A ROLE FOR THE MUSCARINIC ACETYLCHOLINERGIC SYSTEM IN SENSITIVITY TO ETHANOL'S STIMULANT EFFECTS

By

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LIST OF ABBREVIATIONS

ACh – acetylcholine

AUD – alcohol use disorder

B6 – C57BL/6 mouse inbred strain

CPP – conditioned place preference

D2 – DBA/2 mouse inbred strain

DA – dopamine

ES cell – embryonic stem cell

FH+ – family history positive

FH - – family history negative

HIP – hippocampus

KO – knockout

mAChR – muscarinic acetylcholine receptor

mRNA – messenger RNA

NAc – nucleus accumbens

nAChR – nicotinic acetylcholine receptor

PCR – polymerase chain reaction

PFC – prefrontal cortex

RNAi – RNA interference

SN – substantia nigra

SNP – single nucleotide polymorphism

qRT-PCR – quantitative real-time polymerase chain reaction

QTL – quantitative trait locus

VM – ventral midbrain

VTA – ventral tegmental area

WT – wildtype

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ABSTRACT

Initial sensitivity to the stimulant effects of alcohol may be a risk factor for developing alcohol (ethanol) use disorders. The FAST and SLOW selectively bred mouse lines were developed as a model of extreme sensitivity (FAST) and insensitivity (SLOW) to the locomotor stimulant effects of ethanol, and differ at genetic loci that influence the stimulant response to acute ethanol injection. A challenge has been identifying the relevant genes.

A main purpose of this dissertation was to investigate whether the muscarinic acetylcholinergic system differs between FAST and SLOW mice, and could play a role in their differential sensitivity to ethanol. FAST mice had been previously investigated for their response to the peripheral administration of the muscarinic antagonist scopolamine, and ethanol. The first study in this thesis investigated this drug combination in SLOW mice. SLOW-1 and -2 mice were differentially sensitive to the depressant effects of ethanol, but the results seen following the combination of scopolamine and ethanol in each replicate resembled the ethanol alone group. As the stimulant effect of scopolamine was attenuated once combined with ethanol in SLOW-1 mice, it was hypothesized that scopolamine stimulation and ethanol depression may be competing behaviors. A follow-up rotarod study revealed that scopolamine and ethanol together resulted in increased ataxia in SLOW-1 mice compared to ethanol alone. FAST-2 mice, when given scopolamine into the nucleus accumbens

(NAc) core, displayed an accentuated stimulant response when given a moderate dose of ethanol compared to mice given ethanol alone. However, this accentuation was additive, and not synergistic as we had hypothesized, based on the results of the previous peripheral experiment. FAST-1 mice were not significantly stimulated by scopolamine or ethanol. Therefore, muscarinic antagonism in the NAc was not responsible for the previously observed synergism.

To further examine the contribution of the cholinergic system in risk for developing alcohol use disorders, we investigated the m4 and m5 muscarinic acetylcholine receptor subtypes, which had been previously implicated as candidate genes for acute locomotor stimulation to ethanol in quantitative trait locus studies. FAST-2 and SLOW-2 mice differed in two non-synonymous polymorphisms for the *Chrm5* gene, but no differences were found between the lines for the *Chrm4* gene. In the ventral midbrain, the origins of the mesolimbic dopamine pathway, FAST mice displayed greater expression of the *Chrm5* gene than SLOW mice. Studies utilizing mouse models lacking either the m4 or m5 receptor gene revealed that the m5 receptor was necessary for a stimulant response to ethanol. However, in order to reveal these effects, the mice needed to first be backcrossed to a background strain that was sensitive to the stimulant effects of ethanol. The m4 gene had no effect on ethanol-induced stimulation, and neither the m4 or m5 null mutant genotypes consumed ethanol differently. Overall these data suggest that greater expression of *Chrm5* may confer greater

sensitivity to ethanol-induced stimulation, and that this receptor is necessary for an ethanol stimulant response to occur. However, the *Chrm4* is no longer an attractive candidate gene for acute locomotor stimulation to ethanol.

