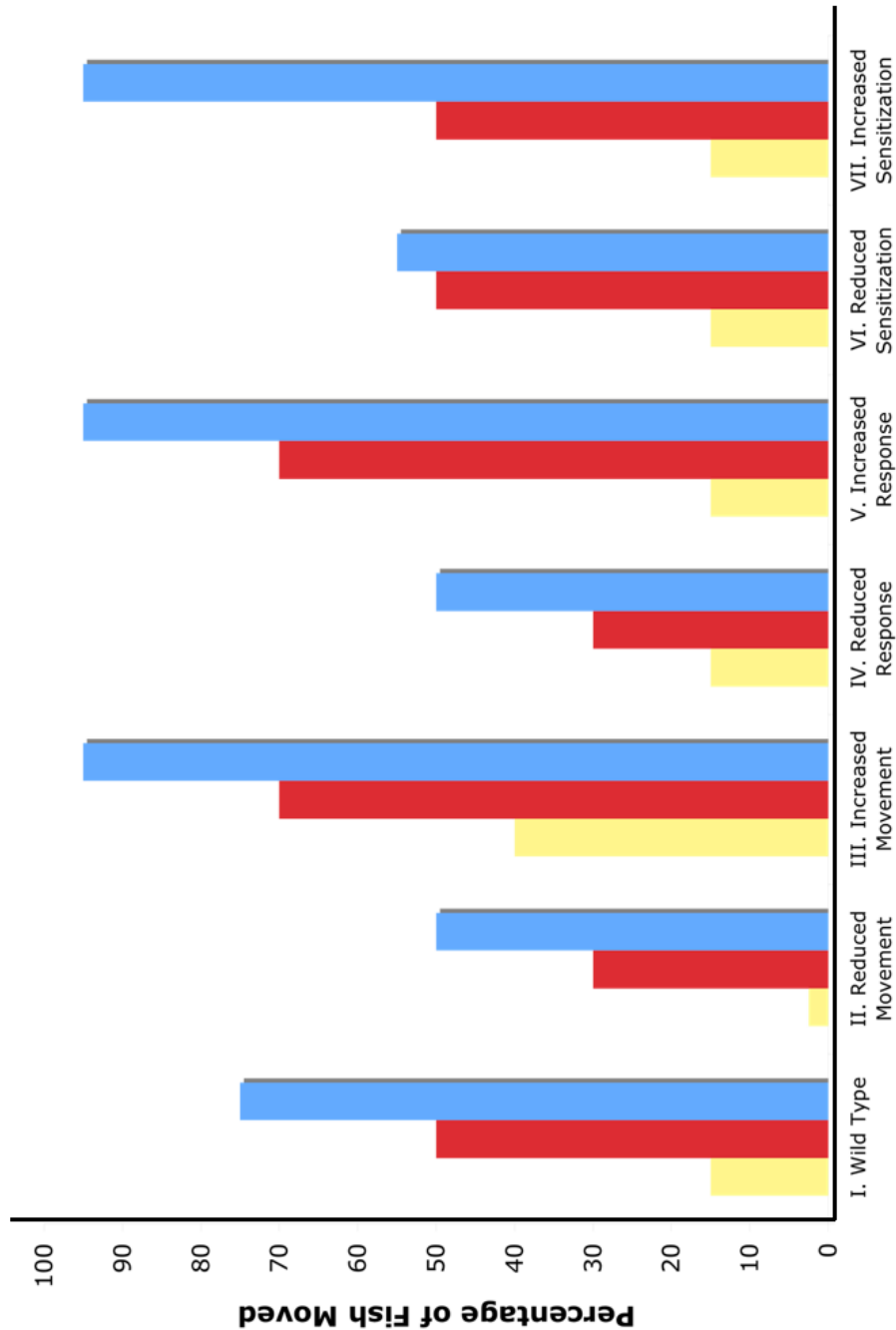
Figure 7. A range of differences in nicotine response within mutant backgrounds is possible. A wild-type response to nicotine (I) can be altered by either changing the entire response or by altering the sensitized response. A movement-deficient (II) or hyperactive (III) zebrafish would produce a shift when compared to the wild type response with a noted change in the basal level of movement (yellow bar) compared to wild-type. A decrease (IV) or increase (V) in total response to nicotine would produce similar ratios of movement to that found in the wild type response, but would be shifted in amplitude. A reduction (VI) or increase (VII) to sensitization would appear normal with an acute response (red) but show an altered sensitized response (blue).

Section 4: *Bette Davis*

In the GBT-P9 screen, the initial pool of GBT-mutagenized fish contained multiple

expressed GBT chromosomes; upon outcrossing and subsequent selection, we

established a zebrafish line with only a single expressed GBT whose offspring exhibited

a markedly reduced nicotine response profile linked to GFP expression from the GBT

(*bdav*; Figure 8A). Interestingly, we note that the reduction in the total (acute and

sensitized) nicotine response of *bdav* heterozygous fish is comparable to that noted in

the cholinergic receptor mutant, *chrna1* (compare to Figure 3E). In contrast to the

reproducible differences in the nicotine response, *bdav* mutant fish react normally to

other tested behavioral stimulatory processes including locomotor activation due to

external physical stimulation, to the noxious chemical AITC (mustard oil) or to extreme

temperatures (Figure 8B). This noted altered nicotine, but normal stimulatory response

profile, in *bdav* animals does not exclude yet-to-be determined effects from other

physical or pharmacological stimuli (see more complete discussion below). The onset of

the reduced nicotine response in *bdav* heterozygous fish is also not delayed compared to

their wild-type siblings (Figure 8C).

The transposon-based molecular tag was used to follow genetic linkage over six

generations to date, demonstrating dominant Mendelian inheritance. Molecular analysis

identified a single, sense strand GBT-P9 insertion in *bdav* fish in the zebrafish

Chaperonin Containing TCP1 subunit 8 (*cct8*; Figure 8D*)*. RT-PCR analysis

demonstrates this GBT insertion results in reduced wild-type *cct8* transcript levels in
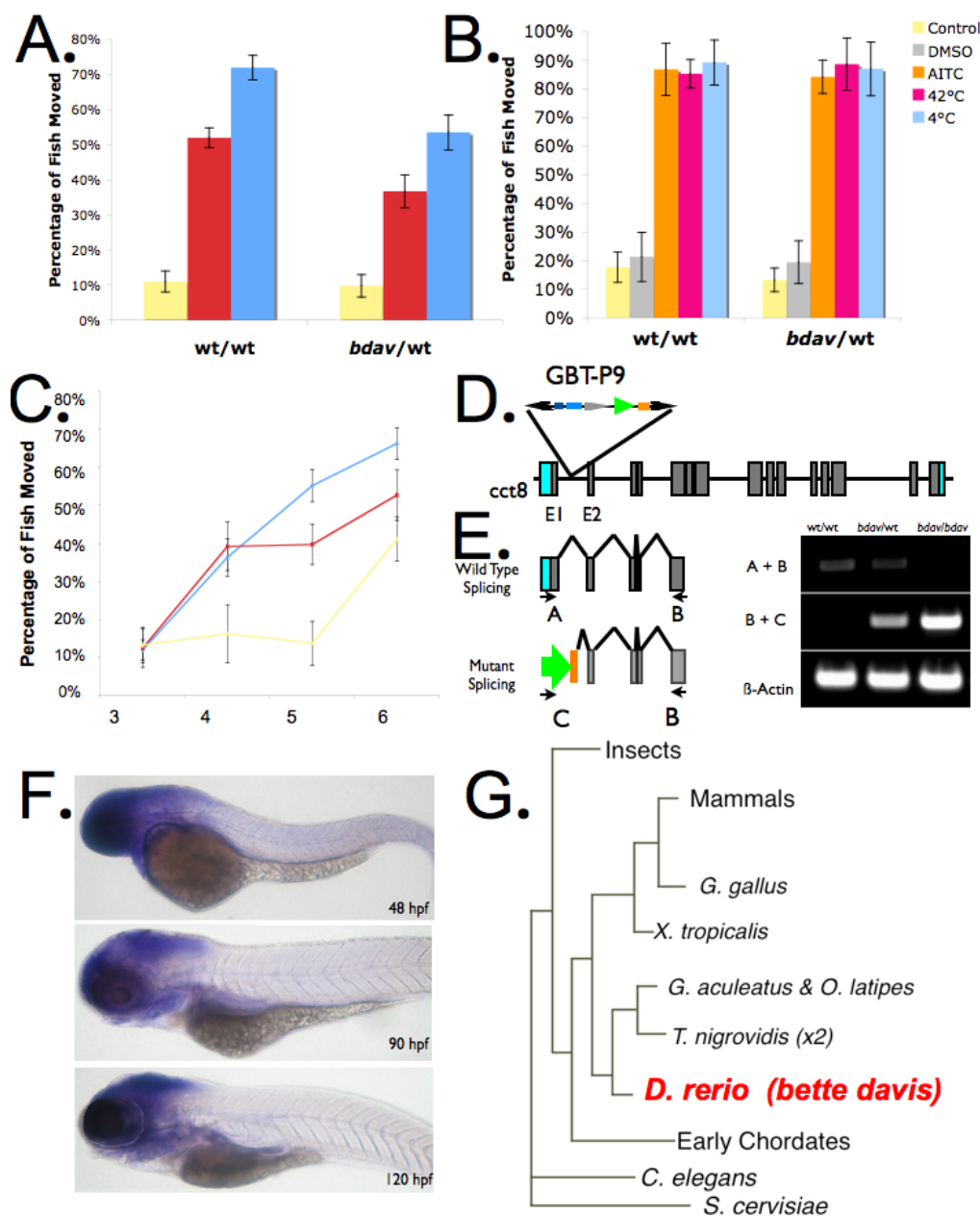
Figure 8. *bette davis (bdav)*, **a GBT-P9 linked mutation in** *cct8* **shows a reduced response to nicotine.** Animals with altered nicotine response profile A. harboring only a single GFP locus were isolated and propagated for six generations formally isolating the *bdav* nicotine response locus. Nicotine was administered at the standard dose of 10 μM. B. *bdav* heterozygous fish show no difference in response to allyl isothiocyanate or extreme temperatures when compared to their wild-type siblings. C. *bdav* heterozygotes show a reduced response to nicotine through development. D. The single expressed GFP locus is encoded by a GBT integration in predicted intron two of zebrafish chaperone containing protein 8 (*cct8*). E. RT-PCR analysis of cct8 transcript in wild-type, heterozygous, and homozygous *bdav* embryos. Primers in exons 1 and 5 were used to detect wild-type transcript of the cct8 gene. Primers in exon 5 and the GFP portion of the transposon were used to determine expression of transposon mRNA. RT-PCR using primers in -actin were performed as an internal control. F. Sagittal imaging of a whole mount in situ hybridization for *cct8* at 2 dpf (top) through 5 dpf (bottom) shows high levels of neural expression. G. Phylogenetic analysis (Ensembl) indicates cct8 is an evolutionarily ancient gene and is encoded by a single ortholog in humans and other mammals. *, P < 0.05 when comparing to control or acute. **, P < 0.05 when comparing to corresponding treatment group.

bdav heterozygous animals and a greatly attenuated level of wild-type *cct8* RNA in *bdav* homozygous animals (Figure 8E). *cct8* RNA expression is found in specific neural tissue during embryonic and larval stages of development (Figure 8F) similar to the pattern previously reported [88].

Traditionally viewed as a protein involved in the regulation of extreme temperature (heat shock [89] and cold shock [90]), the Chaperonin Containing TCP1 complex (cct) is viewed largely as a regulator of protein folding [91]. This evolutionarily conserved protein complex (ontology of *cct8* found in Figure 3G) has been shown to be largely necessary for actin and tubulin folding within in eukaryotes ranging from yeast through humans. These genes, specifically the *cct8* subunit have been implicated as being significantly sensitive to psychoactive drugs including the selective serotonin reuptake inhibitor paroxetine (Paxil, Seroxat) [92]. Despite these findings, the identification of cytosolic chaperonin subunit that plays a role in altering the nicotine response is intriguing. Differential binding of the individual subunits of the cct complex [93] could alter protein kenetics of the nicotine receptor as has recently been observed with other binding processes in work with cultured neurons [94]. Additionally, *cct8* specifically has been shown to be upregulated in the prefrontal cortex after exposure to nicotine in rats further suggesting a role within the human nicotinic response [95]. A determination of the specific role that *cct8* and other protein folding proteins play within the nicotine response could bring forth an additional pathway to examine as an intervention point within the nicotine response, however, additional research is required.

Section 5: *Humphrey Bogart*

We added three technical advances for the GBT-R15 screen (Appendix A). First, we used the miniTol2 transposon vector [96] to simplify the molecular analysys of the tagged loci and to facilitate the addition of complex genetic elements in subsequent GBT vectors. We then modified the critical transcriptional termination cassette with a red fluorescent protein (mRFP) reporter that lacks an initiation codon requiring fusion to the nascent peptide to yield information regarding temporal and spatial regulation of the trapped locus. Third, the core mutagenesis module was flanked by loxP sites for reversion via Cre-mediated recombination as formal proof of causality.

GBT-R15 mutagenized fish were screened for mRFP expression before subsequent nicotine response profiling with the goal of enriching our screening efforts for genes more likely to be involved in neural responses. Thus, unlike the largely unbiased GBT-P9-based screening approach, the GBT-R15 screen allows for a pre-selection screening step. *hbog* fish display neurally restricted mRFP expression and mRNA localization (Figure 9A; Figure 9B). *hbog* heterozygous fish exhibit a strong reduction in the nicotine response profile (Figure 9C). Homozygous fish are adult viable and, in contrast to wild type fish, homozygous larvae display a 65% reduction in the pre-treated total nicotine response as determined by comparing pre-treated movement to control movement rates (Figure 9C). Homozygous *hbog* mutant fish, however, react normally to other tested behavioral stimulatory processes including locomotor activation due to: external physical stimulation, to the noxious chemical AITC (mustard oil) or to extreme temperatures (Figure 9D). This noted altered nicotine but normal stimulatory response
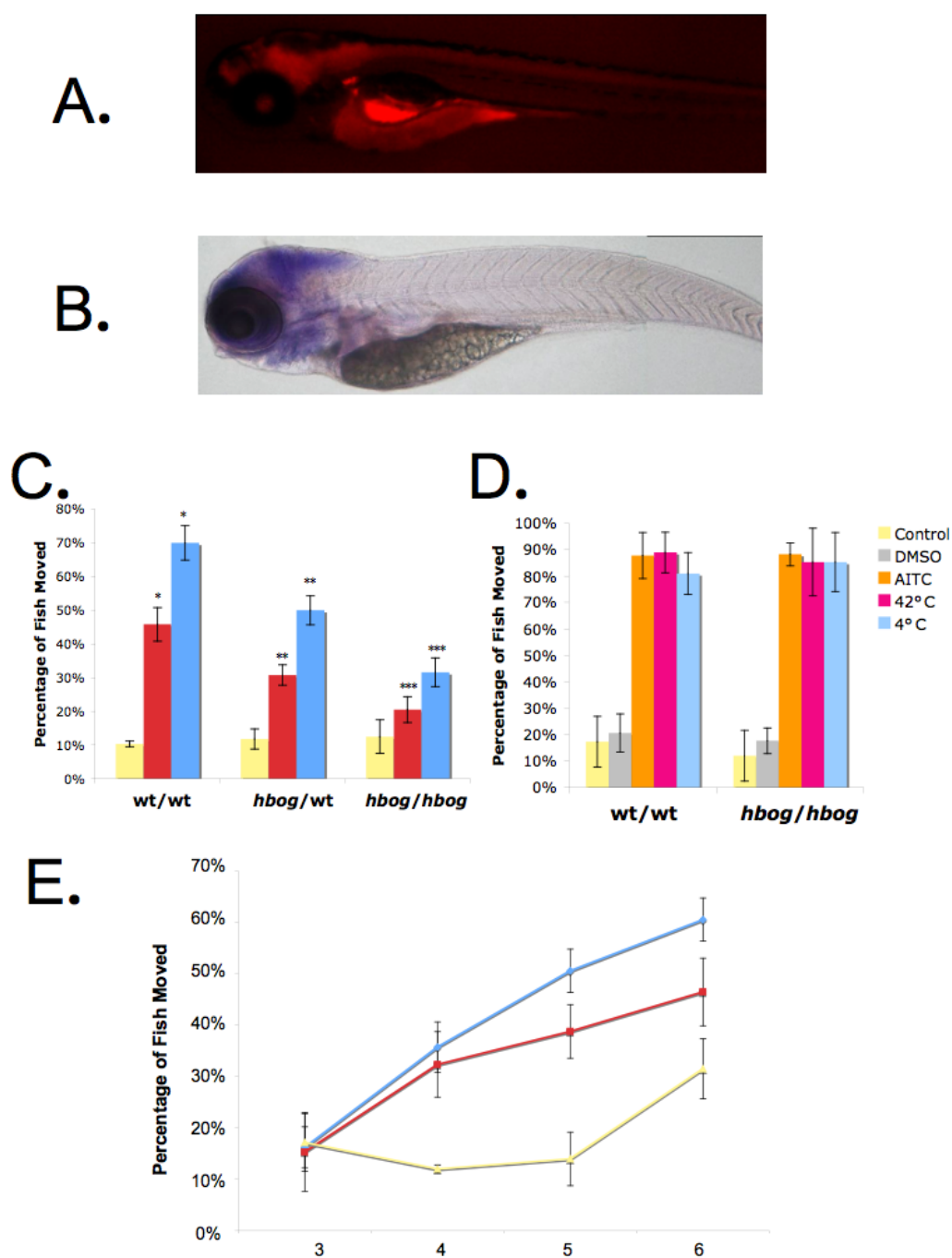
Figure 9. *humphrey bogart (hbog)*, **a GBT-R15 linked mutaiton in zebrafish Gaba-B receptor 1.2 (gabbr1.2) locus shows a reduced response to nicotine.** A. Sagittal fluorescent imaging (anterior to left) shows a general neural expression of mRFP-gabbr1.2 fusion protein at 5 days pf. B. Sagittal imaging (anterior to left) of a whole mount in situ hybridization for *gabbr1.2* at 5 dpf (bottom) shows high levels of neural expression. C.Full nicotine response profile of the *hbog* mutant comparing homozygous and heterozygous mutant animals to wild-type siblings at the standard nicotine dosage of 10 μM. Reduction from wild-type response is seen in both acute and sensitized groups. Reduction of homozygote phenotype is greater than heterozygote phenotype (P < 0.05). D. *hbog* heterozygous fish show no difference in response to noxious chemicals (allyl isothiocyanate) or temperatures (4 and 42 °C) when compared to wild-type siblings. E. *hbog* heterozygotes show a reduction in nicotine response through development and are not delayed. *, P < 0.05 when comparing to control or acute. **, P < 0.05 when comparing to corresponding wild-
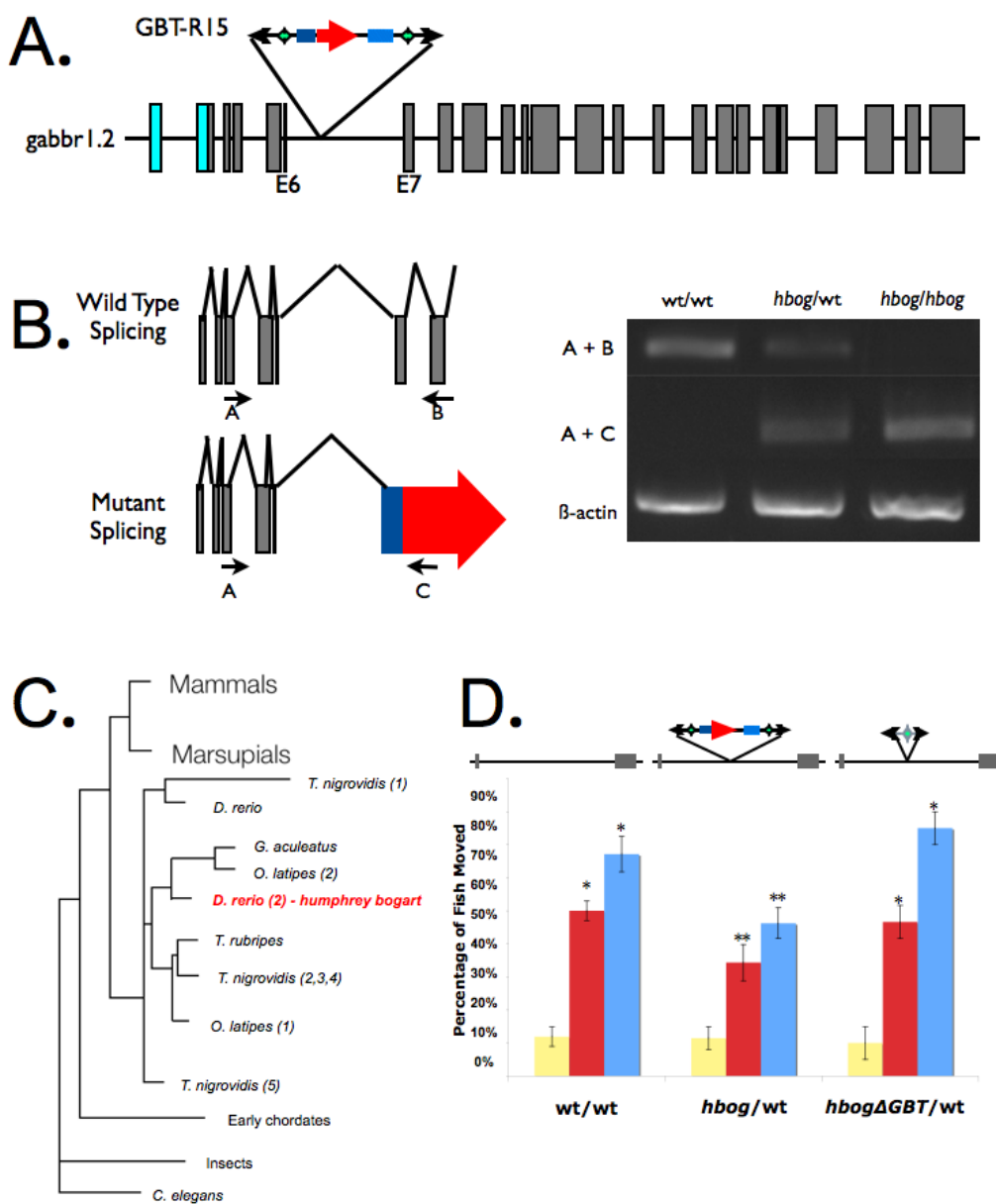
Figure 10. Molecular analysis of *hbog* nicotine response mutant. A. Schematic representation of the GBT-R15 insertion in intron 6 of the zebrafish *gabbr1.2* gene. Location of exons 4 and 9 of the gabbr1.2 gene and primers A, B, and C used for RT-PCR analysis are indicated. B. RT-PCR analysis of gabbr1.2 transcript in wild-type, heterozygous, and homozygous hbog fish. Primers in exons 4 and 9 were used to detect wild-type transcript of the gabbr1.2 gene. RT-PCR using primers in -actin were performed as an internal control. C. Simplified homology of the gabbr1/ hbog locus shown in red. Humans and other mammals encode a single gabbr1 locus. Derived from Ensembl homology engine. D. Germline propagation of Cre-mediated reversion of GBT-induced *hbog* mutant shows a nicotine response profile indistinguishable from wild-type sibling animals. Drawings of GBT-R15 insertion presence is represented above graphs of nicotine response profile. *, P < 0.05 when comparing to control or acute. **, P < 0.05 when comparing to corresponding wild-type group.

profile in *hbog* animals excludes yet-to-be determined effects from other physical or

pharmacological stimuli (see more complete discussion below). The onset of the

reduced nicotine response in *hbog* heterozygous fish is also not delayed compared to

their wild-type siblings (Figure 9E).

Molecular analysis shows that *hbog* is due to a single GBT-R15 insertion in the sense

GBT orientation of the sixth intron of the zebrafish *gabbr1.2* locus (Figure 10A) that

encodes one zebrafish ortholog of the GABA(B) receptor seven-pass transmembrane

subunit 1. RT-PCR analysis demonstrates this GBT insertion results in reduced wild-

type transcript levels in heterozygous animals and a greatly attenuated level of wild-

type RNA in homozygous animals (Figure 4F). This insertion generates a severely

truncated chimeric mRNA (Figure 10B) that encodes for an mRFP protein fused to the

first 135 amino acids of the *gabbr1.2*-encoded protein, deleting 830 amino acids in this

G-protein coupled receptor. GABA(B) receptors are evolutionarily ancient in origin,

with a single human ortholog of *gabbr1* (Figure 10C).