CHAPTER 1: General Introduction

The burden of alcoholism and alcohol use disorders (AUDs) is widespread among Americans, affecting 8% of the general population (Grant et al., 2004). While only one in twelve individuals identifies himself as having an AUD, nearly one in five Americans has abused alcohol at least one time in his life (Hasin et al., 2007). Understanding the factors that influence the transition from experimentation with alcohol to development of an AUD is of importance in preventing alcoholism. Excluding low-risk drinkers/abstainers, significant users of alcohol can be divided into three groups: at-risk or high-risk drinkers, functional alcoholics, and chronic severe alcoholics (Hasin et al., 2007; Willenbring, 2010). These three groups have characteristics that are similar to those described for the discrete phases of alcoholism that have been suggested: acquisition, controlled-drinking (maintenance), and uncontrolled drinking (dependence) (Spanagel, 2009). The acquisition phase is of unique interest, as individuals may be identified who over-consume alcohol, but have not yet met criteria for an AUD (Willenbring, 2010). These individuals, by virtue of their overconsumption, are at a greater risk for alcohol-related health issues.

Most of the studies on the acquisition of AUDs have focused on adolescents, and the highest rates of alcohol use are for those in their early 20s (Brown et al., 2008; Jacob et al., 2009). Studies have shown that the earlier one initiates drinking, the more likely one is to develop an AUD later in life (Hingson et al., 2006; Jacob et al., 2009). It is clear from the statistics that many

consumers of alcohol are able to self-regulate their drinking patterns either initially or over time, while others may not be able to do so. Understanding how individuals may be differentially sensitive to the initial effects of alcohol during the acquisition phase, and how that sensitivity impacts later drinking, is of vital importance to understanding the nature of addiction to alcohol.

The Genetics of AUDs

Since the 1970s, researchers have noticed that alcoholism tends to run in families, thus suggesting a genetic component to the disease (Cotton, 1979). Family studies, as well as twin and adoption studies, are useful in parsing both genetic and environmental contributions to alcohol dependence. Twin studies utilize both monozygotic, or identical, twins, and dizygotic, or fraternal, twins. Identical twins share 100% of their genetic material, while fraternal twins, like siblings, share 50% of their genetic information, on average (Ball, 2007). Twins share not only genetic information, but also some environmental influences, so adoption studies in which related individuals reside in separate environments are crucial in disentangling genetic and environmental effects. Twin studies have consistently calculated the heritability of alcohol dependence as 50 – 60% (Agrawal and Lynskey, 2008; Enoch and Goldman, 1999; 2001; Kaprio et al., 1987). In other words, 50 – 60% of the variation in the alcohol dependence phenotype in the population under study could be attributed to additive genetic variation. Adopted-out children of alcoholics also had increased rates of treatment for alcohol problems (9%) as compared to control individuals (1%),

further indicating a genetic component for alcoholism (Goodwin et al., 1973). Goodwin (1974) also found that sons of alcoholics were just as likely to develop an AUD if they were adopted-out as if they were reared at home with the alcoholic parent. However, environment is able to modulate drinking behaviors, and also interacts with genetics. For example, identical twins who are in close contact with one another drink more similarly than dizygotic twins with close contact, even after the confound of zygosity is removed (Kaprio et al., 1987). There is some evidence that high genetic susceptibility to alcoholism renders some individuals more sensitive to high-risk environments than does lower genetic susceptibility, though the interaction of high genetic susceptibility and criminality differentially affects males depending on the combination (Bohman et al., 1982; Cloninger et al., 1981, but see Haber et al., 2010). While the nature of genetic contributions to alcoholism is complicated, it is clear that they exist.

The field of human genetics of alcohol dependence has recently seen a surge in new candidate genes thought to serve as risk and/or protective factors for alcoholism (Agrawal and Lynskey, 2008; Gelertner and Kranzler, 2009). The identification of risk factors that may signal increased susceptibility to acquisition of an AUD, or to the transition from acquisition to a chronic disorder, would be of supreme importance in identifying those individuals at risk before they progress to an AUD. There have been genetic factors repeatedly associated with alcohol dependence, identified through the family linkage study, *Collaborative Study on the Genetics of Alcoholism*, which may ultimately be proven to be genetic risk factors. These include polymorphisms in the γ-aminobutryic acid receptor type A

(*GABRA2*), the cholinergic muscarinic receptor subtype 2 (*CHRM2*), and alcohol dehydrogenase (*ADH4*) genes (Edenberg and Foroud, 2006). There are also some identified intermediate phenotypes, perhaps caused by genetic factors shared in common with AUDs (in which case these would be referred to as endophenotypes). Some researchers have looked at functional magnetic resonance imaging (fMRI) scans of drug-naïve adolescents, both with and without family histories of alcoholism (Schweinsburg et al., 2004). These individuals are useful to study, as in the Schweinsburg et al. (2004) study, they were investigated prior to ever having any alcohol. Those with a family history of alcoholism displayed less frontal brain activity associated with neural inhibition than did those without a family history of alcoholism, despite never experiencing alcohol's effects (Schweinsburg et al., 2004). Other intermediate phenotypes have included distinct phases (P300) of event-related potentials (Hill, 2004). Sons of alcoholics had reduced P300 portions of event-related potentials as compared to controls, despite never having been exposed to alcohol (Begleiter et al., 1984). These results highlight the contribution of genetic as well as developmental factors that may influence susceptibility to becoming an alcoholic. However, the large overlap in range of response between those with and without these intermediate phenotypes makes them unsuitable for use as diagnostic markers.

The Alcohol Biphasic Dose Response Curve

Responses to alcohol/ethanol in both humans and animals are biphasic in nature. Responses to ethanol at lower doses or initially following administration during the ascending limb of the blood alcohol curve often manifest as feelings of euphoria, loquaciousness, and stimulation. Higher doses of ethanol, or responses at later time points during the descending limb of the blood alcohol curve, are more likely to be sedative and intoxicating. However, an individual's sensitivity to ethanol's stimulant and intoxicating/sedative effects is influenced by genetics as well as environment (Holdstock and de Wit, 1998; Lukas and Mendelson, 1988; Pohorecky, 1977). This is also evident in laboratory animal studies, discussed in more detail below (Dudek et al., 1991; Dudek and Tritto, 1994). Sensitivity to one or more of alcohol's effects might have a role in risk for addiction.

Human Behavioral Sensitivity Risk Markers

Given the societal burden of alcoholism and the knowledge that it has a genetic component (Agrawal and Lynskey, 2008; Cotton, 1979; Enoch and Goldman, 1999; 2001; Kaprio et al., 1987), the identification of genetic risk factors is important for the future prevention, or potential treatment, of AUDs. The need to identify markers that are related to risk for developing alcoholism is important, as evidence for biomarkers in existing alcoholics may be complicated by repeated exposure to alcohol throughout one's life (Newlin and Thomson, 1990). Identification of better risk markers that might be useful in individuals who

have had minimal prior exposure to alcohol may ascertain which individuals are more susceptible to developing AUDs, and in addition, biochemical pathways related to those risk factors may be important therapeutic targets in the future.

Several researchers have made use of a population suspected to be at risk for developing AUDs: those with a family history of alcoholism, or family history positive (FH+) individuals. Ideal for studies of risk are FH+ individuals who have not yet developed patterns of alcohol use associated with the development of an AUD, because risk can then be partially separated from the effects of alcohol. The majority of studies examining risk for alcoholism have focused on a low level of response to the intoxicating effects of alcohol as a sensitivity trait. In a longitudinal study using body sway as the measure, Schuckit found that individuals with a low level of response to the intoxicating effects of alcohol were four-fold more likely to develop an AUD in later years than those with a high level of response to the intoxicating effects of alcohol (Schuckit, 1994). Individuals with a low level of response to alcohol also initiated problem drinking earlier in life than did those with high levels of response to alcohol (Schuckit and Smith, 2001). Furthermore, this response is heritable, with a genetic contribution of 40 – 60% in the population studied (Wilhelmsen et al., 2003). Another laboratory reported that FH+ individuals experienced greater acute intoxicant effects and greater tolerance to a steady state blood alcohol level than family history negative (FH-) individuals (Morzorati et al., 2002). However, to reconcile these findings with those from Schuckit's lab, one must consider that "intoxication" to FH+ individuals might be pleasurable, and also that FH+ individuals might continue drinking to