The use of GBT-R15 facilitated two analyses in *hbog* not possible for the GBT-P9 –

based *bdav* allele. First, we deployed Cre recombinase to delete the core mutagenicity

cassette at the *hbog* locus and tested the nicotine response profile of this reverted

chromosome (Figure 10D). Cre-mediated reversion could be obtained using either

somatic delivery of Cre or after germline propagation followed by molecular

confirmation of the excision process (germline reversion is diagrammed in Figure 10D).

The Cre-reverted chromosome results in animals with a nicotine response profile

indistinguishable from wild-type, providing strong evidence of causality for this specific GBT insertion in the *hbog* behavioral mutation. Second, we used confocal imaging analysis to determine the specific location of the gabbr1.2/mRFP fusion protein expression and compared this with RNA distribution using whole-mount *in situ* hybridization (Figure 11). Note the strong expression of gabbr1.2 RNA in specific neural tissue; the gabbr1.2/mRFP analysis indicates enhanced expression in neuronal subsets that appear largely as a subset of the RNA-expressing cells (Figure 11). The GBT-R15 vector system thus provides new options over our prior gene-breaking approach [37] by the generation of reversible genetic alleles that can also display information on expression of the trapped loci.

GABA(B) receptors are assembled from multiple subunits [97], indicating that changes in any subunit may result in GABA(B) signaling changes. Genetic variations in the GABA(B) receptor subunit 2 are associated with nicotine dependence [98]providing evidence for a conserved role for GABA(B) signaling in the human nicotine response. GABA(B)-targeted interventions may be of therapeutic value as the addition of a GABA(B) agonist or positive modulator reduced self-administration of nicotine in rats [99, 100], though clinical testing alone or in conjunction with nicotine replacement strategies has not been conducted to date. Additionally, these GABA(B) agonists have been shown to produce an addictive result as well, making its use as a clinical option for nicotine cessation fleeting[101-103].
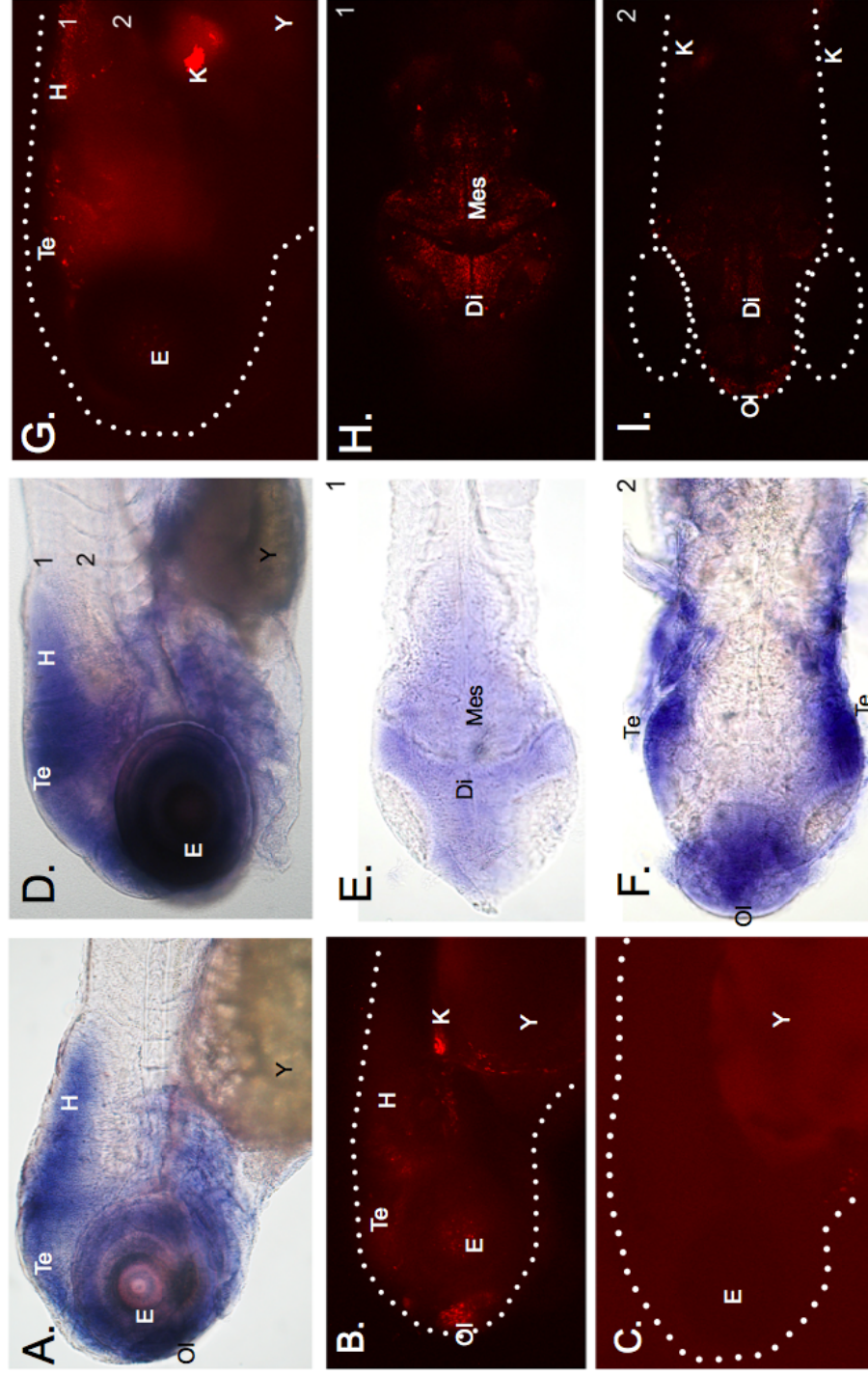
Figure 11. The mRFP/gabbr1.2 Fusion Protein in *hbog* Transgenic Fish Localizes to a Subset of Neural Tissue. A. Whole mount *in situ* hybridization for mRNA expression of the *gabbr1.2* locus at 3 days post fertilization. **B.** *hbog*-mRFP fusion protein expression at 3 days post fertilization. **C.** Wild-type mRFP background at 3 days post fertilization. **D.** Whole mount *in situ* hybridization for RNA expression of the *gabbr1.2* locus at 5 days post fertilization. 1 and 2 are sections represented in panels E and F, respectively. **G.** *hbog*-mRFP fusion protein expression at 5 days post fertilization. The observed expression in the kidney is due to accumulation of this secreted mRFP fusion after filtration. 1 and 2 are sections represented by panels H and I respectively. Abbreviations used: E Eye, K Kidney, Y yolk, H hindbrain, Mes mesencephalon, Di diencephalon, Ol olfactory bulb, Te Tectum. White dotted line shows the general outline of the zebrafish.

Section 6: *Yul Brynner*

With the combination trap vector GBT-RP2 we combined the protein trapping ability of

GBT-R15 with the polyadenylation trapping of GBT-P9 (see Figure 2 and Appendix

A). This allowed the implementation of both a biased RFP-based screen as well as an

unbiased GFP based screen for mutant lines. Through the unbiased screening portion of

the GBT-RP2 screen an additional candidate mutant line was determined. This line,

named *yul brynner*, displayed a unique nicotine response when examining the full

nicotine response profile (Figure 12A). This unique response profile has been present

but inconsistent in appearance with alterations in either the pre-treated or acute response

of mutant fish when compared to their wild-type siblings (Figure 12B). In all

generations the line has harbored more than one transposon insertion (with greater than

80% GFP positive) and has shown an altered response to nicotine with acute and pre-

treated larvae showing a similar response (Figure 12). Additionally, in more recent

generations, segregation between the two characteristics has taken place with an altered

response occurring both in the acute and the pre-treated populations (Figure 12C). The

presence of multiple expressed transposons presents a complication in a fluorescent-

based sorting technique. Although it is possible to purify a line to a single expressing

transposon, the presence of additional expressing transposons in a line of interest will

dampen the results of the

altered expression due to the presence of an un-altered yet fluorescently tagged

transposon line of fish.
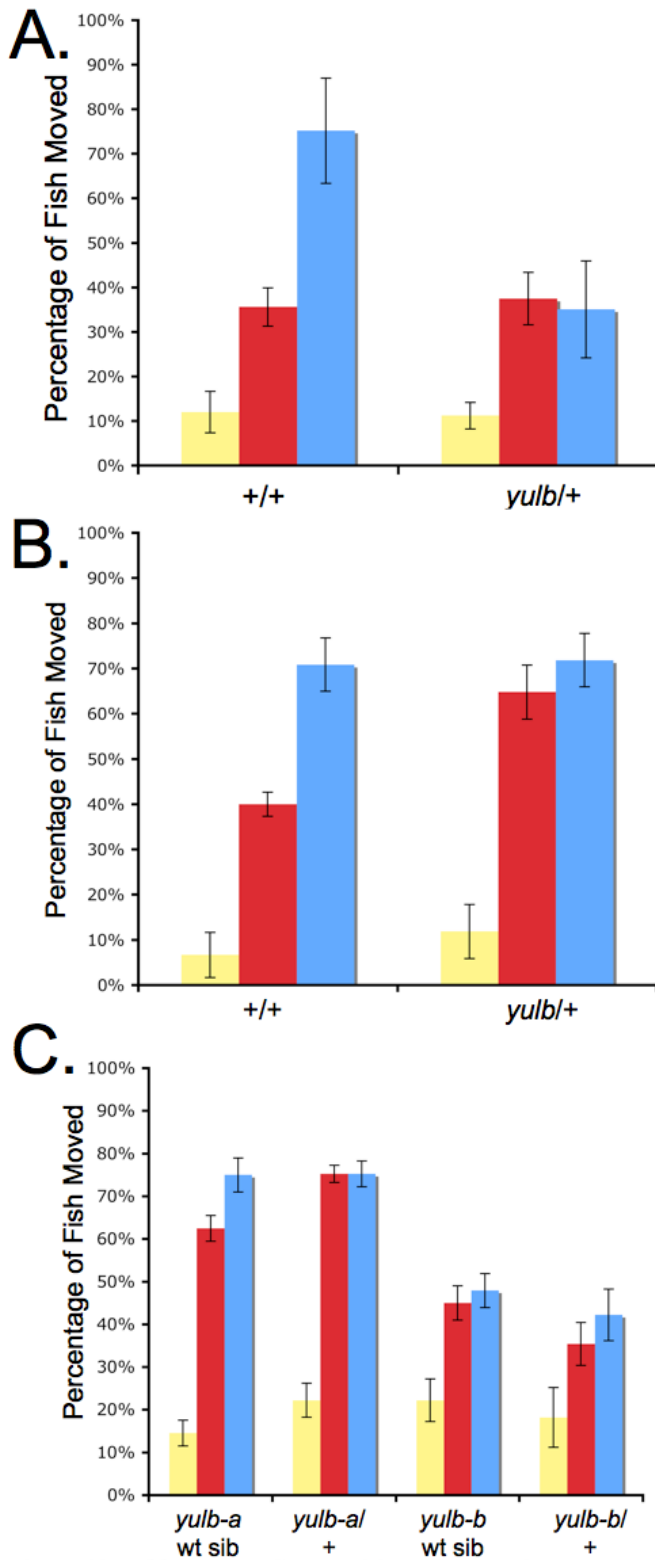
Figure 12. *yul brynner*, **a GBT-RP2 insertion provides a novel type of nicotine response mutant.** A. *yulb* founding offspring show a reduction in treated response to nicotine compared to wild-type siblings. B. A second generation of *yulb* shows an increase in acute response compared to wild-type siblings. C. Third generation *yulb* show differing responses to nicotine when in both wild-type and mutant siblings.

Due to the number of expressing transposon insertions in the background that harbors the *yulb* locus, its identity remains unknown to this point. The *yulb* line is considered a "jackpot" line of fish— a line that has incorporated a very large number of insertions for a widely unknown reason, speculated to be an early integration event or an overdosing of the injected transposon [Ekker lab observations, unpublished]. Due to the number of transposon insertion events required to produce an in-frame mutation within the transcriptional unit of a protein (roughly 6 successful insertions), many transposons are initiall injected into the one-cell embryo. This can create lines of fish that carry multiple transposons, occasionally upwards of 20 or more. It is estimated, through Mendialian inheritance of the chromosomes, that the initial *yulb* progenitor harbored over five expressing GFP-linked insertions. This number of insertions has two specific and unique outcomes: the dampening of the specific nicotine response mutation and the possibility of a multigenic-trait. Dampening occurs due to the sorting due to GFP-linkage when initially examining the line for differences in nicotine response. GFP-linked insertions that do not associate with the insertion that causes the phenotype will still be sorted as GFP positive while being phenotype negative. It is also possible to create a mutant that has multiple altered genes that, in combination, produce a phenotypic response. The resolution of either of these problems is possible through molecular identification of the insertion loci or continued out-breeding of the line of interest. Due to the interest in the nicotine response profile of the *yulb* line, the removal of additional loci is on going.

Chapter 3

Conditioned Place Preference in Larval Zebrafish

Section 1: Introduction to Conditioned Place Preference in Teleosts

Worldwide, more than 55 million people die annually due to causes either directly or

indirectly attributable to an addiction to either a legal or illicit drug of abuse[104]. This

addiction is defined as a complex set of traits that result in a dependence to a substance

despite the knowledge of the adverse effects of the substance upon its user. These traits

are further classified by their relation to internal and external influences: the genes of

the user, the environment within which the user is immersed and the physiological

affects of the drug itself [68]. Due to the complexity of drug abuse and the cellular,

molecular and organismal mechanisms involved in forming this addictive behavior,

model organisms have been used to deconstruct addiction into its basal components.

Specifically, model organisms have been used to examine the physiological affects of

the drug itself [105, 106], the genes involved in these pathways[107, 108], the

interaction between the reward pathway of the drug and the organisms' environment or

a combination of multiple interactions[109, 110].

To examine the interactions between the association of a stimulus and the rewarding

affects the stimulus has on its user, whether specific to drug abuse or not, an assessment

paradigm was developed based on the traditional Pavlovian conditioned response[109].

This paradigm of study is termed "Conditioned Place Preference" or CPP, specifically

referring to the repeated exposure to a stimulus with a paired environment to develop a

change in preference based upon the rewarding aspects of the stimulus. In the past,

CPP has been used to examine a wide variety of stimuli from drugs of abuse to

pheromones[109, 110]. Additionally, CPP has been shown to be evolutionarily

conserved being successfully deployed in model organisms ranging from

invertebrates[111] to higher vertebrates including rodents[108], primates[112]and even

humans [113]. More recently, CPP has been used as a tool to elucidate the genetic

components of the learned response to a drug of abuse[114-116].

The zebrafish has emerged as a model organism that is easily amenable to behavioral

screening for genes involved in addictive and non-addictive pathways [45, 64].

Additionally, it has been used successfully well as chemical screening for drug

discovery[38], and modifiers of behavior [39]. The combination of these routes of

study makes the zebrafish an ideal model for drug abuse studies.

Although the zebrafish had been used as a model within avoidance and learning studies

from the 1970's onward [117, 118] it was not until the early 2000's that the it was first

used for a traditional CPP study [43, 44](for an excellent review of the use of zebrafish

and its benefits within behavioral research see Miklosi and Andrew, 2006 [119]). These

studies focused on adapting previous research performed upon mice to the zebrafish

model system. Place conditioning was performed by introducing the adult zebrafish into

a novel choice environment, a chamber consisting of two halves differing in visual

appearance. Although the method of visual distinction was different in each case, both

groups of researchers took advantage of innate biological processes of light/dark

preference and fear to create differing initial place preference. This allowed researchers

to examine the ability of the adult zebrafish to associate presence of a stimulus, alcohol

or cocaine respectively, with the novel environment. It was also possible to determine if

the rewarding effects of the drug was stronger than the innate biological processes

involved in their initial preference (For a visualization of testing apparatus and

paradigm see Figure 13A). Additionally, each study included an examination into the

genetic component to the CPP paradigm, with one of the studies identifying a number

of mutants (*dum* and *jpy*) that were differentially affected by the addition of cocaine

[44]. To control for the possibility of a deficit in learning or in sight, the researchers

also employed a visual acuity test and a simple t-maze test for learning ability (See

Figure 13B). These studies would set the precedence within the behavioral community

of zebrafish for the use of CPP for the study of genes involved in drug abuse and

addiction. Following these seminal studies using the adult zebrafish as a model for CPP,

the variety of drugs of abuse tested quickly diversified, with researchers examining the

amphetamine [47, 48], opiates (morphine [46] and salvinorin [120]), and the legal drugs

of nicotine and ethanol [45, 54].

Section 2: Conditioned Place Preference in Larval Zebrafish

Using the work that has been pioneered using the zebrafish for conditioned place

preference [43, 44] as a guide, we examined the onset of the learned response to

nicotine using larval zebrafish. This simple assay was accomplished by placing larval

zebrafish into a choice environment consisting of a petri dish with one side markedly
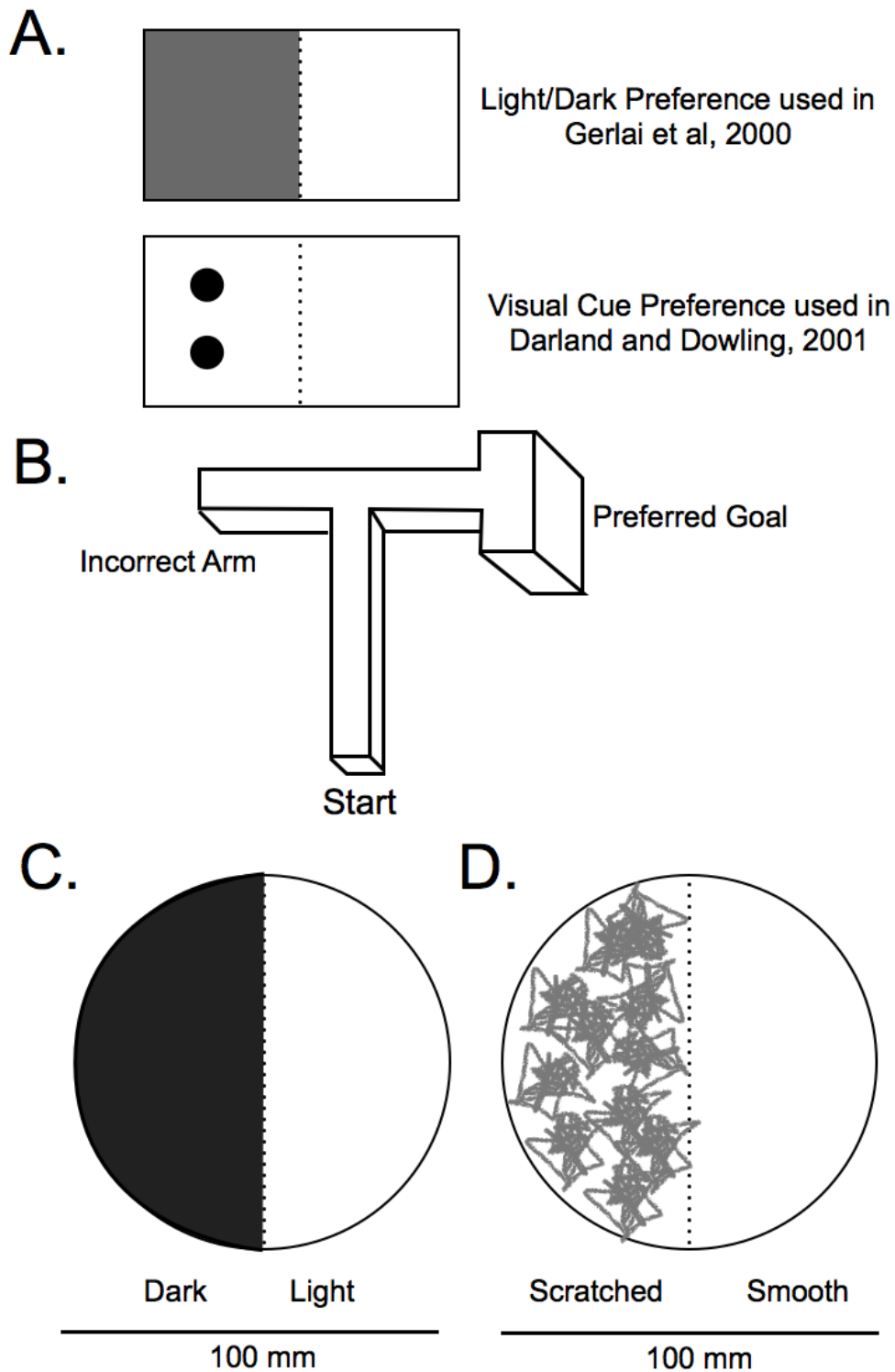
Figure 13. Apparatuses used for conditioned place preference in zebrafish. A. Gerlai et. al. [19] used a tank sectioned into visually distinct halves, one being dark, the other light. B. Darling and Dowling [18] used a tank sectioned into visually distinct halves with a visual cue of two dark dots representing the distinction. C. A T-maze used to test for learning ability in the adult zebrafish in a number of studies [20]. D. A 100mm Petri dish is separated into two sides with a dark visual cue on one half. E. A 100 mm Petri dish is separated into two sides with a scratched tactile cue on one half.

different from the other in ambient light (light/dark) or texture (smooth/rough) (Visual

representation in Figure 13 C and D). This initial testing was followed by a short

recovery time and a single or series of training sessions (Figure 14 A; Chapter 4 for

details). Place preference was determined as a percentage of larvae on a given side of

the dish over time. This method of quantification was chosen over the traditional

method of time spent on a given side due to the natural tendency of a larval zebrafish to

remain still at 5 days post fertilization [57]. With the larval zebrafish being allowed to

randomly arrange themselves within the testing environment, a lack of preference

would be visualized as a value of 50% of the fish located on the given counted side. A

preference would be visualized as a value different from 50% with values higher

representing a preference towards the counted environment and less than 50%

representing a preference against the given counted environment. In their initial

exposure to the testing plates, the larval zebrafish (4-6 dpf) showed a slight preference

towards an environment similar to that in which they were raised: smooth or light, over

that of a novel environment: dark or rough (Figure 14, B and C). This preference

change was enhanced when the two stimuli were combined (smooth and light versus

rough and dark) (Figure 14 D). After an initial preference was determined, zebrafish

larvae were exposed to the least-preferred environment paired with 10μM nicotine for a

number of training opportunities. This paired exposure shifted the preference of the

larvae towards the previously less preferred side in a training-dependent manner (Figure

14 E). For the remainder of the tests using a stimulus as a rewarding or deterring factor,

the change in preference from control or baseline will be demonstrated within the
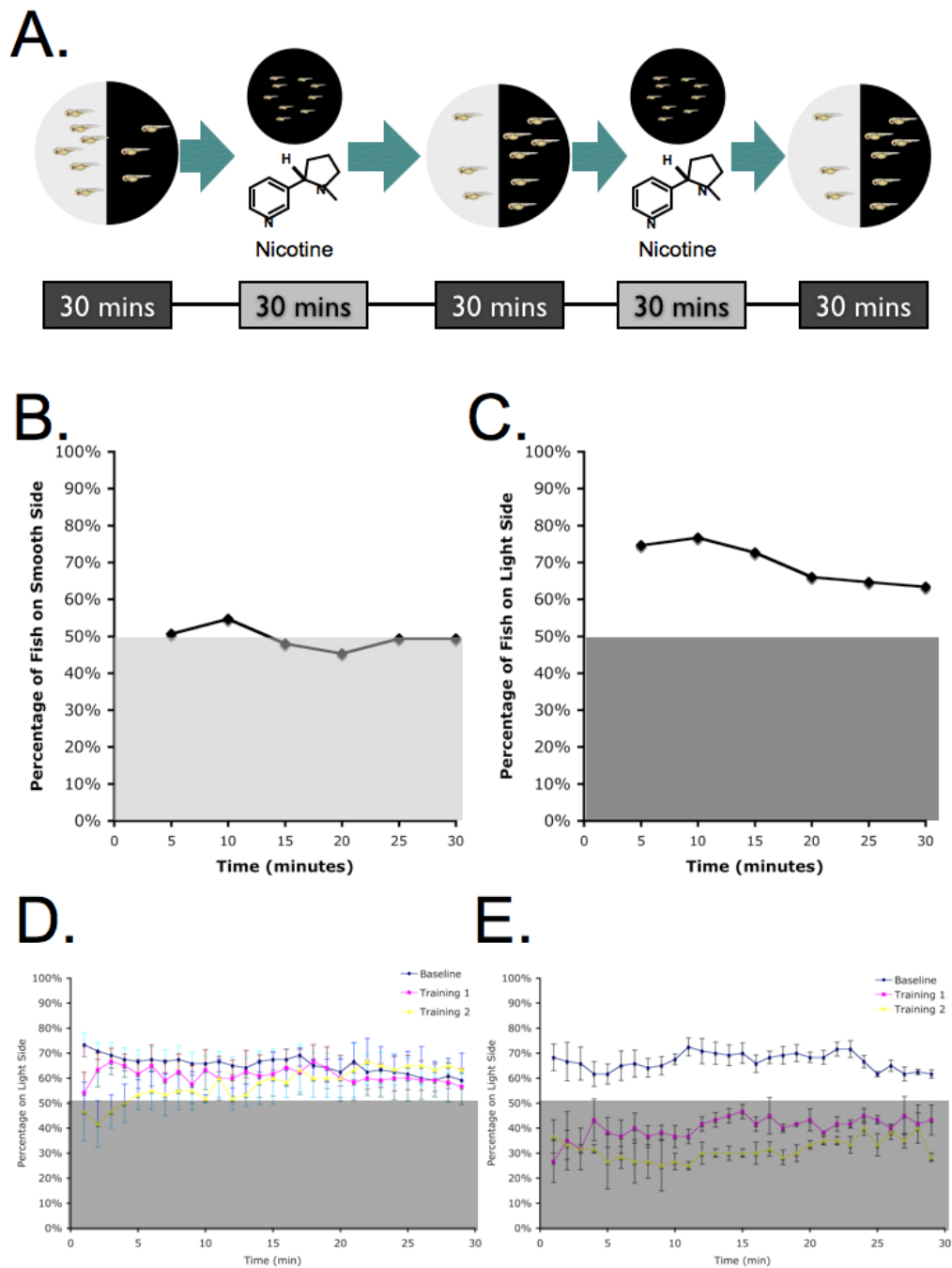
Figure 14. Larval zebrafish show a preference to a known environment when exposed to a novel environment. A. Testing paradigm for induction of conditioned place preference to a given cue (dark is presented, however any cue can be used). B. Larvae show a preference at 5 dpf to a clear plate compared to a darkened plate. C. Larvae show a preference at 5 dpf to a smooth plate compared to a rough plate. D. Larvae show an increase preference to the combination of a smooth/clear plate when compared to a rough/dark plate. E. This preference can be overcome with training accompanied with 10 μM nicotine paired with a dark/rough cue.

yellow

figures or text. In all cases, this will be a representative of the difference between the final testing session and the initial testing session.