“feel” the drug if they are more susceptible to acute tolerance. This could put FH+ at risk for the development of an AUD because of increased consumption of alcohol.

On the other hand, rather than insensitivity to intoxicating effects, some researchers have explored behavioral stimulation to alcohol, as those who experience stimulating, euphoric effects may be more likely to seek them in the future. In a seminal review, Newlin and Thomson (1990) performed an analysis of the existing alcohol research on acute response to alcohol in those with (FH+) and without (FH-) family histories of alcoholism. Aligning the time courses of the various studies, Newlin and Thomson (1990) proposed a differentiator model in which the FH+ individuals displayed acute sensitization to drug action during the rising phase of the blood alcohol curve, and acute tolerance during the falling phase. They suggested that FH+ individuals find alcohol more pleasurable because they experience the euphoric, excitable aspects of alcohol to a greater degree than those without a family history of alcoholism (FH-). According to the model, FH+ individuals also experience the dysphoric, intoxicating aspects of alcohol to a lesser extent than the FH- individuals. In addition, those classified as moderate or heavy drinkers experienced the euphoric and stimulating effects of alcohol more strongly during the ascending phase of the blood alcohol curve than those classified as light drinkers (Holdstock et al., 2000; King et al., 2002). Heavy drinkers also reported less severe sedative and dysphoric effects of alcohol (King et al., 2002). Accordingly, greater levels of stimulation to alcohol along the ascending limb of the blood alcohol curve have been associated with

greater consumption of alcohol (Erblich and Earleywine, 2003). Sensitivity to the initial subjective effects of ethanol has been shown to be associated with certain allelic variants (for example, *GABRA2*) in healthy social drinkers (Roh et al., 2010). Taken together, these data indicate that a blunted ataxic response, and sensitivity to the behavioral stimulant effects of ethanol are sensitivity traits relevant for risk for alcohol abuse. These traits are influenced by genetics and family history, and may promote an increase in alcohol consumption because they confer greater sensitivity to the subjective and behavioral effects of alcohol.

Of Mice and Men: Using Mice as a Model of Human Behavioral Stimulation

Mice and humans have been inextricably linked since the dawn of surplus agriculture (Silver, 1995). The transition of mice from the home to the lab began at the turn of the 20th century, when Castle used them to investigate patterns of inheritance (Silver, 1995). He helped to found The Jackson Laboratory in 1929, to ensure easy access to mice (Silver, 1995). Besides the ease of obtaining them, and their relatively low cost, there are several other reasons why mice are an excellent model for the study of behavioral genetics, and accordingly the genetic underpinnings of alcoholism. Average estimates indicate roughly 85% synteny, or conservation of gene order, between mouse and human coding exons (Flicek et al., 2003). Technology surrounding the use of genomic markers for sequencing and mapping has evolved, enabling researchers to pinpoint differences in sequence down to single nucleotides (single nucleotide polymorphisms, or SNPs). There are also multiple unique mouse gene mapping

populations, as well as the ability to manipulate single genes using knockout, transgenic, and other molecular technologies (Lasek and Azouaou, 2010; Palmer and Phillips, 2002b). Another major benefit to using mice as a model of the human condition is the vast array of behavioral assays that have been developed and tested over multiple years and across multiple laboratories (Crabbe, 2008; Crawley, 2007). A variety of public databases exist which allow researchers to identify and compare resources, oriented from genomic to behavioral (Peters et al., 2007). Obviously mice are not humans; this necessitates apportioning distinct features of disease into definable traits for manipulation in mice.