To test the specificity of a preference to the raised environment, larval zebrafish were raised in varying conditions of light and tested in a choice plate consisting of these differences in light (conditioning paradigm displayed in Figure 15 A). Shade of light was controlled for by equalizing the grayscale value for each shade of color and only adjusting the hue applied to the color. We used colors of red, green and blue, corresponding to the long, medium and short wavelength photoreceptors found within the zebrafish eye. However, due to the maintenance of the grayscale value, an optimal output of wavelength for each photoreceptor could not be obtained. When raised in a neutral light environment of full spectrum-white light, larvae showed no preference towards an individual shade of light (Figure 15 B). After raising the larval zebrafish in a single colored environment starting at 48 hpf, larvae were tested for preference at 4, 5 and 6 days post fertilization. This was accomplished by placing the larvae in groups into a petri-dish placed upon a transparent colored sheet with equal portions of red, green and blue (Figure 15 A). To control for environmental differences, the orientation of the colored spaces was arranged randomly for each dish while maintaining the equal prevalence of the individual colors. Additionally, the color intensity of each of the given colors was matched both by using equal saturation and value (on an HSV scale as determined by Adobe Photoshop) and by equalizing grayscale values when converted. Rather than counting a singular side of preference, a percentage of the total number of larvae in a given colored environment was determined. Because of the equal area
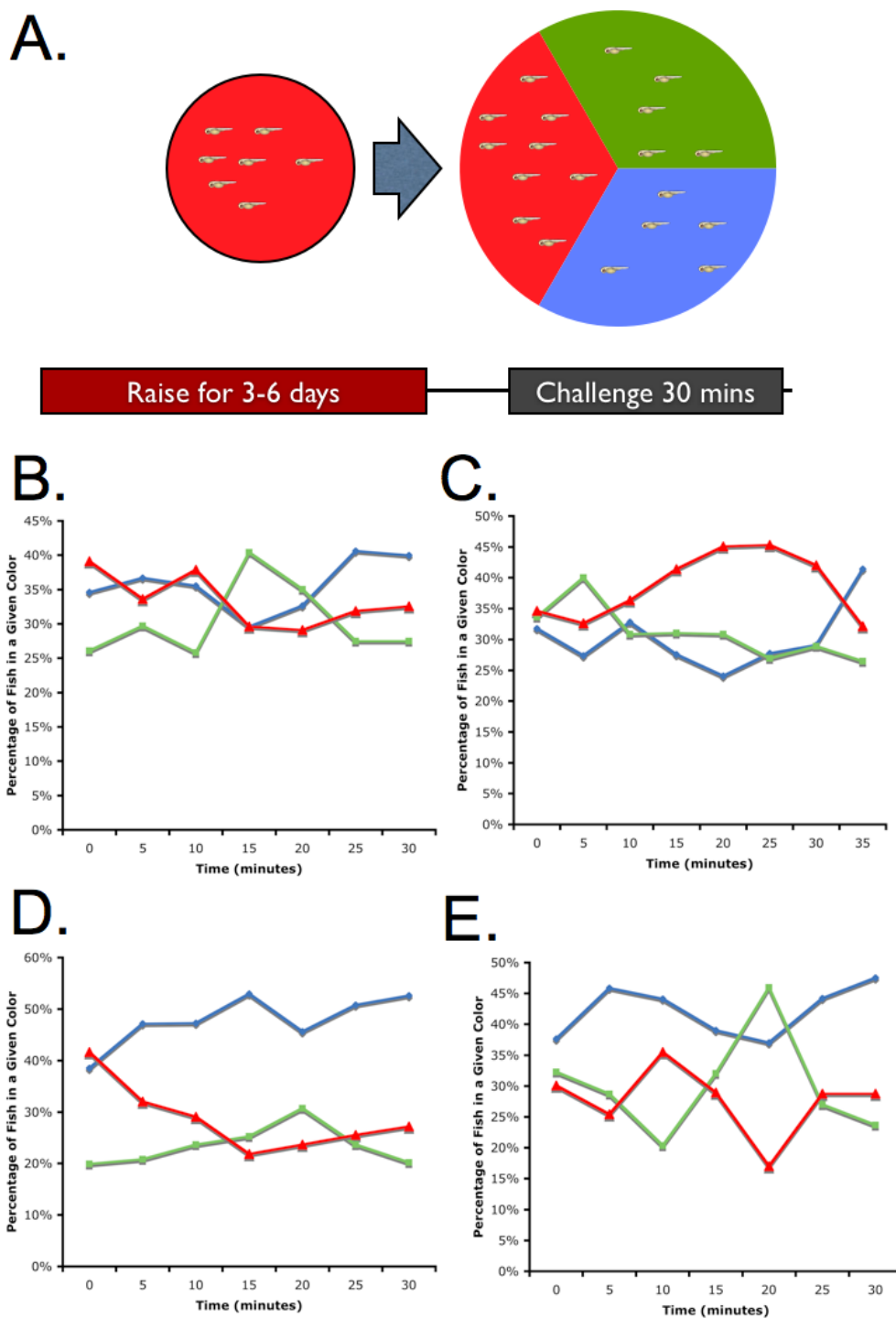
Figure 15. Larval zebrafish can be trained to prefer a colored background. A. Paradigm of color raising protocol (red shown, but all colors were used). All color tests were performed with a randomized arrangement of the red/blue/green disc shown (rotated from the arrangement shown). B. Larvae at 5 dpf do not show a preference to any color. C. When raised in a red environment, larvae show a preference to red at 5 dpf. D. When raised in a green environment, larvae show a preference to a blue environment at 5 dpf. E. When raised in a blue environment, larvae show a preference to blue at 5dpf

arrangements of the colored environments, a preference for a given environment was

considered a value higher than 33.3%. A preference against a specific environment was

considered a value lower than 33.3%. No preference was considered a value of 33.3%.

For all graphical representations of the preference data determined from this testing

(Figure 15), the color of the line represents the color of the given environment. No

change to the texture or intensity of light was provided. Larval zebrafish raised in a red

environment showed a preference to a red environment over blue or green (5 dpf

shown; Figure 15 C), however larval zebrafish raised in a blue or green environment

showed a preference to both blue and green but not red (5 dpf shown; Figure 15 D and

E). This change in preference can most likely be attributed to the developmental

patterning of the cone cells within the retina of the larval zebrafish. Initially, all

photoreceptors develop in a small, centralized patch of the retina allowing for minimal

color differentiation beginning at 40-50 hpf [121]. Following this, cones develop

sequentially with blue developing at 4 dpf, red at 7dpf and green developing last at 10

dpf [122]. Full color vision is developed between 15 and 40 dpf [122]. Despite the

inability of the larvae to distinguish the color of the red light, we speculate it is able to

visualize the blue and green colors due to the early development of the blue cone. When

presented with a novel environment, the larvae prefer to stay in the known environment,

in this case, the red[123]. Due to the overlap in absorbed spectrum of the blue and green

photoreceptors, the larvae are unable to distinguish between the green and blue,

presenting a preference away from the red areas. Although these results are promising,

the change in preference between the colored environments was not as striking as that

seen with the combination of the tactile stimulus paired with the luminescence, thus the combinatory stimulus was used for further development of the larval CPP assay.

The requirements of the learned response were tested by examining both developmental timing and the frequency and number of training sessions (see Figure 14 A for visual representation of testing paradigm). An examination of the length of the testing sessions was also examined by placing larval zebrafish into the testing environment for an extended period of time (Figure 16 A). When placed into the testing environment, larvae spent the first amount of time developing a preference. After this initial exploratory period of approximately 5-10 minutes a preference was made and fish tended to stay on this preferred side for upwards of two hours (Figure 16 A). Following an extended period of stay within the preferred side, the larvae once again entered a period of exploration (not shown). Because of this window of preference, a testing period was considered to be 30 minutes, encompassing the initial expiration and development of preference. To streamline the data analysis process, and for better comparison of data, it was determined that the optimal method of data collection and analysis was to average the percentage of larval fish on a given side between 15 and 20 minutes. This allows for an assurance that the larvae have left the exploration phase and have not yet begun to begin exploring once again. For the remainder of this section, all wild-type comparisons will be represented as a bar graph of this average with error being representative of differences within a given population. Once the time-course of exploration had been established, the number of training sessions and the length of training sessions were tested. It was determined that larval zebrafish could develop a

change in place preference when being paired with 10μM nicotine after a single training session of 30 minutes, however this preference is greater with an additional training session (Figure 16 B). As more training sessions were added upon the same day, non-nicotine treated larvae began to be habituated to the novel environment, slowly removing the preference towards the nascent environment (Figure 16B; 3-4 training sessions). It should be noted that while a habituation to the novel environment occurced, the preference of the larvae never passed the point of a preference to the novel environment over the initial environment (not shown). The length of training sessions was also examined using two training sessions of equal length. These sessions ranged in time from 5 minutes to 60 minutes. It was determined that between 30 and 60 minutes was an effective time for training due to the greatest difference between the nicotine paired and non-nicotine controls (Figure 16 C). For all other assays, a 30-minute training period was used.

To create a robust assay that is specific for a preference towards an individual stimulus rather than simply testing for a habituation to a novel environment, a number of specific control situations were explored: environment paired with a non-rewarding stimulus (cinnamon oil), disruption of mechanism of the stimulus with a competitive inhibitor and the disruption of rewarding aspects of the stimulus with an inhibitor of reward. Using the optimal training and testing conditions outlined above, larval zebrafish were trained in the presence of mecamylamine, a competitive inhibitor to nicotine [83]. Larval zebrafish were expose to mecamylamine one hour prior to being exposed and trained to nicotine in the dark environment. We found that in the presence of 150μM
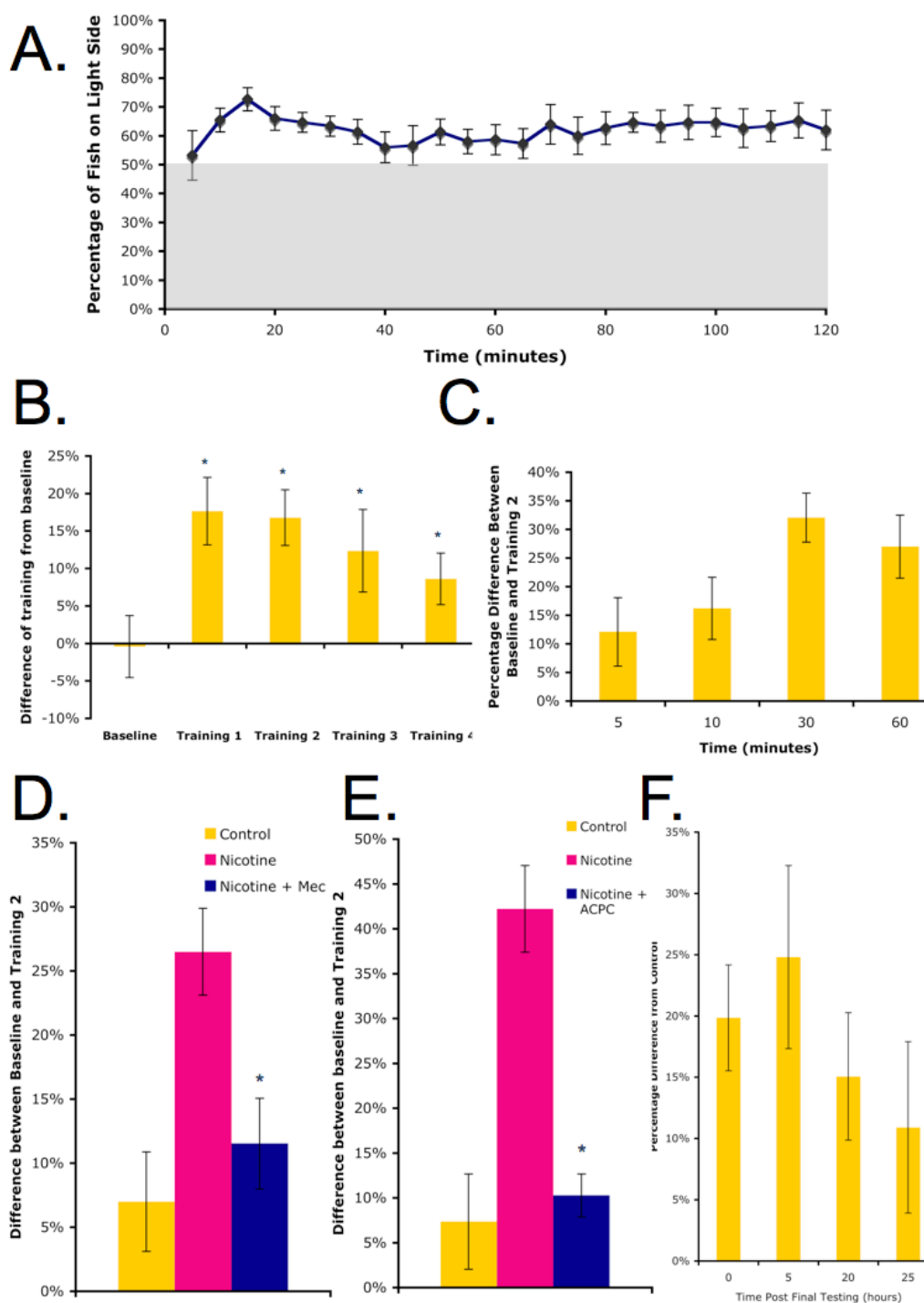
Figure 16. Place preference conditioning is dependent on the rewarding aspects of nicotine. A. When placed into an environment containing both familiar and novel areas, larvae develop a preference within the first 5-10 minutes. Following this, they prefer to stay on the known side for an extended period. **B.** Comparison of control to nicotine treated with varying numbers of 30 minute training session. **C.** Time of training session varying from 5 minutes to 60 minutes. A training session of 30 minutes provides the optimal change in preference. **D.** Competitive nicotinic receptor antagonist, mecamylamine, diminishes the change in preference that is found with exposure to nicotine. **E.** NMDA partial agonist ACPC diminishes the change in preference that is found with exposure to nicotine. **F.** Training induced place preference lasts over 20 hours before being extinguished.

mecamy

lamine the change in preference that is associated with nicotine was diminished when compared to groups that did not receive the additional inhibitor (Figure 16 D). Additionally, larvae that received the mecamylamine alone saw no significant change in preference when compared to control larvae (Figure 16 D; mecamylamine and control). Accompanied with the differences between the nicotine treated fish and the non-treated fish (Figure 14 D and 14 E), it can be determined that the nicotine is the driving stimulus that encourages the larval zebrafish to alter place preference. To examine the learning capability of the larval zebrafish and to ensure that the change in preference was specific to a conditioned place preference paradigm, larvae were trained in the presence of 1-aminopropanecarboxylic acid (ACPC), a known non-biologically relavant partial agonist of the NMDA receptor that has been shown to block the acquisition of place preference conditioning to a variety of psychoactive drugs in rats[124]. Using rodent work as a guide, larval zebrafish were exposed to a concentration of 150μM ACPC 30 minutes prior to drug administration. We found that in the presence of the ACPC the change in preference that is associated with nicotine was diminished when compared to groups that did not receive the inhibitor (Figure 16 E). A third method of examining the rewarding aspects within the confines of a CPP assay is to examine the extinction of training[125]. This can be accomplished with the larval zebrafish by examining the change in preference over time without including additional training sessions. It was found that the larval zebrafish retain the change in preference over a period of 24 hours (Figure 16 F). The combination of these data provide that this larval CPP assay can be as affective as those previously described in both rodent models and

adult zebrafish. Additionally, this represents one of the first associative-learning based larval zebrafish assays amenable to a forward genetic screen.

To explore additional aspects of the learned response to a stimuli, we examined whether the larval zebrafish could also respond to a negative stimulus in a conditioned place avoidance (CPA) mechanism (Figure 17 A). It has been shown previously that larval zebrafish react in an adverse manner when exposed to low levels (0.5%) of cinnamic acid, commonly found in cinnamon oil[87]. This stimulus was used in a similar method to that described above with cinnamon oil being paired to the novel environment. The dark side was used as the environment paired to the cinnamon oil due to the housing of the larvae in a light environment. When trained with the cinnamon oil paired to the novel environment, larval zebrafish increased their preference to the light-smooth side (Figure 17 B). Additionally, this increased avoidance of the novel environment persists for at least 6 hours (Figure 17 C). Although this is not as strong as the change in preference associated with a positive stimulus, it demonstrates the utility of the larval zebrafish in a CPA assay.

Section 3: Conditioned Place Preference in GBT-mutant lines

The conditioned place preference assay in larval zebrafish presents the opportunity to explore further aspects of the addiction process that are not able to be seen using the nicotine response assay alone. Specifically, a mutant of interest that has been found using the nicotine response screen could still acquire an addictive response to nicotine while having a lessened response. This would show that while necessary for the full
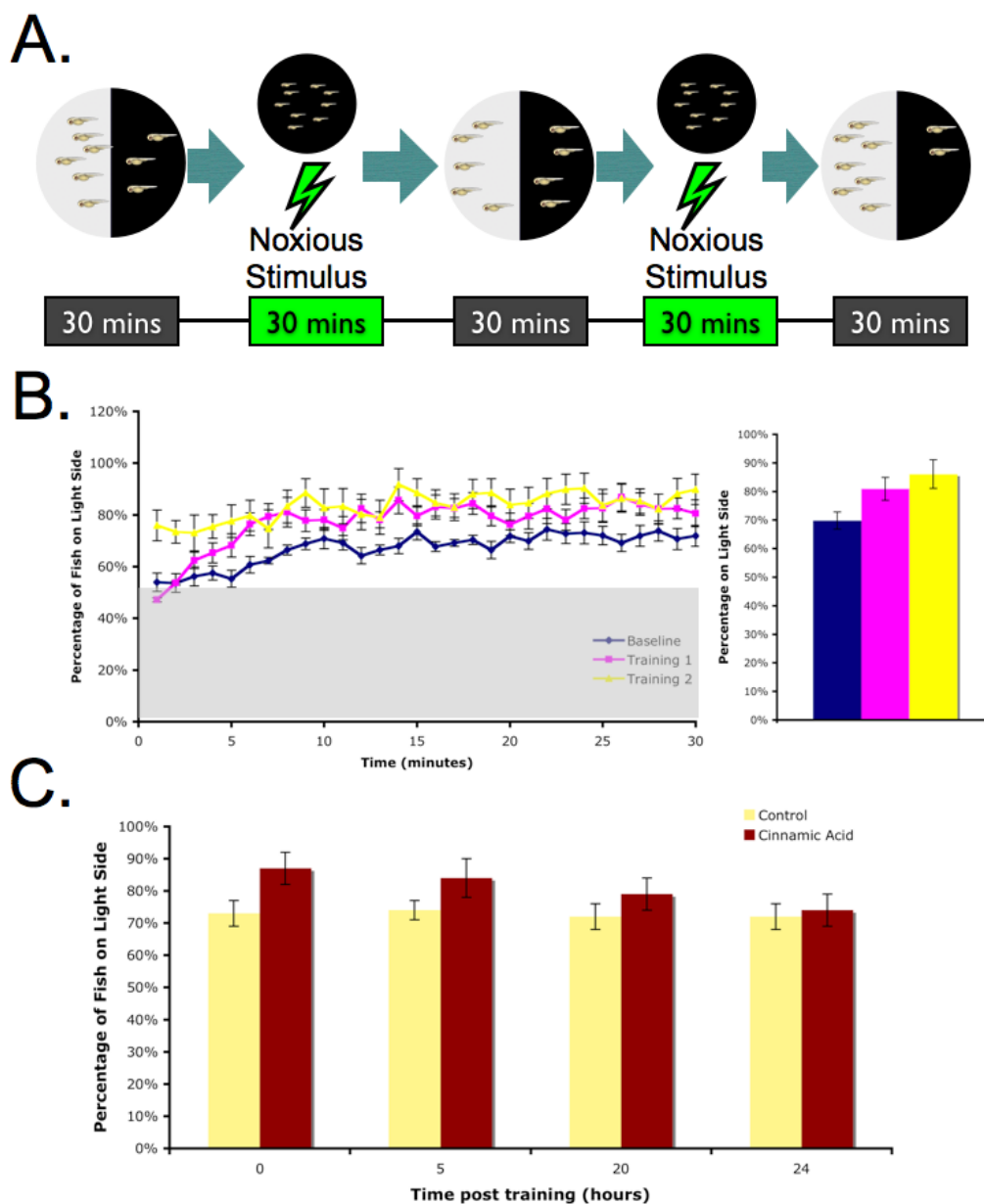
Figure 17. Conditioned place aversion can be obtained by replacing a rewarding stimulus with an aversive stimulus. A. Testing paradigm for the induction of conditioned place aversion to a given cue (dark is presented, however any cue can be used). The cue is paired with a noxious stimulus for a period of time with a testing session immediately following. B. Larvae exposed to a novel environment paired to 0.5% cinnamic acid increase their preference for the known environment. Shown are entire time course and exemplary bar graph. C. This increased avoidance of the novel environment persists for approximately 24 hours.

nicotine

response, the gene in question would not play a role in development and maintenance of the rewarding aspects of the stimulus. Additionally, the inclusion of a CPP assay on previously described nicotine response mutants provides the opportunity ot separate the reward pathway from the physiological response to nicotine. To this end, we have explored the conditioned place preference responses of *hbog* and *bdav* the GBT mutants described previously (Chapter 2 and Petzold, et al. 2009[64]). To better demonstrate the differences between the response of the mutant larvae compared to their wild type siblings, the entire thirty minute response period was included within these graphs along side the averaged comparison. Coloration of the graphs corresponds to the other graphs throughout this chapter.

Although *bdav* and *hbog* showed a similar reduction in global nicotine response, they differed greatly when tested in the nicotine conditioned place preference assay. In the presence of nicotine, with the standard treatment and training, *bdav* heterozygote larvae were shown to not develop a change in preference (Figure 18A). Wild type siblings of these *bdav* heterozygotes showed a change in preference towards the training side (Figure 18A). In contrast, *hbog* heterozygotes developed a change in preference towards the training side at the same rate and manner as their wild type siblings (Figure 18A and 18B respectively). This suggests that *cct8* plays a role in the mechanism of action of the nicotine reward pathway. Additionally, *gabbr1.2* may not play a role in the mechanism of action of the nicotine reward pathway. The duplication of the *gabbr1* gene within the zebrafish genome may provide enough of a gene product to allow for the development

Figure 18. Previously described GBT mutant larvae show differing responses when examining conditioned place preference to nicotine. A. *bdav* heterozygotes shows a reduction in place preference when trained to nicotine in a dark/rough environment when compared to wild-type siblings. B. *hbog* heterozygotes shows a similar change in preference to nicotine when trained in a dark/rough environment when compared to wild-type siblings.

and

maintenance of the reward aspects of nicotine while still being disrupted within the initial response to nicotine.

Section 4: Conclusions and Future Directions

Understanding the developmental and evolutionary mechanisms of complex behavioral traits, such as addiction and learning, will likely lead to a greater understanding of human addiction and drug abuse. Additionally, with the great strides of genetic and chemical modification within teleost organisms, such as the zebrafish, a wider array of conditioned place preference assays can be used to provide a genetic link to these processes. Whether key genes in the process or modifiers, the development of a conditioned place preference assay in larval zebrafish lays the groundwork to allow for a new wave of studies to help provide the genetic basis of the learned aspects of addiction. Furthermore, with emerging technologies in the zebrafish field, such as the targeted zinc finger nuclease[126]and large-scale chemical screenings[127], an even wider array of genes and chemical modifiers can be targeted within these conditioned place preference assays using a reverse genetic approach. Additionally, with the added screening power of the forward-genetic GBT-based mutagenic screen presented in Chapter 2, a combinatory approach can be taken to further examine the place genes hold within the nicotine addiction pathway. Used as a follow-up assay upon mutants discovered in the nicotine-response assay, the conditioned place preference assay provides a mechanism to determine the learned aspects of addiction.

The additional merits of using a novel environment-testing situation as described above allows for the exploration of both conditioned place preference (nicotine) and avoidance (cinnamic acid). This suggests that similar testing situations that have been examined in rodents can be used within the larval zebrafish system allowing for a further exploration into complex behavioral systems. Larval-based conditioned place preference screening provides a mechanism for the discovery of genes involved within learning, plasticity and novelty seeking behavior. With the addition of the conditioned place avoidance assay, the developmental and genetic mechanisms that drive adverse effects of drugs of abuse, stress and pain could be examined. The use of a non-locomotor based assay provides the opportunity to study a variety of stimuli including depressants such as alcohol. This approach also allows the researcher to examine both shared and individual pathways to drug abuse within an extensive array of stimuli, both stimulating and depressant.

With simple alterations to the current protocol, it is possible to greatly enhance the CPP and CPA assays. Using current technologies available, it would be possible to create an ever-changing environment with a video played on an LCD screen. This approach enables larval zebrafish to be "trained" to follow a moving spot associated with the drug or stimulus of choice allowing for a testing paradigm that is both dynamic and ever changing. Additionally, this learning based paradigm would provide an opportunity to explore the genetic pathways that create learning, memory and plasticity.

Chapter 4

Methods

Locomotion observation of Nicotine Response Assay.

Movement was assessed by digital snapshot capture of the fish using a "multiplex"

mode of a either a Nikon Coolpix 990 or Nikon Coolpix SR10 digital camera 30

seconds after the testing dose of nicotine. This 16-shot mode generates photographs

over a period of six seconds with each photograph occurring every 0.4 seconds. This

time window was selected to maximize the nicotine response signal compared to the

basal movement while also providing a reproducible assessment of the basal movement

capability of the tested animals.

Movement changes were determined by manually overlaying the initial photograph to

the 4th photograph (a period of 1.6 seconds) in each series using Adobe Photoshop.

Other frames in the sequence were used to address any ambiguity in the initial

movement assessment. The percentage of fish that visibly changed position was

recorded for each treatment group.

Statistical analysis

The behavioral data were analyzed using Microsoft Excel 2004 for Macintosh with

Statistical Package (Microsoft). Percentage of fish moved was ascertained by

determining the number of fish moved and dividing by the total number of fish for each

treatment group. The mean of the percentages was then determined along with the

standard error. All error bars in figures represent this derived standard error. Statistical significance of movement differences was assessed using two tailed t-tests with control fish being compared to either the acute treatment group or the pre-treated group. To determine statistical significance between mutant groups (Figure 6), we determined the mean of the mean between the pre-treated mutant and wild-type siblings. Statistical significance of the difference of means was assessed using a two-tailed z-test comparing the overall mean to the individual mean tested. Statistical significance of the addition of a drug to the water of the fish was assessed using analysis of variance (ANOVA) with treatment as a grouping factor followed by a Student Newman-Keuls post hoc test using KaleidaGraph 4.1 for Macintosh (Synergy Software). Statistical significance of place preference was determined as a difference in place vs a random distribution ($H_0 = .5$) using a binomial distribution. A $p<0.05$ was considered statistically significant.

Nicotine administration.

A dose range up to 100 micromolar of nicotine was used on day five zebrafish for assessing the main nicotine response profile (Description found in Chapter 2; Figure 3B). The ten micromolar dose was selected as the 'standard treatment' used in the genetic screen due to the strong behavioral signal detected in both the acute and pre-treated animals. Fish in a particular "Pre-treatment" group were given a defined dose nicotine solution with an incubation time of one minute. Fish were then transferred into fresh embryo water. After an incubation of eight hours, fish in both the "Pre-treatment" and "Acute" groups were administered the same defined nicotine dose combined with 500μl of embryo water. Fish in the "Control" group were given a 500μl dose of embryo

water to control for the physical action of administering nicotine. All fish were allowed

a 30 second incubation period before behavioral assessment via digital imaging,

described above.

GBT transposons

For a discussion of GBT transposons used, please refer to Appendix A; Figure 21.

Zebrafish collection and preservation

Embryos were collected at day zero, approximately one-hour post fertilization (pf) and

placed into 100x15 mm Petri dishes with no more than 100 embryos per dish. These

dishes were then stored in a 30° C incubator. Dead and unfertilized embryos were

removed. At three dpf, fish were then transferred into 60x15mm Petri dishes with no

more than 20 fish per dish for fluorescent-based genotyping according to Davidson et

al.[36]

All data points represent at least three independent measurements on independent

biological samples with at least ten animals measured for each replicate.

NMDAR inhibitor administration. To test the effect of APV, an NMDAR competitive

inhibitor, on the nicotine response (28, 29), fish in "Pre-treatment" and "Acute" groups

were each placed in a solution of ten micromolar APV immediately prior to receiving

either a nicotine pre-treatment ("Pre-treatment" group) or embryo water ("Acute"

group) on day five at 9 AM. After a one-minute incubation, the water was replaced

with fresh embryo water. "Pre-treatment" and "Acute" groups were administered

nicotine after an eight-hour incubation as described above.

Mecamylamine administration. To test the affect of mecamylamine, a nicotinic

receptor competitive inhibitor on the nicotine response[83] fish in the "Pre-treatment"

and "Acute" groups were each placed in a solution of 10 micromolar mecamylamine

(Sigma Aldrich) one hour prior to receiving a testing dose of nicotine on day five at 5

PM. After the incubation, the water was replaced with fresh embryo water. The groups

were administered nicotine as described above. When used with the conditioned place

preference assay, larval zebrafish were treated with mecamylamine as described above

one hour prior to the initial training with nicotine. This administration of

mecamylamine was performed while larvae were in their initial housing.

Response to noxious chemicals. To test the response of each mutant to noxious

chemicals, allyl isothiocyanate (AITC - mustard oil; 377430; Sigma Aldrich; diluted to

a final concentration of $100\mu M$ in 1% DMSO[87]) was administered to the zebrafish at

five days post fertilization. 1% DMSO served as vehicle control. Fish were sorted into

mutant and wild type clutches (sorting by fluorescence) and were given a dose of AITC.

Observation of movement was determined after 30 seconds using a digital camera as

described for the nicotine administration above.

Response to noxious temperatures. To determine the noxious temperature response

profile[87] for each mutant, fish were sorted fluorescently into mutant and wild-type

groupings with minimal embryo water (2 ml). These plates of fish were then filled to normal levels (10 ml) with either hot embryo water (42°C) or cold embryo water (4°C). Locomotor response was observed after 30 seconds in the adverse condition as described above.

chrna1[b107] *heterozygous zebrafish*. Heterozygotes carrying a hypomorphic allele in the cholinergic receptor, nicotinic, alpha polypeptide 1 *chrna1* ([84]; obtained from the Zebrafish International Resource Center) were out-crossed to generate mixed clutches of sibling animals of wild-type and *chrna1* heterozygous genotypes for nicotine response assessment. In a parallel and blinded analyses, individual animals were then isolated and individually PCR genotyped as either wild-type or *chrna1* heterozygotes.

GBT mutant screening

Mutant zebrafish were generated by the co-injection of plasmid DNA encoding each GBT plus Tol2-encoding synthetic mRNA as described [37]. Adult fluorescent protein-expressing F_1 transposon heterozygous fish were out-crossed to wild-type fish to generate a heterogeneous population of F_2 larvae. The embryos were then separated into fluorescent-positive and fluorescent-negative groups and placed into similarly sized fish groupings for nicotine response assessment as described above (Figure 5). The response of the pre-treated GBT-positive larvae was subtracted from the pre-treated GBT-negative response to determine the response differential in these lines. To ensure that GBT-mutations did not alter movement response, the basal movement rate in the control response was also monitored. Using expression linkage analysis to estimate

copy number[36], we conservatively estimate at least 213 GBT-expressed loci were screened using this approach, 178 GBT-P9 and 35 GBT-R15. Candidate loci were subsequently out-crossed at least 4 times, recovering the noted linked nicotine response profile in each subsequent generation. The resulting alleles have been named $bdav^{mn30}$, $hbog^{mn31}$, and $yulb$ selected after Bette Davis, Humphrey Bogart and Yul Brynner, celebrities who died of tobacco-related causes and to enhance communication that genetic background can and does alter the response to addictive drugs.

Southern blot analysis for linked loci

Genomic DNA isolation and Southern blot hybridization was conducted as previously described(30). Genomic DNA samples were isolated from GFP-positive and GFP-negative embryos for the *bdav* locus digested with a combination of BsoB1 and BsrB1. Blots were probed with a 609 bp GBT-P9-specific probe generated by PCR (5'-GGGTGAAGGTGATGCAACATACGG -3' and 5'-TCATCCATGCCATGTGTAATCCCA -3'). Genomic DNA samples were isolated from RFP-positive and RFP-negative embryos for the *hbog* locus digested with Ase1. Blots were probed with a 585bp GBT-R15-specific probe generated by PCR (5'-GTCATCAAGGAGTTCATGCGCTTCA -3' and 5'-GATGTCCAGCTTGATGTCGGTCTTGT-3').

5' and 3' rapid amplification of cDNA ends (RACE)

Total RNA was isolated from 10 three day- old zebrafish larvae using RNeasy RNA isolation kit (Qiagen). First strand cDNA was synthesized in a 10 μl reaction from

250ng of total RNA using Superscript II reverse transcriptase (Invitrogen). 5' and 3'

RACE were conducted on this cDNA as previously described[37, 128]

RT-PCR

RNA was prepared from clutches of at least ten sibling animals at five days of age.

RNA was reverse transcribed using Superscript II, and two microliters of reverse

transcription reaction was used for PCR. For *hbog*, wild-type primers used were

located in Exon 3 and Exon 9 (Exon 3- 5'-

CGACCACCTAGAGATGGAGGGATCAG-3' Exon 9-

5'GGGAAAACGCTGACGGTTGGAG-3'). Mutant *hbog* primers used the same Exon

3 primer as above along with a GBT-R15 primer located within the mRFP (GBT-R15 –

5'-CGCCCTTGGTCACCTTCAGCTT-3'). For *bdav*, wild-type primers were located in

Exon 1 and Exon 5 (Exon 1- 5'- TGCAGCCTCATCCAGCATCAATCT -3' Exon 5-

5'-TGCAATGAGGTTGGCCAGGAAA -3'). Mutant *bdav* primers used the same

Exon 5 primer as above along with a GBT-P9 specific primer located within the GM2-

GFP (GBT –P9 – 5'- TGGGATTACACATGGCATGGATGAAC -3'). For control

primers, ß-actin primers as previously described (ß-actin-ex4F1 and ß-actin-ex4R1 (*28*))

were used. For determination of knockdown in *bdav* hetereozygous and homozygous

fish, genotyping and semi-quantitative knockdown data was obtained following RT-

PCR of cDNA from individual embryos.

Genetic analysis of bdav.

The number of single chromosome, GFP-linked fish from the initial *bdav* founder line that did not produce offspring with a deficiency in nicotine response was 0 out of 107 total fish, indicating tight genetic linkage. The standard linkage assessment such as the traditional logarithm of odds (LOD) formula as described by Morton [129]is difficult to assess in this screening paradigm (and would yield a nearly infinite LOD score). Consequently, we believe the likelihood that the observed nicotine response mutation is not linked to the GFP-tagged locus is very low.

Cre reversion

Cre coding sequence was PCR amplified using primers BglII/Cre-F1 (5'-ACGAGATCTACAAGATGTCCAATTTAC-3') and Spe/Cre-R1 (5'-AGCACTAGTTTAATCGCCATCTTCCA-3') and peGFP-Cre (Halpern laboratory) as a template. PCR fragment was digested with BglII and SpeI and cloned into BglII-SpeI digested pT3TS[130]. Synthetic Cre-encoding mRNA was synthesized from XbaI-linearized plasmid pT3TS-Cre and 50pg injected into homozygous $hbog^{mn31}$ embryos and raised to adulthood. These animals were out-crossed, and the resulting offspring were molecularly genotyped for Cre-mediated reversion at the *hbog* locus using primers *hbog*InsertF1 (5'-TGGTCCATTCTCATTGAGCAGCG -3') and *hbog*InsertR1 (5'-GCAGAAAGCGGTTCCAGGTTTGA -3') resulting in a shifted gene product. The nicotine response profile was subsequently assessed on this allele ($hbog^{mn32}\Delta$GBT).

In situ *hybridization*

Whole mount *in situ* hybridization was conducted as described[131]. Partial *hbog*

coding sequence was amplified using primers 5'-CTAGGCAAATGGGACTCACC-3'

and 5'-CTCTTGTTCACCAGATCCAG-3' and cloned into Strataclone vector. *hbog*

antisense probe were synthesized by linearizing with Xba1 and using T3 RNA

polymerase. Partial *bdav* coding sequence was amplified using Exon 1 primer as

described above and 5'-TGTGGCGTACAGTTTGGAGATCAG -3' and cloned into

Topo vector (Invitrogen). *bdav* antisense probe were synthesized by linearizing with

Xba1 and using T3 RNA polymerase.

Photography of mutant lines

Photography of mutant lines, both in live imaging of fluorescence and imaging of fixed

embryos, was accomplished using a microscope and a consumer grade camera. For

imaging of the fluorescent pattern of *hbog*, embryos were embedded within a 1.5% low-

melt agarose solution and photographed using a Zeiss Apatome and AxioVision 4.1

software. This imaging procedure was later replaced with SCORE imaging as described

in Petzold et. al 2010[132] found in Appendix B.

Isolation of full coding region for hbog/gabbr1.2

We performed a BLASTN against publicly available sequences in the NCBI database.

The identified sequence was highly similar to accession number XM 682935; a

predicted sequence similar to GABA-B receptor 1. To fully characterize the coding

region of this sequence, we performed a 5'RACE with primers located in Exon 9

(above) and Exon 6 (5'- TGTTCACCAGATCCAGGGCCATT -3') of the predicted

sequence. This resulted in a complete sequence of untranslated Exon 1, and the partially translated Exon 2 upstream of the predicted coding sequence. The predicted full-length coding sequence for *hbog/gabbr1.2* has been submitted to GenBank under accession number XM 682935 .

Phylogenetic analyses

We performed a BLASTN of the coding region of *hbog* against the Ensembl zebrafish genomic DNA database (Zebrafish genome assembly – Zv 6). This result was highly similar to Ensembl Gene ID ENSDARG00000016667. We then used the "ortholog prediction function" to generate a predicted orthologic tree. We repeated this procedure using the *bdav* coding sequence. The resultant BLASTN of the coding region identified as being highly similar to Ensembl Gene ID ENSDARG0000008243.

Environmental Differential Plates

Choice plate environments were created by modifying a standard 100mm petri dish. These modifications created either a differential in light, texture or both. To generate differences in light, an opaque black colored electrical tape was wrapped around the outside of the dish, covering the entire dish for training dishes and one half of the dish (as measured with a ruler) for choice plates. If color choice was being examined, a colored film was applied to the bottom of the plate. Color was normalized by matching both saturation and value (HSV scale) within Adobe Photoshop to create similar intensities of light. To generate differences in texture, plates were scratched using a medium grit sand paper (60 grit) in a random but circular pattern. For environmental

conditioning plates, the entire plate was scratched, for choice plates, a tape barrier was placed along the center line preventing scratches on half of the dish. For combination testing, a similar procedure was followed combining the two environmental changes upon the same dish. After the environmental changes were applied, dishes were washed with embryo water to remove extra particles and possible chemical residues revealed within the environmental modification process.

Place Preference Procedure

To determine initial preference to environmental cues, zebrafish larvae, 5dpf, were placed into a testing environmental plate, testing either differences in response to light, texture or both, in groups of 20 fish per dish (as described above). Placement of zebrafish was analyzed using either photography or videography as described above for a period of 35 minutes. After this exposure to the environmental testing plates, fish were returned into initial housing dishes within the bottom lit incubator for a period of at least 1 hour. After this recovery time, in the event of nicotine conditioning, larvae were administered a single dose of nicotine immediately followed by being placed into a conditioning plate, placed within the photography cabinet for a period of one hour. Control fish were treated in a similar manner, without the exposure to nicotine. A preference was then taken in a similar manner to that described previously. This procedure was then repeated upwards of 4 times, depending on the experimental conditions. (See Figure 13 for a diagrammatic of the procedure)

Determination of place preference

Place preference was assessed by digital snapshot capture of larvae within conditioning plates using a standard point and shoot camera on an interval timer (Nikon Coolpix S10). This setting takes individual photographs upon a set time interval schedule determined by the user. For the purposes of this study, time intervals of either 1 minute or 5 minutes were used. Placement was determined as the percentage of larvae in a given location at the moment the photograph was obtained. With the larval zebrafish being allowed to randomly arrange themselves within the testing environment, a lack of preference would be visualized as a value of 50% of the fish located on the given counted side. A preference would be visualized as a value different from 50% with values higher representing a preference towards the counted environment and less than 50% representing a preference against the given counted environment. For color preference, rather than counting a singular side of preference, a percentage of the total number of larvae in a given colored environment was determined. Because of the equal area arrangements of the colored environments, a preference for a given environment was considered a value higher than 33.3%. A preference against a specific environment was considered a value lower than 33.3%. No preference was considered a value of 33.3%.

Chapter 5

Conclusions and Future Directions

This study presents the larval zebrafish as a model for genetic assesment, within conserved biological paradigms that are a part of complex behavioral pathways of addiction and learning. In particular, the large family size of the zebrafish allows the detection of a single gene's contribution within a multi-loci behavioral response. With the added benefit of the gene-breaking transposon, an efficient and powerful forward genetic screen can be conducted by a single researcher or within a team to examine a specific pathway of study. The robust assays that have been presented within this study serve to highlight only a small number of the possible uses of such an assay within the larger system. The methods described provide the framework to further explore the mechanism of addiction, behavioral disorders and other complex behavioral systems. Some potential routes of study are described below.

Discovery of necessary and sufficient neurons for nicotine response

The innovations implemented within the gene-breaking transposon, such as the inclusion of Cre-recombinase recognition sites for ease of reversion and a protein-trap, allow for a more specific and directed study. Moreover, these sites also allow for the determination of the specific neural structure and function of individual neurons within the zebrafish brain. For example, a determination about which of the many $GABA_B$ receptor-expressing neurons are critical for the observed physiological response to

nicotine can be made. This would be accomplished by engineering a gene-specific

driven Cre-recombinase line of zebrafish to cross to the *hbog* mutant line. The co-

localization of the gene-specific Cre-recombinase and the GBT-R15 *hbog* would

produce a localized reversion within the gene-specific neurons allowing the gene-

specific function to be determined. The current limitation to this research approach is

the lack of gene-specific Cre-recombinase lines of zebrafish.

Development of nicotine based reporter lines to explore NRTs and interventions

The fluorescent based gene-tags and reporters that are included within the GBT protein-

tagging, polyadenilation and combination traps take advantage of the inherent

transparency of the larval zebrafish. With the inclusion of the protein-specific

expression tagging that is present within the GBT-R14, R15 and RP2 vectors (Appendix

A), the possibility of a biased approach to screening was possible. Aside from reaching

beyond the single reading frame that is being utilized currently in these trapping

vectors, other fluorescent-based technologies can take advantage of the transparent

nature of zebrafish larvae. Specifically, it has been shown that transgenic zebrafish

reporter lines can be readily made using the Tol2Kit Gateway-based construction

system[133]. This system takes advantage of the *Tol2* transposon to create a non-

mutagenic expression vector that produces gene specific fluorescent expression. By

using a method similar to this, it is possible to create an expression-tagged line of

zebrafish that is responsive to nicotine. The nicotinic receptor subunits have been

studied extensively [134, 135] and many of the regulatory elements of these subunits

have been characterized in humans and rodents[136, 137]. Taking advantage of this

information would allow researchers to first find the regulatory elements that drive the expression of the nicotinic receptor and then use these elements to drive a fluorescent-based marker. This expression-based line of fish would be able to determine when nicotinic receptors are being upregulated in the presence of nicotine or other stimuli. In combination with the GBT screening process, it would be possible to examine, in real time, the specific effects that the mutated gene would have on nicotine receptor levels. Additionally, with the current strides within the chemical screening field, this type of reporter fish has the potential of aiding in the discovery of a new nicotine replacement intervention (Figure 19). With this line of fish, a researcher has the ability to apply a large number of chemicals in combination with nicotine or alone and observe the resulting fluorescence. A nicotinic receptor agonist, or nicotine alone, would produce a GFP response alone and have an enhanced response in the presence of nicotine due to an upregulation of the nicotinic receptor (Figure 19B). A nicotinic receptor antagonist should lessen the response of GFP in the fish (Figure 19C). Using this information, a panel of drugs could easily be screened using a number of these transgenic fish housed within a 96 well plate (Figure 19D). By monitoring the GFP expression levels of these fish, novel antagonists and agonists to the nicotinic receptor can be discovered.