Acute locomotor stimulation in mice is an animal model of behavioral stimulation to alcohol in humans. Behavioral activation and locomotor activity in mice are related to motivation to obtain reinforcers (Salamone et al., 2007). Indeed, many drugs of abuse, including ethanol, which have reinforcing properties, share the ability to elicit locomotor behavior in mice (Wise and Bozarth, 1987), as do natural reinforcers. There are common neural pathways associated with ethanol/drug self-administration and the locomotor stimulant effects of addictive drugs, to be discussed below (Jones and Mogenson, 1980; Graybiel, 1998; Wise and Bozarth, 1987). Therefore, locomotor activation can be used to investigate the euphorigenic effects of drugs of abuse, such as ethanol (Phillips and Shen, 1996). Locomotor stimulation also provides a convenient measure of alcohol sensitivity that is useful for studies of alcohol's mechanisms of action.

Harnessing Mouse Genetics as a Tool for the Study of Human Behavioral Stimulation

Investigators are able to take advantages of unique differences among mouse strains to use mice as a genetic model of behavioral stimulation to ethanol. Genetic animal models are useful because one can test the behavior of interest, investigate pharmacological pathways that may influence the behavioral response, and look for the underlying genetic factors influencing both pharmacological responses as well as predisposition. In addition, the environment is largely, though not completely, controllable (Crabbe et al., 1994a). Exploring the genetic underpinnings of stimulation to ethanol in mice may provide data that associate genetic stimulation sensitivity with risk for alcoholism, which may serve as a good diagnostic tool for the treatment of AUD in the future.

Inbred Strains

Individuals within an inbred mouse strain are essentially like genetic clones, because they are virtually genetically identical to each other (with the exception of sex chromosome differences, mutations, and/or copy number variants). Therefore, any differences among individuals of an inbred strain are largely due to environmental influences. However, mouse inbred strains vary widely in origin, genetic makeup, and resulting behaviors (Silver, 1995). As such, mean phenotypic differences among inbred strains can be used in correlational analyses to estimate genetic correlations. A significant correlation between two

traits indicates that some of the same genes influence the two traits (provided that the environmental influences among the strains are similar). There are differences among inbred strains in many phenotypes, including differences in sensitivity to the locomotor stimulating effects of ethanol (Crabbe, 1983; Crabbe, 1986; Dudek et al., 1991; Kiianmaa et al., 1983; Tabakoff and Kiianmaa, 1982). By utilizing inbred strains, investigators can compare environmental differences within strains, and genetic differences between strains. Another advantage of inbred strains is that they have remained relatively stable over time, which makes researcher efforts “cumulative” (Crabbe, 2008).

By chance, the two inbred strains C57BL/6J (B6) and DBA/2J (D2) differ drastically in their locomotor response to ethanol and have served as a useful tool for the study of ethanol-induced stimulation. D2 mice display robust stimulation to increasing doses of ethanol, while B6 mice remain largely unactivated, or even sedated, to the same doses of ethanol (Crabbe et al., 1980; Dudek et al., 1991; Dudek and Tritto, 1994; Lister, 1987). The D2 strain also shows a preference for cues previously paired with ethanol in a conditioned place preference (CPP) paradigm, whereas B6 mice do not (Cunningham et al., 1992). Interestingly, these two strains display an opposite relationship in ethanol drinking preference, where B6 mice prefer ethanol over water, and D2 mice avoid consuming ethanol, under oral two-bottle choice situations (McClearn and Rodgers, 1959; Yoneyama et al., 2008). Although these particular mouse strains show an inverse relationship between ethanol-induced stimulation and ethanol preference, it is not accurate to describe this as a correlational relationship

(correlational analyses have n – 2 degrees of freedom, resulting in 0 degrees of freedom for a correlation derived from two inbred strains and therefore a lack of statistical fortitude). Furthermore, recent data from the Cunningham lab, using a unique introgastric delivery system, show that D2 mice will consume considerably more alcohol under these conditions than they do when offered alcohol as a drink solution (Fidler et al., 2011). Taste factors likely play a role in D2 strain avoidance (Fidler et al., 2011).