Furthermore, with the use of a non-*Tol2* based GBT, it would be possible to perform a biased based screen similar to that previously mentioned. This screening approach would be able to discover genes that affect both the expression of the nicotine driven GFP and the location of the GFP providing yet another method for examining the method of determining the mechanism of nicotine response and addiction. The use of a
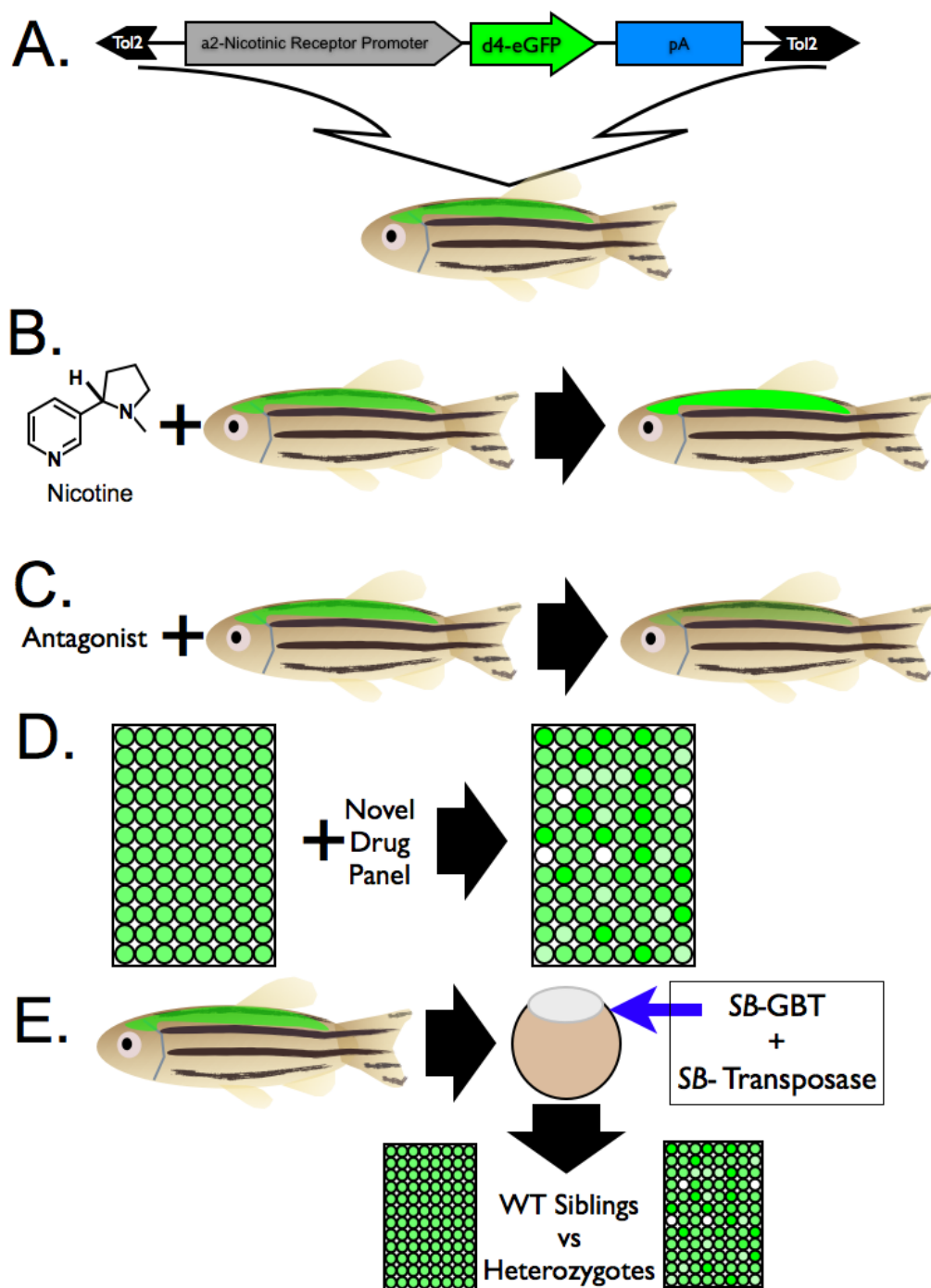
Figure 19. Development of a nicotinic receptor short-half-life-GFP transgenic allows further mutant screening and chemical discovery. A. A short-half-life-GFP based transgenic zebrafish can be developed using the Tol2Kit gateway system to create a transiently expressing GFP fish. A low level of GFP will be on at all times due to the expression of the nicotinic receptor in the nervous system of the zebrafish. B. The addition of nicotine or a nicotinic receptor agonist should increase nicotinic receptor expression leading to an increase in GFP. C. The addition of a nicotinic antagonist should decrease nicotinic receptor expression leading to a decrease in GFP. This line can be employed in novel drug screening by looking for changes in GFP expression (D) or in conjunction with a non-*Tol2* based mutagenesis screen (E).

non-*Tol2* based GBT system would be required dependent on the method of creating the nicotinic reporter line. If the gateway-cloning system was described as mentioned previously, an injection of *Tol2* mRNA that accompanies the GBT construct has the potential of re-mobilizing the Gateway-based *Tol2* ends creating both a localized 8bp duplication and the possibility of concatamerization, localized genome rearrangements and excision of the Gateway-based construct. The use of an alternate transposon system, such as *Ac/Ds*, *PiggyBac*, *Hermes* or *Sleeping Beauty*[138], as a vehicle for either the reporter construct or the GBT construct would allow for a systematic forward genetic based screen within such a background (Figure 19E). Alternatively, the initial reporter line of fish could be created using a non-*Tol2* based method such as an alternative transposon or simply injection of the transgene into a once-cel embryo. Additionally, once a stable line of fish was created by any of the previously mentioned methods, these reporter fish could be crossed into the screening lines of fish at an earlier time point, either at the F1 or F2 generations (See Figure 5 for initial screening paradigm details)

Use of larval assay with putative NRTs or current NRTs (varenacline) to help describe mechanism of action.

An immediate potential possible use of the larval-based nicotine response assay is the exploration of current and putative nicotine replacement therapies. Many studies have show that the larval zebrafish is amenable to the discovery of novel drugs when deployed in a screening approach[127, 139, 140]. The simplicity of the nicotine response assay allows the user to screen through a large number of potential drugs in an expedited manner. Additionally, drugs previously approved by the Food and Drug

Administration that play a role within proposed mechanisms of addiction, such as the

serotonergic, dopiminergic and gabaergic pathways can be tested in a similar manner to

that described with mecamylamine (Methods found in Chapter 4, results in Chapter 2

and 3). This would provide an accelerated approach to examine the potential use of

drugs as a nicotine replacement therapy or intervention without the need for phase 1

clinical trials. Furthermore, the specific route of action of currently used drugs such as

varenacline (Chantix[141]) can be examined to elucidate a mode of function. Learning

more about the mechanism of action of such drugs could provide insight into the

addiction process as a whole as well as providing additional mechanisms for improved

intervention technologies.

Nicotine paired with other drugs to determine common genes within pathways of

addiction

Drug abuse as a whole accounts for more than 55 million annual deaths worldwide

[104]. Despite these overwhelming statistics, the majority of drug users began use with

abuse of a legal substance such as nicotine or alcohol [104, 142]. One of the benefits of

performing a larval-based behavioral screen is the amenability of such a screen to the

use of alternative stimuli including non-addictive substances, legally obtainable

substances and illicit substances. With slight alterations to the methods presented, a

screen could be developed to search for genes involved in the response mechanism to a

number of stimuli (Figure 20A). Once a gene is determined to have an affect upon the

response to any of these stimuli, alternative stimuli could be administered either as

larvae in a similar method to that described here or as adults, utilizing previously

described methods [28, 143].

The idea that the use of a drug of abuse can lead to progressive illicit drug use has been

known since the early 1970s with the term "gateway drug" being first coined by Robert

Dupont in 1984 [144]. The "gateway drug" hypothesis states that an individual is more

likely to become a user of illicit drugs when exposed to "weaker" drugs at an early age.

These "weaker" drugs of addiction (nicotine, alcohol and marijuana) provide a

"gateway" for the user to experience more addictive and illicit drugs of abuse (cocaine,

methamphetamine, morphine etc)[145]. It is thought that these "gateway" drugs act to

train juveniles into the complex behaviors that are associated with use of illicit drugs of

abuse. The added combined pathways of reward that are stimulated by use of tobacco

help to create a perceived need for more rewarding stimuli as well as enhancing the

rewarding attributes of a secondary stimulus[146]. The specific genes that create this

combined response to stimuli can be examined with the methods described. A

combinatory approach to addictive studies would allow the researcher to examine

shared pathways in addiction as well as a direct exploration into the "gateway drug"

hypothesis in a model organism. This approach can be used to elucidate the sufficient

and necessary genes that allow for such an addiction to occur. Additionally, a

combinatory approach could be employed in conjunction with a small molecule based

screening process to for initial experiments in drug discovery for the dampening of

addiction in general or to specific drugs of abuse.

Nicotine assay paired with stress assay to examine linked pathways between behavioral disorders and addiction (Developed by Clark et al. in preparation)

The current trend towards an individualized approach to medicine requires an increased examiniation of the role that genetic and epigenetic factors play on complex behaviors. The develompental and early life stage environmental interactions combined with the genetic background of an individual can prime an individual for later reactionary responses including and trigger the onset of a behavioral disorder [147]. Addiction has been shown to share many common pathways to other behavioral disorders and is often found to present itself along these disorders [148]. For example, addiction to alcohol or nicotine has often been diagnosed in patients that suffer from schizophrenia, depression and anxiety [149, 150]. Predisposition to addiction within major depressive disorder and generalized anxiety disorder has been shown to share upwards of a 70% and 50-60% inheritance due to genetic variations within the population [151-153]. Additionally, the exploration of the role that the stress response plays within the onset and progression of addiction will permit the development of novel combinatory interventions. With the use of the nicotine response assay in larval zebrafish, it would be possible to further explore how these eraly stressing events can lead to differences within either conditioned place preference or general nicotine response. Such a method could attempt to compare stressed and unstressed larvae in the presence of nicotine; specifically examining further locomotor changes into adulthood. Additionally, a similar larval based screen could be developed to examine the genes involved in such shared pathways. Understanding the role the stress response plays within the onset and maintenence of addiction would

allow for more complex indicators of disease and improved medical and psychological

intervention.

Assays in juveniles and adults

Withdrawal occurs in an individual after cessation of use of a drug of dependence or

abuse. Nicotine withdrawal produces a variety of physiological and psychological

changes in a subject. Physiological changes include decreased adrenaline, cortisol, heart

rate and an increase in weight, resting metabolic rate, startle response and drug

metabolism[154] among many other symptoms. Behavioral changes include an increase

in aggression, anxious behavior and eating as well as a decrease in performance and

focus in precision-based tasks. The combinations of these symptoms provide the major

cause of relapse into drug use from members who have been abstaining from

nicotine[155-157]. Of these symptoms, in adult zebrafish, there has been some research

into anxious behaviors[158, 159] including a study examining withdrawal to

amphetamine[48] and cocaine [160], but little else that has been directly correlated with

withdrawal. While these studies could be recapitulated using nicotine as the addictive

substance rather than amphetamine, the advantage of the present screening technique

would be lost. Alternatively, it is possible to examine the startle response of larval

zebrafish beginning at 48hpf [161]. A similar approach could be used with juvenile

zebrafish exposed to chronic, low doses of nicotine, acute high-doses of nicotine or

multiple doses of nicotine over time (Figure 20B). A true larval-based system could not

be employed within these studies due to the need for a withdrawal response to be

developed over time[162]. However, the use of a larval system for the initial exposure
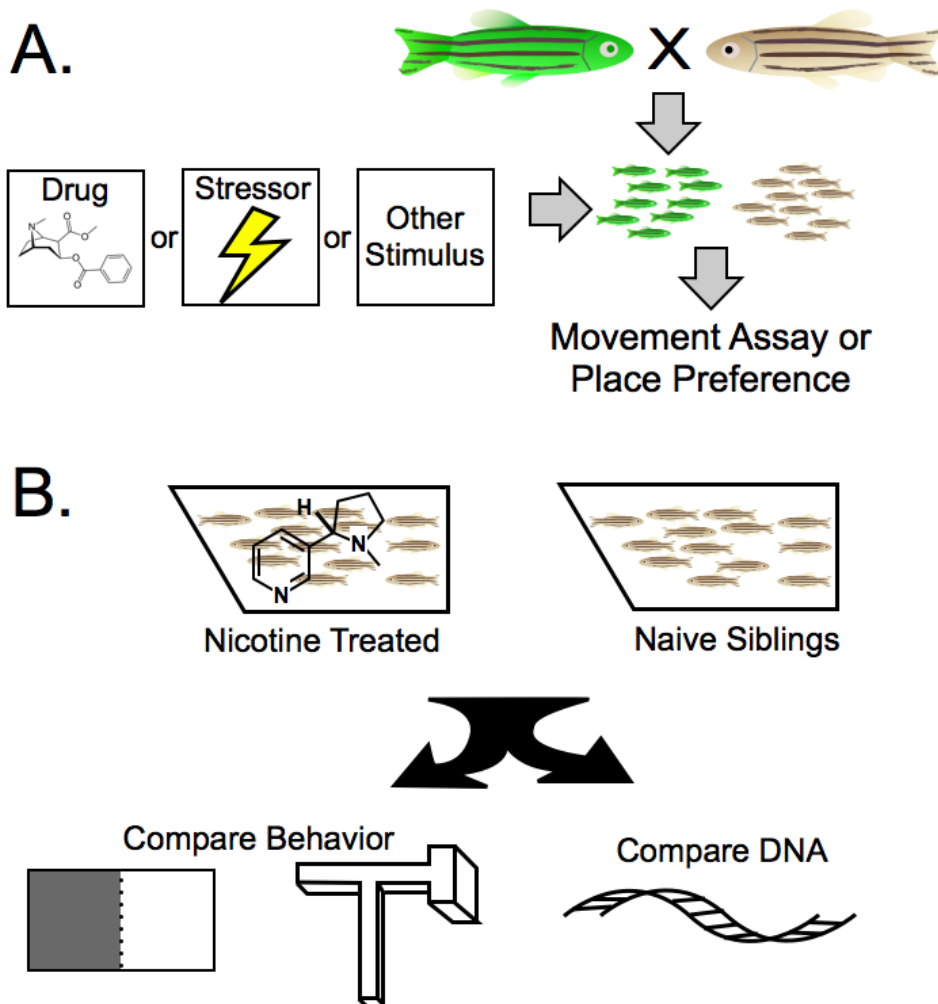
Figure 20. Use of the presented assays with slight alterations allow for greater depth of study. A. With slight alterations to the protocols presented allow testing of a variety of complex behavioral traits including stress, illicit drug addiction, legal drug addiction and their shared pathways of action. B. Longitudinal studies within larval and juvenile zebrafish exposed to acute or chronic levels of nicotine allow for direct comparisons of genome transcript levels and complex adult behaviors previously described.

and

training of a zebrafish presents additional advantages and opportunities. One of the benefits to working with a developmental model is the ability to look at the long-term effects of early exposure to a drug of abuse[163]. Combined with the GBT screen, a long-term longitudinal study examining the permanent genetic and behavioral changes associated with an early exposure to a drug such as nicotine is possible. This longitudinal study would raise larvae that have been exposed to multiple doses of nicotine through their development and compare their movement, behavior and specific genetic markers to their non-nicotine exposed siblings (Figure 20B). Such a study, paired with microarray and genome-wide sequencing technology, could indicate genes that are directly linked to addiction to nicotine. This would in turn provide a genetic marker that could be used within a human population to determine both risk involved in smoking and susceptibility to addiction and indications as to affectivity of intervention.

Genome-wide association studies to examine genetic and epigenetic changes in response to nicotine

The behavioral sensitization of the larval zebrafish that is described following multiple doses of nicotine presents additional avenues of discovery. Although untested as of yet in the zebrafish model system, in other models, sensitization has been demonstrated to persist after prolonged periods of nicotine abstinence[164]. This persistence of phenotypic response suggests an epigenetic regulation of the nicotine response. These epigenetic modifications occur by modifying chromatin structure which in turn alters the transcriptional machinery and binding of transcriptional proteins to the DNA. There have been several studies exploring the modification of histone acetylation that have

shown this as a regulator of sensitization in cocaine addiction.. Furthermore, a pharmacological inhibition of histone deacetylation (HDAC) prior to administration of cocaine has shown to quench the sensitization response[165]. Using the nicotine response model presented here, it would be possible to examine the effects of HDAC inhibitors both upon the action of sensitization and longterm upon nicotinic receptors and epigenetic markers such as FosB. Additionally, next generation sequencing techniques can be employed to examine genome wide transcriptional level changes associated with nicotine sensitization. This information could then be used to identify genes that play a role in the development of sensitization, which could in turn be used as a genetic and molecular marker for smoking addiction.

Conclusions

This study has brought to light the complexity of the genetics of behavior. This behavioral diversity must be viewed as major players within the specific response (ChRNA complex units) that are altered by modifiers to this response. Similar genetic controls contribute to the diversity that is seen within pigmentation. For example, much of the natural variation in pigmentation lies in a genetic locus, SLC24A5, that subtly alters melanocyte function rather than within the TYR locus, the latter which removes all pigmentation completely[166]. The discovery of this type of gene pathway presents the possibility of many complex genetic pathways following a similar paradigm of a central pathway of genes that are modified by the actions of many other interacting genes that together encode the range of variations found in a population for a specific phenotype. Complex behaviors such as stress, addiction and learning seem to be

obvious candidates for multi-genic complexly controlled pathways. This study presents the larval zebrafish as a mechanism to explore these alterations of complex genetic pathways in a forward genetic screen.

Within the context of behavioral science as a whole, the use of a developmental model organsism within a forward genetic screen for novel loci is not a novel concept [167-170]. However, *bdav* and *hbog* represent the first fully characterized mutant lines of zebrafish that have been found in a forward genetic behavioral screen. Additionally, *bdav*, *hbog*, and the previously characterized *nic1*, represent the first dominant behavioral mutants presented in the zebrafish. Furthermore, *hbog* represents the first published conditional insertional mutant in the zebrafish and the first insertional behavioral mutant in the zebrafish.

The inclusion of other modifiers to behavior, such as the response to temperature, touch and other noxious stimuli, not only adds power to the specificity of the assay but also allows the researcher to examine shared pathways within the behavioral response as a whole. Additionally, by examining a combination of drugs of abuse, like opioids and depressants, paired with nicotine this assay could allow for a determination of the action of a previous addiction within the behavioral addictive pathways as a whole. Exploration of shared pathways would allow for the discovery of independent mechanisms of action within traditional behavioral pathways possibly providing more insight into complex behavioral and psychological based disease paradigms. Alternatively, the exploration of stimulus specific pathways allows for a greater

understanding of the mechanism of action of the pathway being studied providing more

options for a chemical intervention or treatment. This inclusion would also allow for the

discovery of a broader range of genes involved within the behavioral diversity that is

seen not only within zebrafish but also within life as a whole. Additionally, the

conditioned place preference assay outlined within this work represents an opportunity

to examine the behaviors represented within the learning and plasticity of memory and

the pathway it shares with the response to drugs of abuse. The combination of these

assays presents the researcher with a more complete paradigm of study of a number of

behavioral pathways including the stress response, cue based learning, memory and

other complex behaviors.

Cited References

1.   Jha, P. and F.J. Chaloupka, *Curbing the epidemic : governments and the economics of tobacco control*. Development in Practice. 1999, Washington DC: The World Bank.

2.   WHO, *WHO Report on the global tobacco epidemic, 2008: The MPOWER package*. 2008, Brazil: World Health Organization.

3.   Society, A.C., *Cancer Facts and Figures 2010*. 2010, American Cancer Society: Atlanta Georgia.

4.   ANRF, *Summary of 100% SmokeFree State Laws and Population Protected by 100% US Smokefree Laws*. 2010, American Nonsmokers' Roghts Foundation: Berkeley, California. p. 1-2.

5.   Repace, J.L., J.N. Hyde, and D. Brugge, *Air pollution in Boston bars before and after a smoking ban*. BMC Public Health, 2006. **6**: p. 266.

6.   Young, W.F., et al., *Economic impacts of the Pueblo Smoke-Free Air Act*. Am J Prev Med, 2010. **38**(3): p. 340-3.

7.   Dani, J.A. and M. De Biasi, *Cellular mechanisms of nicotine addiction*. Pharmacol Biochem Behav, 2001. **70**(4): p. 439-46.

8.   Hoffmann, D. and I. Hoffmann, *Significance of exposure to sidestream tobacco smoke*. IARC Sci Publ, 1987(81): p. 3-10.

9.   FAO, *Projections of Tobacco Production, Consumption and Trade to the year 2010*. 2003, Food and Agriculture Organization of the United Nations: Rome.

10.   CDC, C.f.D.C.a.P., *Cigarette smoking among adults--United States, 2007.*, in *MMWR Morb Mortal Wkly Rep*. 2008, Centers for Disease Control and Prevention: Atlanta, GA. p. 1221-1226.

11.   Bonnie, B., K. Stratton, and R. Wallace, *Ending the Tobacco Problem: A Blueprint for the Nation*. 2007, Washington, DC: National Academies Press.

12.   Shiffman, S., et al., *Use of smoking-cessation treatments in the United States*. Am J Prev Med, 2008. **34**(2): p. 102-11.

13.   McCarthy, W.J. and T. Kuo, *Support for benefit of physical activity on satiety, weight control, and diabetes risk*. Arch Intern Med, 2009. **169**(6): p. 634-5; author reply 635.

14.   Shiffman, S., *Smoking-cessation treatment utilization: The need for a consumer perspective*. Am J Prev Med, 2010. **38**(3 Suppl): p. S382-4.

15.   Smith, S.S., et al., *Comparative effectiveness of 5 smoking cessation pharmacotherapies in primary care clinics*. Arch Intern Med, 2009. **169**(22): p. 2148-55.

16.   Raw, M. and A. McNeill, *The prevention of tobacco-related disease*. Addiction, 1994. **89**(11): p. 1505-9.

17.   Davies, G.E. and T.J. Soundy, *The genetics of smoking and nicotine addiction*. S D Med, 2009. **Spec No**: p. 43-9.

18.   Edwards, A.C., et al., *A population-based twin study of the genetic and environmental relationship of major depression, regular tobacco use and nicotine dependence*. Psychol Med, 2010: p. 1-11.

19.   Spitz, M.R., et al., *The CHRNA5-A3 region on chromosome 15q24-25.1 is a risk factor both for nicotine dependence and for lung cancer*. J Natl Cancer Inst., 2008. **100**(21): p. 1552-6.

20.   Thorgeirsson, T.E., et al., *A variant associated with nicotine dependence, lung cancer and peripheral arterial disease*. Nature, 2008. **452**(7187): p. 638-42.

21.   Weiss, R.B., et al., *A candidate gene approach identifies the CHRNA5-A3-B4 region as a risk factor for age-dependent nicotine addiction*. PLoS Genet, 2008. **4**(7): p. e1000125.

22.   Thorgeirsson, T.E., et al., *Sequence variants at CHRNB3-CHRNA6 and CYP2A6 affect smoking behavior*. Nat Genet, 2010. **42**(5): p. 448-53.

23.   *Genome-wide meta-analyses identify multiple loci associated with smoking behavior*. Nat Genet, 2010. **42**(5): p. 441-7.

24.   Liu, J.Z., et al., *Meta-analysis and imputation refines the association of 15q25 with smoking quantity*. Nat Genet, 2010. **42**(5): p. 436-40.

25.   McKinney, E.F., et al., *Association between polymorphisms in dopamine metabolic enzymes and tobacco consumption in smokers*. Pharmacogenetics, 2000. **10**(6): p. 483-91.

26.   Caporaso, N.E., et al., *Current insight on trends, causes, and mechanisms of Hodgkin's lymphoma*. Cancer J, 2009. **15**(2): p. 117-23.

27.   Sokolowski, M.B., *Drosophila: genetics meets behaviour*. Nat Rev Genet, 2001. **2**(11): p. 879-90.

28.   Guo, S., *Linking genes to brain, behavior and neurological diseases: what can we learn from zebrafish?* Genes Brain Behav, 2004. **3**(2): p. 63-74.

29. Creaser, W., *The Technic of Handling the Zebra Fish (Brachydanio rerio) for the Production of Eggs Which are Favorable for Embryological Research And Are Available at Any Specified Time Throughout the Year*. Copeia, 1934(4): p. 159-161.

30. Kimmel, C.B., R.M. Warga, and T.F. Schilling, *Origin and organization of the zebrafish fate map*. Development, 1990. **108**(4): p. 581-94.

31. Caporaso, N., et al., *Genome-wide and candidate gene association study of cigarette smoking behaviors*. PLoS ONE, 2009. **4**(2): p. e4653.

32. Granato, M. and C. Nusslein-Volhard, *Fishing for genes controlling development*. Curr Opin Genet Dev, 1996. **6**(4): p. 461-8.

33. Driever, W., et al., *A genetic screen for mutations affecting embryogenesis in zebrafish*. Development, 1996. **123**: p. 37-46.

34. Haffter, P., et al., *The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio*. Development, 1996. **123**: p. 1-36.

35. Amsterdam, A. and N. Hopkins, *Retrovirus-mediated insertional mutagenesis in zebrafish*. Methods Cell Biol, 1999. **60**: p. 87-98.

36. Davidson, A.E., et al., *Efficient gene delivery and gene expression in zebrafish using the Sleeping Beauty transposon*. Dev Biol, 2003. **263**(2): p. 191-202.

37. Sivasubbu, S., et al., *Gene-breaking transposon mutagenesis reveals an essential role for histone H2afza in zebrafish larval development*. Mech Dev, 2006. **123**(7): p. 513-29.

38. Pichler, F.B., et al., *Chemical discovery and global gene expression analysis in zebrafish*. Nat Biotechnol, 2003. **21**(8): p. 879-83.

39. Kokel, D., et al., *Rapid behavior-based identification of neuroactive small molecules in the zebrafish*. Nat Chem Biol, 2010. **6**(3): p. 231-237.

40. Chen, E. and S.C. Ekker, *Zebrafish as a genomics research model*. Curr Pharm Biotechnol, 2004. **5**(5): p. 409-13.

41. Foley, J.E., et al., *Targeted mutagenesis in zebrafish using customized zinc-finger nucleases*. Nat Protoc, 2009. **4**(12): p. 1855-67.

42. Nasevicius, A. and S.C. Ekker, *Effective targeted gene 'knockdown' in zebrafish*. Nat Genet, 2000. **26**(2): p. 216-20.

43. Gerlai, R., et al., *Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects*. Pharmacol Biochem Behav, 2000. **67**(4): p. 773-82.

44. Darland, T. and J.E. Dowling, *Behavioral screening for cocaine sensitivity in mutagenized zebrafish*. Proc Natl Acad Sci U S A, 2001. **98**(20): p. 11691-6.

45. Lockwood, B., et al., *Acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening*. Pharmacol Biochem Behav, 2004. **77**(3): p. 647-54.

46. Lau, B., et al., *Dissociation of food and opiate preference by a genetic mutation in zebrafish*. Genes Brain Behav, 2006. **5**(7): p. 497-505.

47. Ninkovic, J. and L. Bally-Cuif, *The zebrafish as a model system for assessing the reinforcing properties of drugs of abuse*. Methods, 2006. **39**(3): p. 262-74.

48. Ninkovic, J., et al., *Genetic identification of AChE as a positive modulator of addiction to the psychostimulant D-amphetamine in zebrafish*. J Neurobiol, 2006. **66**(5): p. 463-75.

49. Webb, K.J., et al., *Zebrafish reward mutants reveal novel transcripts mediating the behavioral effects of amphetamine*. Genome Biol, 2009. **10**(7): p. R81.

50. Svoboda, K.R., S. Vijayaraghavan, and R.L. Tanguay, *Nicotinic receptors mediate changes in spinal motoneuron development and axonal pathfinding in embryonic zebrafish exposed to nicotine*. J Neurosci, 2002. **22**(24): p. 10731-41.

51. Levin, E.D. and E. Chen, *Nicotinic involvement in memory function in zebrafish*. Neurotoxicol Teratol, 2004. **26**(6): p. 731-5.

52. Levin, E.D., et al., *Timing of nicotine effects on learning in zebrafish*. Psychopharmacology (Berl), 2006. **184**(3-4): p. 547-52.

53. Eddins, D., et al., *Nicotine effects on learning in zebrafish: the role of dopaminergic systems*. Psychopharmacology (Berl), 2009. **202**(1-3): p. 103-9.

54. Kily, L.J., et al., *Gene expression changes in a zebrafish model of drug dependency suggest conservation of neuro-adaptation pathways*. J Exp Biol, 2008. **211**(Pt 10): p. 1623-34.

55. Bencan, Z., D. Sledge, and E.D. Levin, *Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety*. Pharmacol Biochem Behav, 2009. **94**(1): p. 75-80.

56. Parker, B. and V.P. Connaughton, *Effects of nicotine on growth and development in larval zebrafish*. Zebrafish, 2007. **4**(1): p. 59-68.

57.   Spence, R., et al., *The behaviour and ecology of the zebrafish, Danio rerio*. Biol Rev Camb Philos Soc, 2008. **83**(1): p. 13-34.

58.   Wilson, J.F., et al., *Population genetic structure of variable drug response*. Nat Genet, 2001. **29**(3): p. 265-9.

59.   Wienholds, E., et al., *Efficient target-selected mutagenesis in zebrafish*. Genome Res, 2003. **13**(12): p. 2700-7.

60.   Pickart, M.A., et al., *Functional genomics tools for the analysis of zebrafish pigment*. Pigment Cell Res, 2004. **17**(5): p. 461-70.

61.   Sivasubbu, S., et al., *Insertional mutagenesis strategies in zebrafish*. Genome Biol, 2007. **8 Suppl 1**: p. S9.

62.   Bill, B.R., et al., *A primer for morpholino use in zebrafish*. Zebrafish, 2009. **6**(1): p. 69-77.

63.   Koolhaas, J.M., et al., *Neuroendocrinology of coping styles: Towards understanding the biology of individual variation*. Front Neuroendocrinol, 2010.

64.   Petzold, A.M., et al., *Nicotine response genetics in the zebrafish*. Proc Natl Acad Sci U S A, 2009. **106**(44): p. 18662-7.

65.   Frohman, M.A., *Rapid amplification of complementary DNA ends for generation of full-length complementary DNAs: thermal RACE*. Methods Enzymol, 1993. **218**: p. 340-56.

66.   Balciunas, D., et al., *Harnessing a high cargo-capacity transposon for genetic applications in vertebrates*. PLoS Genet, 2006. **2**(11): p. e169.

67.   Crabbe, J.C., *Genetic contributions to addiction*. Annu Rev Psychol, 2002. **53**: p. 435-62.

68.   Nestler, E.J. and D. Landsman, *Learning about addiction from the genome*. Nature, 2001. **409**(6822): p. 834-5.

69.   Perkins, K.A., *Individual variability in responses to nicotine*. Behav Genet, 1995. **25**(2): p. 119-32.

70.   Feng, Z., et al., *A C. elegans model of nicotine-dependent behavior: regulation by TRP-family channels*. Cell, 2006. **127**(3): p. 621-33.

71.   Shim, I., et al., *Nicotine-induced behavioral sensitization is associated with extracellular dopamine release and expression of c-Fos in the striatum and nucleus accumbens of the rat*. Behav Brain Res, 2001. **121**(1-2): p. 137-47.

72.   Robinson, T.E. and K.C. Berridge, *The neural basis of drug craving: an incentive-sensitization theory of addiction*. Brain Res Brain Res Rev, 1993. **18**(3): p. 247-91.

73.   Vezina, P., *Sensitization of midbrain dopamine neuron reactivity and the self-administration of psychomotor stimulant drugs*. Neurosci Biobehav Rev, 2004. **27**(8): p. 827-39.

74.   Sakoori, K. and N.P. Murphy, *Enhanced nicotine sensitivity in nociceptin/orphanin FQ receptor knockout mice*. Neuropharmacology, 2009. **56**(5): p. 896-904.

75.   Correll, J.A., et al., *Nicotine sensitization and analysis of brain-derived neurotrophic factor in adolescent beta-arrestin-2 knockout mice*. Synapse, 2009. **63**(6): p. 510-9.

76.   Pickering, C., et al., *Sensitization to nicotine significantly decreases expression of GABA transporter GAT-1 in the medial prefrontal cortex*. Prog Neuropsychopharmacol Biol Psychiatry, 2008. **32**(6): p. 1521-6.

77.   Matta, S.G., et al., *Guidelines on nicotine dose selection for in vivo research*. Psychopharmacology (Berl), 2007. **190**(3): p. 269-319.

78.   Wise, R.A., *The role of reward pathways in the development of drug dependence*. Pharmacol Ther, 1987. **35**(1-2): p. 227-63.

79.   DiFranza, J.R. and R.J. Wellman, *Sensitization to nicotine: how the animal literature might inform future human research*. Nicotine Tob Res, 2007. **9**(1): p. 9-20.

80.   Picciotto, M.R., *Nicotine as a modulator of behavior: beyond the inverted U*. Trends Pharmacol Sci, 2003. **24**(9): p. 493-9.

81.   Clarke, P.B. and R. Kumar, *The effects of nicotine on locomotor activity in non-tolerant and tolerant rats*. Br J Pharmacol, 1983. **78**(2): p. 329-37.

82.   Ksir, C., et al., *Exposure to nicotine enhances the behavioral stimulant effect of nicotine and increases binding of [3H]acetylcholine to nicotinic receptors*. Neuropharmacology, 1985. **24**(6): p. 527-31.

83.   Rose, J.E., et al., *Mecamylamine combined with nicotine skin patch facilitates smoking cessation beyond nicotine patch treatment alone*. Clin Pharmacol Ther, 1994. **56**(1): p. 86-99.

84.   Sepich, D.S., et al., *An altered intron inhibits synthesis of the acetylcholine receptor alpha-subunit in the paralyzed zebrafish mutant nic1*. Genetics, 1998. **148**(1): p. 361-72.

85. Kelsey, J.E., et al., *Low doses of dizocilpine block the development and subsequent expression of locomotor sensitization to nicotine in rats*. Psychopharmacology (Berl), 2002. **161**(4): p. 370-8.

86. Shoaib, M. and I.P. Stolerman, *MK801 attenuates behavioural adaptation to chronic nicotine administration in rats*. Br J Pharmacol, 1992. **105**(3): p. 514-5.

87. Prober, D.A., et al., *Zebrafish TRPA1 channels are required for chemosensation but not for thermosensation or mechanosensory hair cell function*. J Neurosci, 2008. **28**(40): p. 10102-10.

88. Thisse, B., et al., *Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening*. Methods Cell Biol, 2004. **77**: p. 505-19.

89. Ojima, N., M. Yamashita, and S. Watabe, *Quantitative mRNA expression profiling of heat-shock protein families in rainbow trout cells*. Biochem Biophys Res Commun, 2005. **329**(1): p. 51-7.

90. Somer, L., et al., *The eukaryote chaperonin CCT is a cold shock protein in Saccharomyces cerevisiae*. Cell Stress Chaperones, 2002. **7**(1): p. 47-54.

91. Valpuesta, J.M., et al., *Structure and function of a protein folding machine: the eukaryotic cytosolic chaperonin CCT*. FEBS Lett, 2002. **529**(1): p. 11-6.

92. Ericson, E., et al., *Off-target effects of psychoactive drugs revealed by genome-wide assays in yeast*. PLoS Genet, 2008. **4**(8): p. e1000151.

93. Lund, P., *Molecular Chaperones in the Cell*. 2001, Oxford: Oxford University Press.

94. Nashmi, R. and H. Lester, *Cell autonomy, receptor autonomy, and thermodynamics in nicotine receptor up-regulation*. Biochem Pharmacol, 2007. **74**(8): p. 1145-54.

95. Li, M.D., et al., *A genome-wide scan to identify loci for smoking rate in the Framingham Heart Study population*. BMC Genet, 2003. **4 Suppl 1**: p. S103.

96. Balciunas, J.K. and B. Villegas, *Laboratory and realized host ranges of Chaetorellia succinea (Diptera: Tephritidae), an unintentionally introduced natural enemy of yellow starthistle*. Environ Entomol, 2007. **36**(4): p. 849-57.

97. Emson, P.C., *GABA(B) receptors: structure and function*. Prog Brain Res, 2007. **160**: p. 43-57.

98. Beuten, J., et al., *Single- and multilocus allelic variants within the GABA(B) receptor subunit 2 (GABAB2) gene are significantly associated with nicotine dependence*. Am J Hum Genet., 2005. **76**(5): p. 859-64.

99. Paterson, N.E., W. Froestl, and A. Markou, *The GABAB receptor agonists baclofen and CGP44532 decreased nicotine self-administration in the rat*. Psychopharmacology (Berl), 2004. **172**(2): p. 179-86.

100. Paterson, N.E., et al., *Positive modulation of GABA(B) receptors decreased nicotine self-administration and counteracted nicotine-induced enhancement of brain reward function in rats*. J Pharmacol Exp Ther, 2008. **326**(1): p. 306-14.

101. Terrence, C.F. and G.H. Fromm, *Complications of baclofen withdrawal*. Arch Neurol, 1981. **38**(9): p. 588-9.

102. Peng, C.T., et al., *Prolonged severe withdrawal symptoms after acute-on-chronic baclofen overdose*. J Toxicol Clin Toxicol, 1998. **36**(4): p. 359-63.

103. Malhotra, T. and I. Rosenzweig, *Baclofen withdrawal causes psychosis in otherwise unclouded consciousness*. J Neuropsychiatry Clin Neurosci, 2009. **21**(4): p. 476.

104. Rehm, J., B. Taylor, and R. Room, *Global burden of disease from alcohol, illicit drugs and tobacco*. Drug Alcohol Rev, 2006. **25**(6): p. 503-13.

105. Stewart, J. and A. Badiani, *Tolerance and sensitization to the behavioral effects of drugs*. Behav Pharmacol, 1993. **4**(4): p. 289-312.

106. Koob, G.F. and E.J. Nestler, *The neurobiology of drug addiction*. J Neuropsychiatry Clin Neurosci, 1997. **9**(3): p. 482-97.

107. Mohn, A.R., W.D. Yao, and M.G. Caron, *Genetic and genomic approaches to reward and addiction*. Neuropharmacology, 2004. **47 Suppl 1**: p. 101-10.

108. Yan, Y. and T. Nabeshima, *Mouse model of relapse to the abuse of drugs: Procedural considerations and characterizations*. Behav Brain Res, 2009(196): p. 1-10.

109. Tzschentke, T.M., *Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues*. Prog Neurobiol, 1998. **56**(6): p. 613-72.

110. Tzschentke, T.M., *Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade*. Addict Biol, 2007. **12**(3-4): p. 227-462.

111. Kusayama, T. and S. Watanabe, *Reinforcing effects of methamphetamine in planarians*. Neuroreport, 2000. **11**(11): p. 2511-3.

112. Pomerantz, S.M., *Dopaminergic influences on male sexual behavior of rhesus monkeys: effects of dopamine agonists*. Pharmacol Biochem Behav, 1992. **41**(3): p. 511-7.

113. Childs, E. and H. de Wit, *Amphetamine-induced place preference in humans*. Biol Psychiatry, 2009. **65**(10): p. 900-4.

114. Cunningham, C.L., et al., *Genetic differences in the rewarding and activating effects of morphine and ethanol*. Psychopharmacology (Berl), 1992. **107**(2-3): p. 385-93.

115. Chesler, E.J., et al., *Complex trait analysis of gene expression uncovers polygenic and pleiotropic networks that modulate nervous system function*. Nat Genet, 2005. **37**(3): p. 233-42.

116. Niwa, M., Y. Yan, and T. Nabeshima, *Genes and molecules that can potentiate or attenuate psychostimulant dependence: relevance of data from animal models to human addiction*. Ann N Y Acad Sci, 2008. **1141**: p. 76-95.

117. Flanigan, W.F., Jr. and W.E. Caldwell, *Galvanotaxic behavior and reinforcement of fish Brachydanio rerio*. Genet Psychol Monogr, 1971. **84**(1): p. 35-71.

118. Hall, D. and M.D. Suboski, *Visual and olfactory stimuli in learned release of alarm reactions by zebra danio fish (Brachydanio rerio)*. Neurobiol Learn Mem, 1995. **63**(3): p. 229-40.

119. Miklosi, A. and R.J. Andrew, *The zebrafish as a model for behavioral studies*. Zebrafish, 2006. **3**(2): p. 227-34.

120. Braida, D., et al., *Hallucinatory and rewarding effect of salvinorin A in zebrafish: kappa-opioid and CB1-cannabinoid receptor involvement*. Psychopharmacology (Berl), 2007. **190**(4): p. 441-8.

121. Kljavin, I.J., *Early development of photoreceptors in the ventral retina of the zebrafish embryo*. J Comp Neurol, 1987. **260**(3): p. 461-71.

122. Branchek, T. and R. Bremiller, *The development of photoreceptors in the zebrafish, Brachydanio rerio. I. Structure*. J Comp Neurol, 1984. **224**(1): p. 107-15.

123. Raymond, P.A., et al., *Expression of rod and cone visual pigments in goldfish and zebrafish: a rhodopsin-like gene is expressed in cones*. Neuron, 1993. **10**(6): p. 1161-74.

124.   Papp, M., P. Gruca, and P. Willner, *Selective blockade of drug-induced place preference conditioning by ACPC, a functional NDMA-receptor antagonist.* Neuropsychopharmacology, 2002. **27**(5): p. 727-43.

125.   Liu, Y., et al., *Conditioned place preference induced by licit drugs: establishment, extinction, and reinstatement.* ScientificWorldJournal, 2008. **8**: p. 1228-45.

126.   Doyon, Y., et al., *Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases.* Nat Biotechnol, 2008. **26**(6): p. 702-8.

127.   MacRae, C.A. and R.T. Peterson, *Zebrafish-based small molecule discovery.* Chem Biol, 2003. **10**(10): p. 901-8.

128.   Clark, K.J., et al., *Transposon vectors for gene-trap insertional mutagenesis in vertebrates.* Genesis, 2004. **39**(4): p. 225-33.

129.   Morton, N.E., *Sequential tests for the detection of linkage.* Am J Hum Genet, 1955. **7**(3): p. 277-318.

130.   Hyatt, T.M. and S.C. Ekker, *Vectors and techniques for ectopic gene expression in zebrafish.* Methods Cell Biol, 1999. **59**: p. 117-26.

131.   Jowett, T., *Analysis of protein and gene expression.* Methods Cell Biol, 1999. **59**: p. 63-85.

132.   Petzold, A.M., et al., *SCORE imaging: specimen in a corrected optical rotational enclosure.* Zebrafish, 2010. **7**(2): p. 149-54.

133.   Kwan, K.M., et al., *The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs.* Dev Dyn, 2007. **236**(11): p. 3088-99.

134.   Lindstrom, J., R. Schoepfer, and P. Whiting, *Molecular studies of the neuronal nicotinic acetylcholine receptor family.* Mol Neurobiol, 1987. **1**(4): p. 281-337.

135.   Whiting, P. and J. Lindstrom, *Purification and characterization of a nicotinic acetylcholine receptor from rat brain.* Proc Natl Acad Sci U S A, 1987. **84**(2): p. 595-9.

136.   Bessis, A., et al., *Promoter elements conferring neuron-specific expression of the beta 2-subunit of the neuronal nicotinic acetylcholine receptor studied in vitro and in transgenic mice.* Neuroscience, 1995. **69**(3): p. 807-19.

137.   Gotti, C., D. Fornasari, and F. Clementi, *Human neuronal nicotinic receptors.* Prog Neurobiol, 1997. **53**(2): p. 199-237.

138. Ni, J., et al., *Transposon tools hopping in vertebrates*. Brief Funct Genomic Proteomic, 2008. **7**(6): p. 444-53.

139. Rubinstein, A.L., *Zebrafish: from disease modeling to drug discovery*. Curr Opin Drug Discov Devel, 2003. **6**(2): p. 218-23.

140. Zon, L.I. and R.T. Peterson, *In vivo drug discovery in the zebrafish*. Nat Rev Drug Discov, 2005. **4**(1): p. 35-44.

141. Coe, J.W., et al., *Varenicline: an alpha4beta2 nicotinic receptor partial agonist for smoking cessation*. J Med Chem, 2005. **48**(10): p. 3474-7.

142. Uchtenhagen, A., *Substance use problems in developing countries*. Bull World Health Organ, 2004. **82**(9): p. 641.

143. Mathur, P. and S. Guo, *Use of zebrafish as a model to understand mechanisms of addiction and complex neurobehavioral phenotypes*. Neurobiol Dis, 2010.

144. Dupont, R.T., *Getting tough on gateway drugs: A guide for the family*. 1984, Washington, DC: American Psychiatric Press.

145. Kandel, D., *Examining the Gateway Hypothesis: Stages and Pathways of Drug Involvement*, in *Stages and Pathways of Drug Involvement*, D. Kandel, Editor. 2002, Cambridge University Press: New York, NY. p. 3-19.

146. Desai, R.I. and P. Terry, *Evidence of cross-tolerance between behavioural effects of nicotine and cocaine in mice*. Psychopharmacology (Berl), 2003. **166**(2): p. 111-9.

147. de Kloet, E.R., R.H. Derijk, and O.C. Meijer, *Therapy Insight: is there an imbalanced response of mineralocorticoid and glucocorticoid receptors in depression?* Nat Clin Pract Endocrinol Metab, 2007. **3**(2): p. 168-79.

148. DeJong, C.A., et al., *Personality disorders in alcoholics and drug addicts*. Compr Psychiatry, 1993. **34**(2): p. 87-94.

149. Breslau, J. and D. Seidenwurm, *Socioeconomic aspects of spinal imaging: impact of radiological diagnosis on lumbar spine-related disability*. Top Magn Reson Imaging, 2000. **11**(4): p. 218-23.

150. Dalack, G.W., et al., *Nicotine withdrawal and psychiatric symptoms in cigarette smokers with schizophrenia*. Neuropsychopharmacology, 1999. **21**(2): p. 195-202.

151. Kendler, K.S., et al., *A population-based twin study in women of smoking initiation and nicotine dependence*. Psychol Med, 1999. **29**(2): p. 299-308.

152. Neale, M.C., L.J. Eaves, and K.S. Kendler, *The power of the classical twin study to resolve variation in threshold traits*. Behav Genet, 1994. **24**(3): p. 239-58.

153. Zachar, P. and K.S. Kendler, *Psychiatric disorders: a conceptual taxonomy*. Am J Psychiatry, 2007. **164**(4): p. 557-65.

154. Hughes, J.R., et al., *Symptoms of tobacco withdrawal. A replication and extension*. Arch Gen Psychiatry, 1991. **48**(1): p. 52-9.

155. Markou, A., T.R. Kosten, and G.F. Koob, *Neurobiological similarities in depression and drug dependence: a self-medication hypothesis*. Neuropsychopharmacology, 1998. **18**(3): p. 135-74.

156. Koob, G.F., et al., *Substance dependence as a compulsive behavior*. J Psychopharmacol, 1998. **12**(1): p. 39-48.

157. Epping-Jordan, M.P., et al., *Dramatic decreases in brain reward function during nicotine withdrawal*. Nature, 1998. **393**(6680): p. 76-9.

158. Speedie, N. and R. Gerlai, *Alarm substance induced behavioral responses in zebrafish (Danio rerio)*. Behav Brain Res, 2008. **188**(1): p. 168-77.

159. Egan, R.J., et al., *Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish*. Behav Brain Res, 2009. **205**(1): p. 38-44.

160. Lopez-Patino, M.A., et al., *Anxiogenic effects of cocaine withdrawal in zebrafish*. Physiol Behav, 2008. **93**(1-2): p. 160-71.

161. Burgess, H.A. and M. Granato, *Modulation of locomotor activity in larval zebrafish during light adaptation*. J Exp Biol, 2007. **210**(Pt 14): p. 2526-39.

162. Kenny, P.J. and A. Markou, *Neurobiology of the nicotine withdrawal syndrome*. Pharmacol Biochem Behav, 2001. **70**(4): p. 531-49.

163. Malanga, C.J., 3rd and B.E. Kosofsky, *Mechanisms of action of drugs of abuse on the developing fetal brain*. Clin Perinatol, 1999. **26**(1): p. 17-37, v-vi.

164. Villegier, A.S., et al., *Transient behavioral sensitization to nicotine becomes long-lasting with monoamine oxidases inhibitors*. Pharmacol Biochem Behav, 2003. **76**(2): p. 267-74.

165. Romieu, P., et al., *Histone deacetylase inhibitors decrease cocaine but not sucrose self-administration in rats*. J Neurosci, 2008. **28**(38): p. 9342-8.

166. Lamason, R.L., et al., *SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans*. Science, 2005. **310**(5755): p. 1782-6.

167. Burgess, H.A. and M. Granato, *The neurogenetic frontier--lessons from misbehaving zebrafish*. Brief Funct Genomic Proteomic, 2008. **7**(6): p. 474-82.

168. King, D.P. and J.S. Takahashi, *Forward genetic approaches to circadian clocks in mice*. Cold Spring Harb Symp Quant Biol, 1996. **61**: p. 295-302.

169. St Johnston, D., *The art and design of genetic screens: Drosophila melanogaster*. Nat Rev Genet, 2002. **3**(3): p. 176-88.

170. Tarantino, L.M. and M. Bucan, *Dissection of behavior and psychiatric disorders using the mouse as a model*. Hum Mol Genet, 2000. **9**(6): p. 953-65.

Appendix A

Gene Break Transposon Vectors

A variety of Gene-Break Transposon vectors were applied in the screening process. These vectors represented different technological advantages within the trapping and mutation process. The following figure represents the trap vectors with a graphical representation as well as an approximation of the number of mutant chromosomes screened.

The initial vector used in mutant screening, GBT-P9, is a Polyadenylation-trap vector based directly off of GBT-P6, as described in Sivasubbu et al. 2006 [1] (Figure 21A). GBT-P6 is a *Sleeping Beauty*-based (SB) transposon vector including a branch-point splice acceptor followed by a polyadenylation signal with putative boundary element (Ocean Pout Terminator "OPT) [2], a strong promoter (β-Actin) with intron and a green fluorescent protein (GFP) with splice donor. When inserted into an intron in the sense orientation, the endogenous gene splicing is hijacked by the splice acceptor of the GBT-P6, halting the transcription of the gene product creating a truncated protein. Additionally, a carp β-Actin promoter drives GFP expression ubiquitously throughout the fish. Due to the lack of a polyadenylation signal following the GFP, mRNA will stabilize in the presence of a polyadenylation signal from the endogenous gene, thus tagging a mutant product and no other inserts (A graphical representation of this process is described in Figure 2).
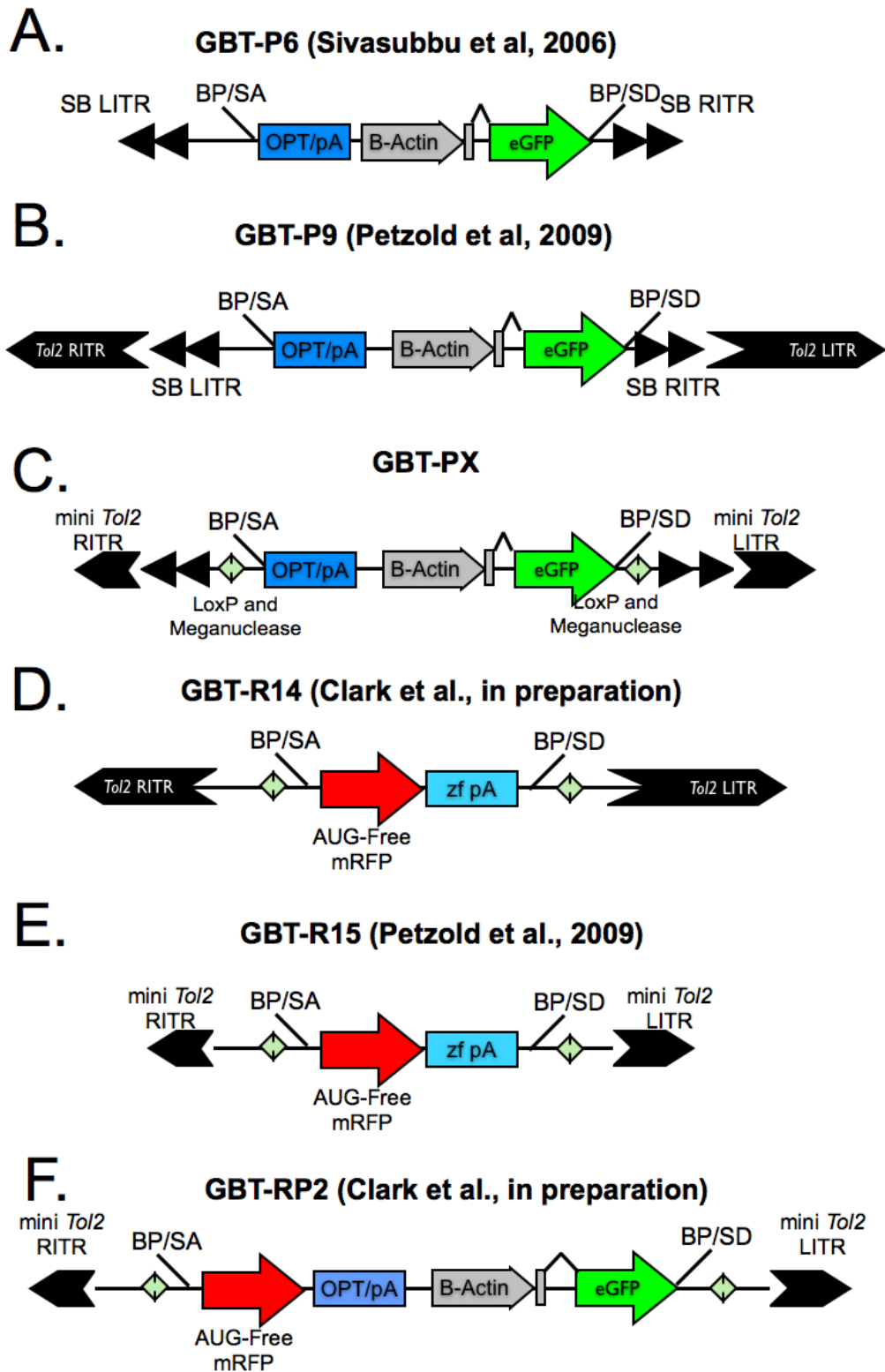
Figure 21. Graphical representation of the differences in GBT-vectors. A. GBT-P6 as published in Sivasubbu et al [1] includes the basal structure of the GBT construct within the *SB* ITR. B. GBT-P9 published in Petzold et al [3] includes GBT-P6 placed into *Tol2* ITR. C. GBT-PX includes LoxP and ISce sites as well as a *miniTol2* ends. D. GBT-R14 acts as a protein trap with an AUG-less mRFP. E. GBT-R15 published in Petzold et al. F. GBT-RP2 combines GBT-R15 and GBT-PX into a single vector.

The next developed GBT vector was GBT-P9 [3].The major difference between GBT-P6 and GBT-P9 is the inclusion of the *Tol2* full inverted-terminal repeats (ITR) flanking the entire GBT-P6 vector in the *cis* orientation (Figure 21B). *Tol2* has been shown to be more active in zebrafish creating earlier transposition and insertion events at a higher affinity when compared to SB [4]. GBT-PX represents two technological advantages over GBT-P9: the inclusion of the *mini-Tol2* ITR[4] and the inclusion of Cre/loxP-recombinase recognition sites and non-matching ISce-meganuclease sites (Figure 21C). The first of these advances allows for a greater rate of transposition and the removal of a cryptic-splice acceptor within the left-ITR as well as a potential internal promoter (Ekker lab unpublished observations). The inclusion of Cre/loxP-recombinase recognition sites allows for an easier and more efficient method of reversion of the mutant locus to wild-type. These sites, located directly internal of the ITR, remove the mutagenic properties of the GBT leaving behind the ITR and a single Cre/loxP recognition site. The inclusion of the ISce-meganuclease sites allows a user to induce a double-stranded DNA break and possibly non-homologous end-joining (NHEJ). If located within a smaller intron, NHEJ can produce localized deletions[5]. Additionally, the remnant SB ITRs have been removed to create a smaller insertion and allow for SB transposon work upon mutant lines.

An additional line of research produced a series of protein-trap vectors. These vectors rely upon the enhancer and promoter of the endogenous gene to produce a fluorescent protein tag. The GBT-R14 vector is a protein-trap vector that includes an AUG-free mRFP followed by the zebrafish β-Actin polyadenylation signal, within the full-length

Tol2 ITR as well as the inclusion of the Cre/loxP-recombinase recognition sites (Figure

21D). GBT-R15 includes a single technological advance over GBT-R14; the

replacement of the full length Tol2 ITR with the mini-Tol2 ITR (Figure 21E).

The final GBT-vector used within this screen is a combination-trap, combining both the

protein-trap and polyadenylation trap. The creation of the GBT-RP2 is accomplished by

combining the GBT-R15 protein trap with the GBT-PX polyadenylation trap (Figure

21F). This represents two technological advances in trapping. First, a ubiquitous

protein-tag allows the notification of a trapping event, combined with the gene-

localization if located in the proper open-reading frame. The addition of the OPT

following the rather than the zebrafish β-Actin polyadenylation following the mRFP

signal provides greater gene breaking and reduces wild-type protein expression (Ekker

lab unpublished observation). A graphical description of the mechanism of the trapping

vector can be seen in Figure 2.

1.   Sivasubbu, S., et al., *Gene-breaking transposon mutagenesis reveals an essential role for histone H2afza in zebrafish larval development*. Mech Dev, 2006. **123**(7): p. 513-29.

2.   Gibbs, P.D. and M.C. Schmale, *GFP as a Genetic Marker Scorable Throughout the Life Cycle of Transgenic Zebra Fish*. Mar Biotechnol (NY), 2000. **2**(2): p. 107-125.

3.   Petzold, A.M., et al., *Nicotine response genetics in the zebrafish*. Proc Natl Acad Sci U S A, 2009. **106**(44): p. 18662-7.

4.   Balciunas, D., et al., *Harnessing a high cargo-capacity transposon for genetic applications in vertebrates*. PLoS Genet, 2006. **2**(11): p. e169.

5.   Sonoda, E., et al., *Differential usage of non-homologous end-joining and homologous recombination in double strand break repair*. DNA Repair (Amst), 2006. **5**(9-10): p. 1021-9.

Appendix B

SCORE Imaging

This appendix is a reprint of the published work:

SCORE Imaging: Specimen in a Optically Corrected Rotational Enclosure

as found in the June 2010 issue of *Zebrafish*

The Use of SCORE Imaging

To facilitate the high-throughput visualization of a variety of expression based mutant embryos, a novel concept for data capture was needed. The development of this method, although not directly used within the scope of this work, has been invaluable to the entire functionality of the screening process and the documentation of these mutant lines. Furthermore, this process has enabled the documentation, presentation and recording of these expression patterns as well as an effective method for teaching members of the community at large about the visualization of the larval zebrafish. The SCORE technique, as described here, has been employed by a variety of users ranging in scientific ability levels of a high school student and elementary school teacher to zebrafish researchers, all of whom have found the process to be useful and at times needed to capture the data that was needed. Furthermore, following presentation at the 9th Zebrafish Conference on Genetics and Development (June 2010), the zebrafish research community at large has found this process to be straightforward, affordable and powerful.

The need for a novel method of imaging the larval zebrafish

Visual data capture is a major part of scientific documentation. The transparency of the

zebrafish (*Danio rerio*) has been an invaluable backdrop to the use of this system for

complex biological research based on relatively simple and classically optical

observation. One of the major hurdles of the zebrafish researcher has been the capture

and meaningful representation of this visual data. Many aspects of zebrafish biology are

better expressed in images and other digital media rather than in written descriptions.

Examples include assessment of morphological mutant phenotypes, protein or nucleic

acid expression patterns, and developmental stages[1-3].

Part of the challenge in obtaining consistent, high quality images of the zebrafish arises

from efforts in attaining static, two-dimensional images of a dynamic, three-

dimensional sample. Additional difficulty comes from the shift of a largely spherical-

shaped early embryo (0 to 24 hpf) to the cylindrical shape of the larvae and adult fish.

Traditionally, this problem has been addressed by sectioning the fish either physically

or optically. However, preparation of the sample is laborious and the depth of field can

be lost. To further increase the photographic challenge, beginning at 72 hpf the

zebrafish larvae inflates its swim bladder[3], providing buoyancy, a challenge that adds

difficulty in manipulating and immobilizing specimens in a purely sagittal or coronal

plane (Cartoon: Figure 22B). The issues of working with this unique shape have

inspired many methods that attempt to remedy these problems and deliver consistent,

high quality image capture [1-4]. However, all of these approaches still require

substantial training to achieve proficiency, are laborious and thus are often not practical
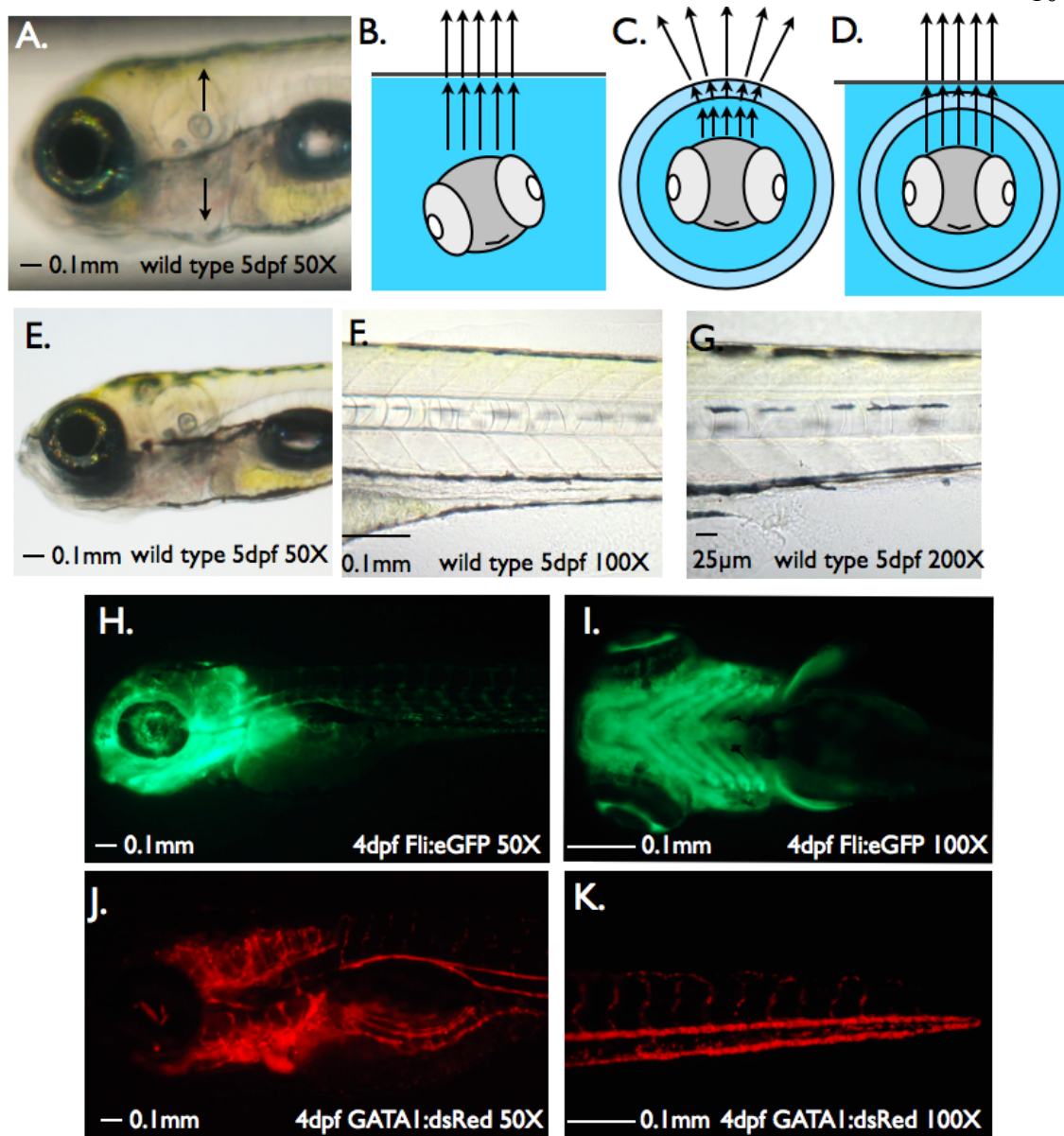
Fig. 22. SCORE imaging of living animals. Zebrafish imaging is facilitated by use of a fluoro-carbon tube for housing. A) Zebrafish images taken in polymer tubing produces a distorted image (arrows) due to the change in refractive index between the tubing and the air. B-D) Cartoon images representing a fish immobilized using traditional techniques on a skewed plane (B), a larval fish within a polymer tube without a corrective solution producing distortion (C) and within a corrective solution to produce a clear image (D). E-G) Fluorinated Ethylene Propylene (FEP) tubing produces a clear image at 50X (E), 100X (F) and 200X (G) of a 5dpf larvae. H-I) A 5 dpf Tg(gata1:dsRed)sd2 larvae in FEP tubing produces a clear, background free image at 50X (H) and 100X (I). (J-K) A 5dpf Tg(fli:eGFP)y1 in FEP tubing produces a clear, background-free image at 50X (J) and 100X (K).

for high-throughput screening or other applications. Quality imaging can be the most critical bottleneck in using the fish for many scientific areas.

We developed a simple and efficient method for holding and photographing embryonic and larval zebrafish that we termed Specimen in a Corrected Optical Rotational Enclosure (SCORE) Imaging. The SCORE method takes advantage of the cylindrical nature of an embryo and allows a specimen to be freely rotated about its longitudinal axis. This rotation, in turn, facilitates image capture of sagittal, coronal or other horizontally angled planes. To accomplish the rotation, larvae are drawn into either a polymer or a borosilicate capillary housing, and are immobilized using a viscous liquid either low melting point agarose or methylcellulose for living larvae or glycerol for fixed embryos. If the fixed embryos are placed in a permanent mounting solution, it is possible that these embryos can be stored indefinitely. The SCORE method uses a capillary tube to allow rotation about the longitudinal axis and combines it with a solution that matches the refractive index of the capillary to eliminate distortion. This process allows the user to quickly obtain publication-quality images in multiple, perfectly aligned imaging planes.

Larval zebrafish imagery within a rotational enclosure

The idea to put a specimen into a capillary tube is not a unique one [2, 5-8] with references to similar approaches dating to the late 1920's (See reference 7 by Moroz et al. and citations therein). Such a method addresses the holding aspect of photography, facilitating the alignment for potentially a full, 360-degree view of the specimen.

However, without modification this approach added major distortion to the light path, especially near the periphery, making the image unfit for data analysis or publication (Figure 22A; arrows). Using the principles of optics as a guide, the main cause of distortion was determined to be the difference in refractive index between the curved outer surface of the capillary and the air surrounding it, creating a bend in light as described previously [9]. Normalizing the refractive index of the capillary housing to the liquid surrounding the housing and the fish greatly reduced this effect (Cartoon diagrams Figure 22C and 22D; example images are Figures 22E-22G). To accomplish a nearly complete refractive index correction, we tested a variety of polymer-based tubes with a refractive index similar to that of water (n=1.33 [10] for matching, see Table 1). We evaluated these materials based on four criteria: 1) distortion present, 2) auto-fluorescence in common wavelengths of light such as deployed in fluorescence microscopy 3) biocompatibility and 4) ease of use.

To determine the best imaging housing for the photography of larval zebrafish, we tested fluorinated ethylene propylene tubing (FEP; n= 1.338 [11]), perfluoroalkoxy tubing (PFA; n = 1.34 [12]) and polytetrafuoroethylene tubing (PTFE; n = 1.35 [11]). While these tubes perform equally well under most levels of magnification in brightfield applications (FEP shown Figure 22E-G; PFA and PTFE not shown), they tend to differ when using red and green fluorescence. We noted that the FEP tubing produced little auto-fluorescence in either the green fluorescent protein (eGFP filter set; Figure 22H-22I) or red fluorescent protein (such as an mRFP filter set; Figure 22J-22K) channels. In contrast, PFA and PTFE tubing showed a higher level of auto-fluorescence

especially with dimmer transgenic fish lines. In addition to the polymer tubing, we also

tested a common zebrafish lab reagent, a borosilicate glass capillary (n=1.48 [9]). For

live embryos, the glass capillary was optically matched to a surrounding solution of

50% sucrose (Table 2). This combination provided a clear and crisp image in brightfield

applications but also produced a dark shadow on the edge of the capillary. In

fluorescent applications there was no auto-fluorescence, however. Overall, when

photographing a live embryo, we recommend the use of FEP tubing due to its modest

cost, lack of auto-fluorescence and crisp optical imaging.

SCORE imaging is also an excellent method for imaging fixed embryos, such as

commonly generated for whole-mount *in situ* hybridization (WISH). 75% glycerol is

commonly used to image WISH embryos and is a suitable optical imaging solution

using borosilicate glass capillaries (Cartoon: Figure 23A). Use of the SCORE method

on fixed samples is ideal for obtaining images in both the sagittal (Figure 23B, 23C,

23D) and coronal planes (Figure 23E). Additionally, the intricacies of the expression

pattern can be displayed in alternative planes of view. For example, if one were to be

studying the nephric duct staining shown by *pax2a* using WISH, the sagittal view does

not show both ducts, and the coronal view is obstructed by additional neural staining

(Figures 23B-23E). A ~30° rotation counter-clockwise view off the coronal plane

(Figures 23F), however, provides the optimal view of the nephric ducts (Figures 23F,

arrows). Use of the SCORE method also allows for a full rotation of a single embryo to

be captured using a video camera attached to the microscope (Figure 23B). If needed,
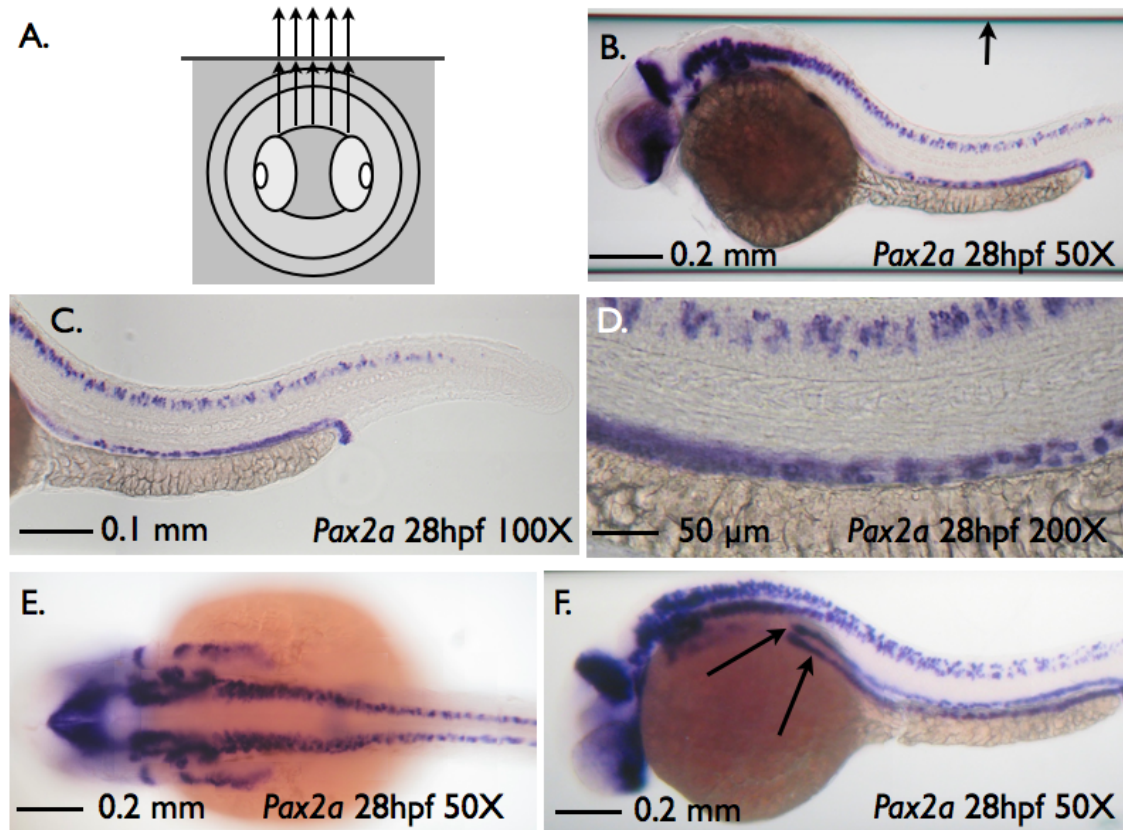
Fig. 23. SCORE imaging of a fixed specimen. Imaging of a pax2a whole-mount *in situ* hybridization embryo and capillary housing in 75% glycerol. A) Cartoon depicting the use of glycerol both inside and outside of the glass tube producing an undistorted image of a fixed embryo. B) Sagittal image of embryo with *pax2* staining at 50X magnification shows no distortion. Note the edges of the capillary that can be readily cropped for publication presentation. Sagittal embryo image of *pax2* staining at 100X (C) or 200X (D) magnification shows no distortion. E) Coronal image of embryo at 50X magnification. F) Angled image of *pax2* staining at 50X magnification. Rotation is angled slightly (~30°) to show a more detailed view of kidney tubule staining (arrow).
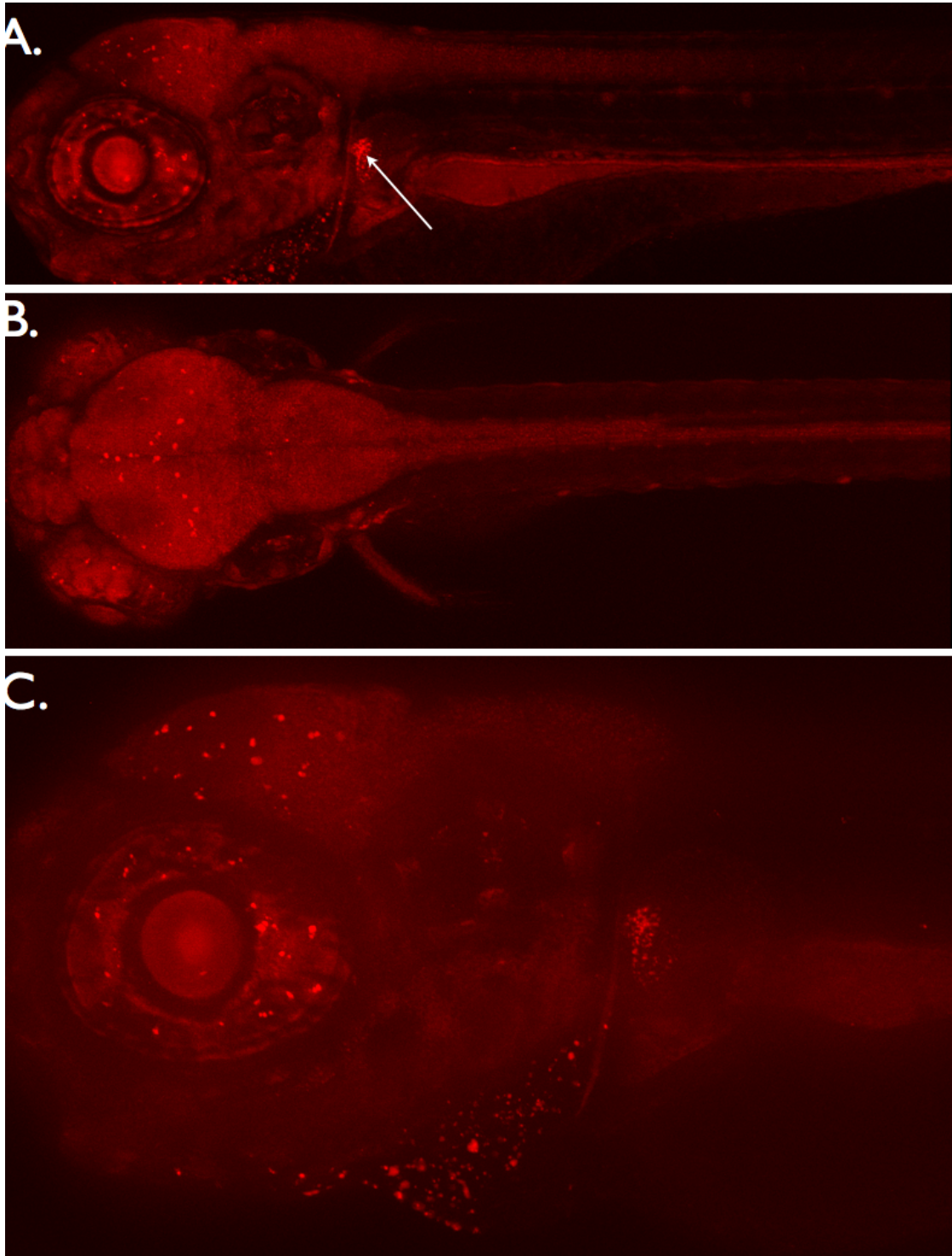
Fig. 3. SCORE imaging of fluorescent, living zebrafish. Imaging SEC0124 of a 5 dpf mRFP-labeled protein trap zebrafish housed in FEP tubing. A) Sagittal image of an mRFP-labeled protein trap zebrafish shows enhancement in specific locations in the neural region as well as a bright kidney (Arrow). B) Coronal image of an mRFP-labeled protein trap zebrafish includes ubiquitous neural expression with enhancement in specific locations. C) Sagittal image of an mRFP-labeled protein trap zebrafish shows a general neural haze with enhancement in specific locations at 100X magnification.

a precise angle of rotation can be attained with the use of a mechanical aid to ensure

reproducibility.

The SCORE method is also a simple and efficient system for creating high quality

wide-field images using standard microscopes at 50X and 100X magnifications. With

the ease and speed of preparation of samples, this procedure, for example, allows the

user to quickly obtain maximum intensity projections of z-stacks in the major planes

(sagittal; Figure 24A, dorsal; Figure 24B) using a single zebrafish and an ApoTome

Grid Microscope. The image was of high quality up to 100X magnification using this

imaging setup (sagittal; Figure 24C). The greatest limitation to deploying the SCORE

method of imaging for whole animal imaging at higher magnification is the available

working distance of most common objectives.

Benefits of SCORE Imaging

The key to the SCORE approach is optical correction of an easy specimen housing

method. This approach could be applied to chemical genetic testing and other, related

high-throughput screens. In addition, the use of this method with smaller capillaries

could be used for imaging single cells, especially over time with a biocompatible

housing solution. Deploying larger capillaries opens the door to the imaging of many

other specimens, including synthetic samples where a full, 360 degree viewing would

be desirable. Finally, the housing method developed here could be adapted for use in

precisely aligning fixed samples for downstream study such as histological preparation.

Applying the SCORE method at higher magnification is largely limited by two issues –

the distortion by air between the samples' cover slip and the lens, and the moderate to

low working distance of traditional lenses, including oil immersion optics. One

potential option would be the use of optical imaging solution compatible immersion

lenses, such as those that work with glycerol and those that work with water. The main

limitation will be the thickness of the specimen in relation to the working distance of

the objective.

Finally, the ready ability to generate high resolution and multi-media content for

scientific papers challenges the contemporary publishing paradigm. The current patch

solution is normally a form of supplementary material provided either by the publisher,

the authors or both. Instead, we strongly feel that moving forward means the use of a

new generation of scientific format that directly integrates such imaging data in the

main manuscript.

Methods used for SCORE image capture

Immobilization of the zebrafish embryos.

For all of our procedures, we have used the anesthetic tricaine (MS-222) at standard

concentrations[13]. Fish were further immobilized using either a 2% methylcellulose

solution or a Low Melting Point (LMP) agarose solution (at approximately 0.5% in

embryo water). If fixed larvae were imaged (such as from a whole mount *in situ*

hybridization experiment*)*, a 75% glycerol solution was used due to its viscosity and similar refractive index to that of borosilicate glass.

Preparation for SCORE imaging

After fish were immobilized (Figure 25A), a capillary adapter was placed in a pipette-pump and used to draw an embryo into an appropriate-sized borosilicate capillary (Figure 25B). When using LMP agarose, the capillary is then allowed to rest for 2 minutes, letting the LMP agarose solidify. Multiple fish can be loaded into a single capillary, allowing for greater variety in sample choice and for data analyses (Figure 25C). Note that the inner liquid must almost fill the entire capillary to lessen the chance of mixing with the potentially different external optical imaging solution. A paraffin wax plug can be added to the end of the capillary by way of scraping or a 'stick-and-twist' method to block this possible contamination and to eliminate sample drift.

Choice of capillary size

Embryos begin as a bulbous shape and straighten as they age. Consequently, the size of the capillary opening depends inversely on the age of embryonic, larval and young fish. Capillaries with a 0.60 mm inside diameter work well for 48+ hpf embryos (WPI #1B120-3 or WPI #TW100-3 for thin walled applications). For younger embryos (0 – 48hpf), an inside diameter of at least 1.12 mm was used (WPI #TW150-3). When choosing the size of the polymer tubing, the smallest readily available tubing (ID-.8mm, ColeParmer EW-06406-60) was used for all embryos 24hpf and older; however, all younger embryos needed to be dechorionated. Within the size range of the capillaries

tested, the outer diameter of the capillary seems not to factor in any distortion of the

embryo, however the width of the wall of the capillary may. Thus it is recommended

that thin-walled capillaries be used when possible.

Choice of plastic tubing to match refractive index of water

We tested a variety of plastic tubing to best match the refractive index of the tubing to

that of water for the greatest ease of use and image clarity. We determined that

fluorinated ethylene propylene tubing is optimal for use with water as an optical

imaging solution and either LMP agarose or Methylcellulose as an immobilization

media. (Table 1).

Table 1. Refractive indices of polymer tubing used.

Solution	Refractive Index	Comments about use
Water (surrounding fluid)	1.34 [15]	Used to cover tubing. Matched for tubing
FEP	1.338[16]	Recommended for live embryos
PFA	1.35[17]	Works well for live embryos
PTFE	1.35-1.38[16]	Not recommended

Matching the refractive index of borosilicate glass using optical imaging solutions

We tested a variety of imaging solutions to match the refractive index of borosilicate

glass. We determined that a 75% glycerol solution is best for use when paired with

fixed embryos. Due to a fear of harm to a living embryo, a 50% sucrose solution is

recommended if it is preferred to use a borosilicate capillary over plastic tubing. A more

in-depth analysis of these choices is presented in Table 2.

Table 2. Refractive indices of borosilicate glass capillaries and optical imaging
solutions used.

Solution	Refractive Index	Comments About Use
Borosilicate Glass Capillary	*1.47-1.49 [14]*	*Used for housing.*
Water	1.34 [15]	Slight distortion, but can work if needed (not shown).
Vegetable Oil (Soybean)	1.47 [14]	Not much distortion, slight GFP background.
Mineral Oil	1.48 [14]	No distortion creates bubbles easily.
75% Glycerol	1.46	High viscosity. May harm living fish. Best for fixed larvae.
Light Corn Syrup (BRIX=80)	~1.5 [18]	High Viscosity.
50% Sucrose	1.464 [18]	Recommended Optical Imaging Solution for live embryos if using Borosilicate Glass

SCORE imaging

The capillary containing the sample is placed onto a slide with a channel with edges at

least as high as the outer diameter of the capillary, allowing at least one end of the

capillary to extend beyond this channel to allow rotation. This barrier can be made by

affixing capillaries of a slightly larger diameter, or craft foam (Crafts Etc, 2mm Funky

Foam) onto a glass slide. The appropriate optical imaging solution (water, 50% sucrose,
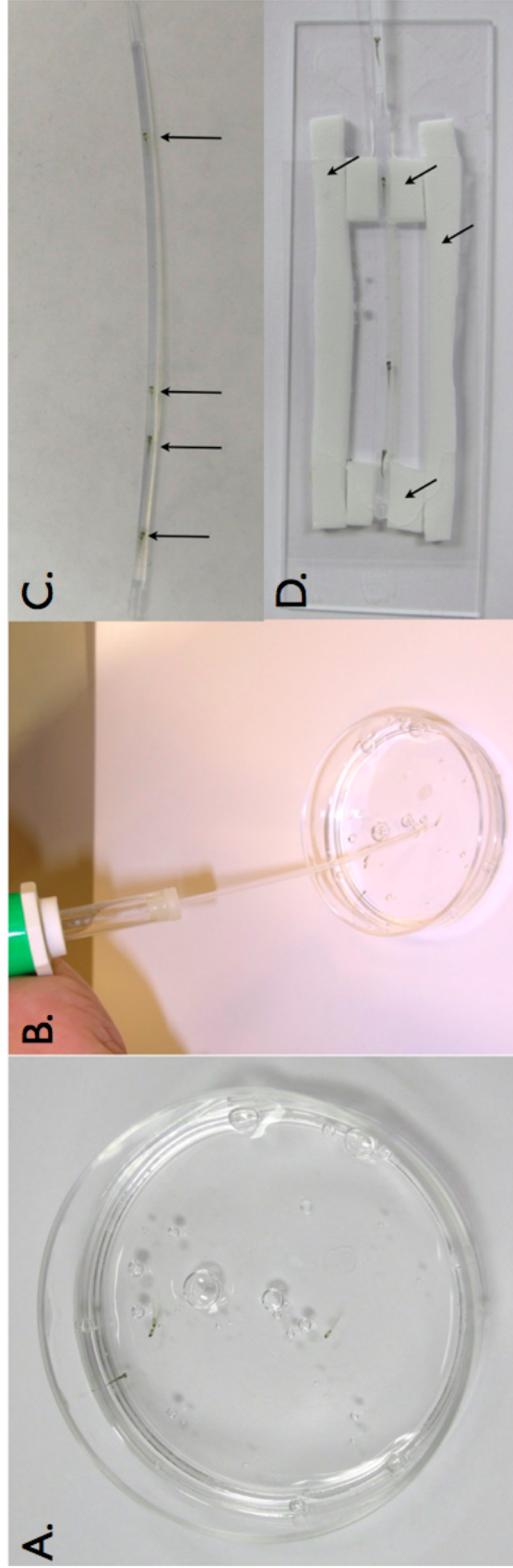
Fig. 4. SCORE methodology. A) Anesthetized larval zebrafish are placed into a methylcellulose (pictured) or LMP agarose solution before being loaded into the capillary housing. B) A pipette pump with capillary adaptor is used to facilitate loading of zebrafish into the capillary housing. C) Multiple larvae (arrows) can be imaged in a single capillary, allowing for rapid screening of mutants. D) A corrective solution (water pictured) is placed upon the capillary to reduce distortion. Foam barriers, marked by arrows, are placed upon the glass slide to help contain corrective solution and to provide a friction surface for ease of rotation.

or 75% glycerol) should be applied over the capillary so that the entire height of the

capillary is covered (Figure 25D). Care must be taken to ensure that no excess optical

imaging solution spills onto the microscope optics. A cover slip is then placed on top of

the capillary immersed in the corrective solution. Once the cover slip has been

deployed, the slide can be examined under a microscope. The orientation of the embryo

can be adjusted by simply rotating the capillary, the ends of which extend past the

viewing area. The slides may be reused after the images have been obtained, if cleaned

fully. To recover the embryos from the capillary, carefully snap off the wax plugs and

expel the embryos by applying pressure to the side of the capillary. All rotation of the

capillary is based off of 0° being a coronal image with the dorsal side upward and the

lens of the specimen aligned, from this mark, sagittal is defined as a 90°

counterclockwise rotation and ventral is defined as a 180° rotation. Any other rotational

angle is approximate and is noted as such.

Digital photography and editing

Images were obtained using a Nikon D3 (Figure 22), Canon Powershot A640 digital

camera (Figure 23), Zeiss ApoTome Grid Microscope (Figure 24) or a Canon G10

(Figures 25 and 26). Bright-field images were captured using a shutter speed of 1/200

(Figures 22 and 23). Green fluorescent photographs were captured using a shutter speed

of 1/60 (Figure 22). Red fluorescent photographs were captured using a shutter speed of

1/25 (Figure 22). Photographs were edited with Keynote 09 for Macintosh OSX (Apple

Inc) to allow for differing focal planes and to remove shadow caused by the capillary

housing; no parts of the image of the fish were removed in this editing process.

Zebrafish collection and preservation

Embryos were collected at day zero, approximately one-hour post fertilization (pf) and placed into 100x15 mm Petri dishes with no more than 100 embryos per dish. These dishes were then stored in a 30° C incubator. At three dpf, larvae were then transferred into 60x15mm Petri dishes with no more than 20 fish per dish for fluorescent-based genotyping according to Davidson et al.[14].

Transgenic fish

The doubly transgenic Tg(*fli-1:eGFP*)y1[15] /Tg(*gata-1:DsRed*)sd2[16] fish were as described by Pickart et al., 2006 [17]. Protein trap fish line Tg(*SEC0124*)mn124 shown in Figure 24 was generated in a similar manner to that described previously [18]. Zebrafish were injected with 25 ng of plasmid containing the gene-break protein trap transposon GBT-R14.5. This construct is the same as GBT-R15 [19] with a full-length Tol2 right inverted terminal repeat instead of the mini Tol2 end found in GBT-R15.

Microscope ease of use

To facilitate longer hours on a fluorescent microscope required for high-throughput screening without producing the eyestrain of a traditional microscope, we employed the camera port of an inverted *Zeiss* fluorescent microscope to its fullest potential by connecting a *Sony HDR-HC9* high definition (HD) video camera (Camera: Figure 26A, Microscope: Figure 26B). The output of the HD video camera can either be run directly into a video screen (Figure 26C) or a computer for video capture (Figure 26D). Notably,
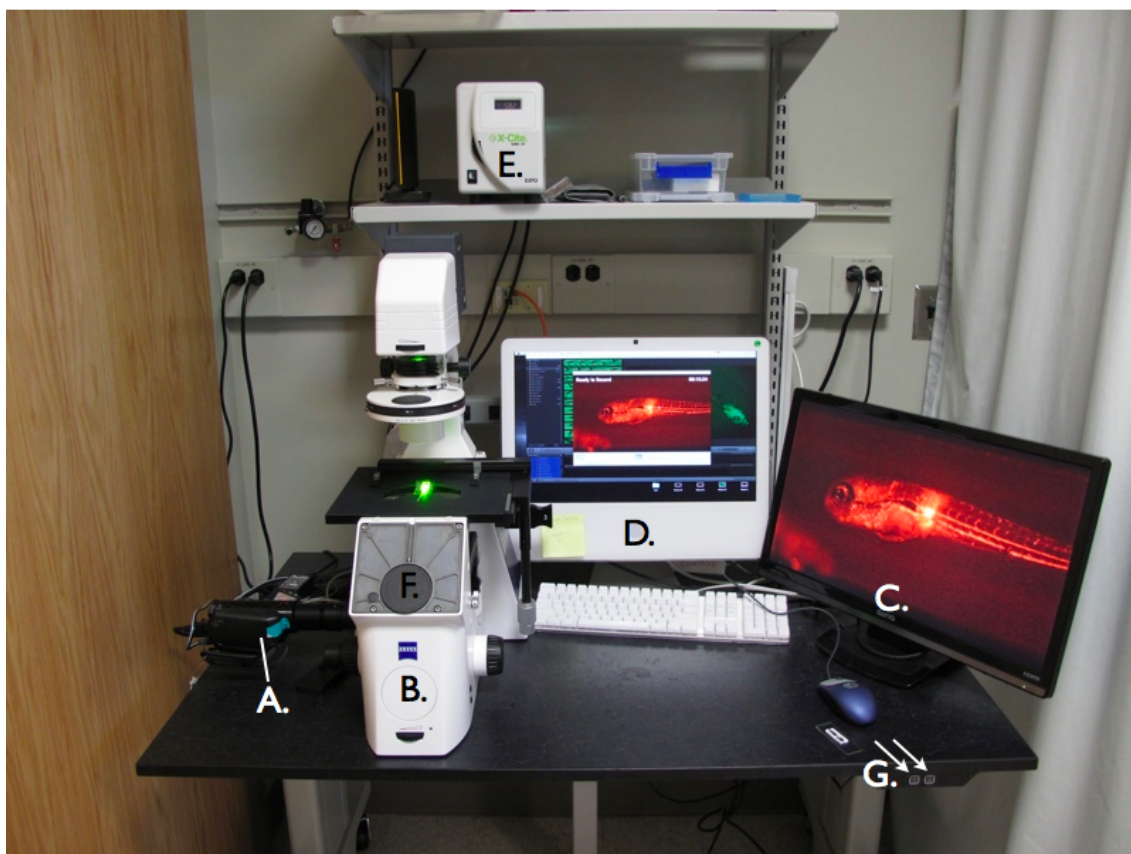
Fig. 5. Ease of use microscope – the "headless" microscope setup. A) A *Sony HDR-HC9* high definition video camera is attached to the camera port of the B) *Zeiss* inverted microscope to facilitate a greater ability to visualize, record and present visual data. C) A high definition monitor allows for reduced eyestrain and ease of presentation to other workers. D) Attaching the video camera to a computer allows for direct recording of the sample in a purely digital format. E) *X-Cite* light source allows for fluorescent microscopy. F) Removal of the eye-pieces of the *Zeiss* inverted microscope allow for a reduction in the light bleaching of the sample and provide a less cluttered working area with no obstructions to the stage. G) The microscope has been placed on a *BostonTec* adjustable lab table to facilitate the ease of movement of the entire scope system for greater ability of access.

we have removed the eyepieces of the microscope to ensure that no unwanted light

bleaches the image when working with fluorescence (Figure 26E) and to vastly improve

access to the samples (Figure 26F). This microscope setup also allows colleagues to

easily and simultaneously observe phenotypes and patterning in the zebrafish, an

environment that facilitates scientific discussion in real-time. Although not necessary

for the SCORE approach, this microscope set-up vastly improves the accessibility to

embryos in SCORE enclosures and eases high-throughput imaging.

1.   Bryson-Richardson, R.J., et al., *FishNet: an online database of zebrafish anatomy.* BMC Biol, 2007. **5**: p. 34.

2.   Buckley, C.E., P. Goldsmith, and R.J. Franklin, *Zebrafish myelination: a transparent model for remyelination?* Dis Model Mech, 2008. **1**(4-5): p. 221-8.

3.   Kimmel, C.B., et al., *Stages of embryonic development of the zebrafish.* Dev Dyn, 1995. **203**(3): p. 253-310.

4.   Sabaliauskas, N.A., et al., *High-throughput zebrafish histology.* Methods, 2006. **39**(3): p. 246-54.

5.   Preibisch, S., et al., *Towards digital representation of Drosophila embryogenesis.* Proceeding of 5th IEEE International Syposium on Biomedical Imaging, 2008: p. 324-327.

6.   Funfak, A., et al., *Micro fluid segment technique for screening and development studies on Danio rerio embryos.* Lab Chip, 2007. **7**(9): p. 1132-8.

7.   Moroz, P., *Microscopy and Manipulation of Cells in Elastic Capillaries.* Trans Am Microsc Soc, 1994. **113**(3): p. 412-4.

8.   Zackson, S.L., *Cell clones and segmentation in leech development.* Cell, 1982. **31**(3 Pt 2): p. 761-70.

9.   Budwig, R., *Refractive index matching methods for liquid flow investigations.* Experiments in Fluids, 1994. **17**: p. 350-355.

10.   Schiebener, P., et al., *Refractive Index of Water and Steam as Function of Wave length, Temperature and Density.* J. Phys. Chem. Ref. Data, 1990. **19**(3): p. 706.

11.   Seferis, J.C., *Refractive Indicies of Polymers.* Polymer Handbook, 1999. **4th ed.**: p. VI/574, VI/575.

12.   Kerbow, D.L. and C.A. Seperati, *Physical Constants of Fluoropolymers.* Polymer Handbook, 1999. **4th ed.**: p. V/43.

13.   Westerfield, M., *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio).* 4 ed. 2000: Univ. of Oregon Press, Eugene, OR.

14.   Davidson, A.E., et al., *Efficient gene delivery and gene expression in zebrafish using the Sleeping Beauty transposon.* Dev Biol, 2003. **263**(2): p. 191-202.

15.   Lawson, N.D. and B.M. Weinstein, *In vivo imaging of embryonic vascular development using transgenic zebrafish.* Dev Biol, 2002. **248**(2): p. 307-18.

16.   Traver, D., et al., *Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants.* Nat Immunol, 2003. **4**(12): p. 1238-46.

17.   Pickart, M.A., et al., *Genome-wide reverse genetics framework to identify novel functions of the vertebrate secretome.* PLoS One, 2006. **1**: p. e104.

18.   Sivasubbu, S., et al., *Gene-breaking transposon mutagenesis reveals an essential role for histone H2afza in zebrafish larval development.* Mech Dev, 2006. **123**(7): p. 513-29.

19.   Petzold, A.M., et al., *Nicotine response genetics in the zebrafish.* Proc Natl Acad Sci U S A, 2009. **106**(44): p. 18662-7.

Appendix C

PERMISSION TO INCLUDE PREVIOUSLY PUBLISHED MATERIAL

Portions of the material within this document have been published previously:

Much of Chapter 2 has been previously published in PNAS as:

"Nicotine Response Genetics in the zebrafish" publication number 0908247106 pages 18662-18667 of vol 106 no 44 from November 3, 2009.

According to the copyright policy of PNAS, the Author retains rights to re-publish works within a thesis or dissertation (Screen Capture from Author FAQs included below)

1. **Since I retain copyright but give PNAS an exclusive license to publish my article, what rights do I have?**

   As a PNAS author, you and your employing institution or company retain extensive rights for use of your materials and intellectual property. You retain these rights and permissions without having to obtain explicit permission from PNAS, provided that you cite the original source:

   - The right to post a PDF of your article on your Web site or that of your employer's institution (provided that the institution is nonprofit).

   - The right to make electronic or hard copies of articles for your personal use, including classroom use, or for the personal use of colleagues, provided those copies are not for sale and are not distributed in a systematic way outside of your employing institution.

   - The right to post and update a preprint version of your article on a public electronic server such as the Web. See the information on electronic preprints below.

   - The right to permit others to use your original figures or tables published in PNAS for noncommercial and educational use (i.e., in a review article, in a book that is not for sale), provided that the original source is cited. Third parties need not ask PNAS for permission to use figures and tables for such use.

   - The right, after publication in PNAS, to use all or part of your article in a printed compilation of your own works, such as collected writings or lecture notes.

   - If your article is a "work for hire" made within the scope of your employment, your employer may use all or part of the information in your article for intracompany use.

   - The right to include your article in your thesis or dissertation.

PERMISSION TO INCLUDE PREVIOUSLY PUBLISHED MATERIAL

Portions of the Material included within Appendix B was previously published within

MaryAnneLiebert's *Zebrafish* as:

"SCORE Imaging: Specimen in a Corrected Optical Rotational Enclosure" from the

June 2010 issue Volume 7, number 2 pages 149-154

This article is published as an Open Access article and has been given permission for

reprint within this document. A copy of this is included on the following page:

Mary Ann Liebert, Inc. publishers

7/16/10

Andrew Petzold
Guggenheim 13
Mayo Clinic
201 2nd Street SW
Rochester, MN 55902

Dear Mr. Petzold:

This letter states that copyright permission has been granted to reprint the following article:

Petzold AM, Bedell VM, Boczek NJ, et al. SCORE Imaging: Specciman in a Corrected Optical Rotational Enclosure. *Zebrafish.* 2010;7:149-154.

The requested permission extends to any future revisions and editions of your dissertation and to the public circulation of your dissertation at the University of Minnesota library and online depository. Should you have any further questions, please do not hesitate to contact me.

Sincerely yours,

Karen Ballen
Manager, Reprint Department
kballen@liebertpub.com
Tel: (914) 740-2194
Fax: (914) 740-2108