Hence, researchers have made use of inbred strain panels for the comparison of acute locomotor stimulation as well as ethanol consumption, finding a wide range of responses across inbred strains (Crabbe, 1986; Yoneyama et al., 2008). An inbred strain panel includes a number of inbred strains, which when tested for two phenotypes provides adequate power for estimation of genetic correlation. Mouse populations originating from D2 and B6 progenitor strains demonstrate an array of responses to ethanol. For example, acute locomotor stimulation and 3 and 10% ethanol consumption were significantly negatively correlated in B6 x D2 recombinant inbred (BXD RI) mice, but only the correlation with drinking of 3% ethanol remained significant when the extreme progenitor D2 and B6 mice were excluded (Phillips et al., 1995). Short-term selected lines for high and low ethanol drinking, originating from an F2 population of B6 x D2 intercross mice (B6D2F2), did not differ in magnitude of ethanol-induced activation (Phillips et al., 2005). These data suggest that the opposite relationship between ethanol consumption and locomotor stimulation to ethanol in D2 and B6 mice is merely a chance effect of fixation of different alleles

that influence each trait that do not have an influence on both traits, and not a firm genetic association. The inbred strain panel analyses performed in Crabbe et al. (1994b) indicated that generally speaking, sensitivity to one ethanol domain, such as locomotor stimulation, did not correlate with other ethanol traits (although ethanol consumption was not measured).

Targeted Genetic Models

Single gene mutants, also known as knockout (KO) mice, have been useful in identifying single gene effects on behavioral traits. The technique allows one to produce a deleted gene product by targeted mutation. Multiple different receptor systems and receptor-associated proteins have been implicated in acute locomotor stimulation to ethanol using single gene mutant mice (Crabbe et al., 2006), including α7 nicotinic receptors (Bowers et al., 2005), adenosine 2a receptors (Houchi et al., 2008), dopamine D4 receptors (Rubinstein et al., 1997), metabotropic glutamate 4 receptors (Blednov et al., 2004), γ-aminobutryic acid A (GABA-A) receptors (Kralic et al., 2003), μ-opioid receptors (Ghozland et al., 2005), cannabinoid-1 (CB1) receptors (Naassila et al., 2004), dopamine-and adenosine 3':5'-monophosphate-regulated neuronal phosphoprotein (DARPP-32) (Risinger et al., 2001), and vesicular monoamine transporter 2 protein (Wang et al., 1997). Interestingly, many of these KO models are on a B6 background, which does not readily show activation to ethanol. In fact, many of the receptor knockouts described above show enhanced ethanol-induced locomotor activity as compared to their wildtype (WT) counterparts,

indicating that KO of that particular gene, or the genes linked to the mutant allele, strongly influence the response to ethanol.

There are well-known issues with KO models. Some KOs may be homozygous lethal, necessitating the testing of heterozygotes, which only contain one copy of the mutated allele, in the place of homozygous KOs (e.g., Wang et al., 1997). Also, developmental compensation for the lack of the particular gene may occur [for example, CB1 knockouts show an upregulation of the dopamine D2 receptor (Houchi et al., 2005)], leading to spurious conclusions if the compensatory effect modulates the phenotype being tested. The creation of KO mice requires that the targeted mutation be inserted in donor embryonic stem (ES) cells, which once incorporated, are typically implanted in a blastocyst from a commonly used mouse inbred strain, such as B6. Because of technical difficulties with stem cells from most inbred strains, the ES cells most commonly used have been from the 129 substrains (Simpson et al., 1997). There can be unpredictable gene x gene interactions from passenger genes in the ES cell line, which flank the targeted gene even after several generations of backcrossing, or even specific gene effects linked to the ES cell line on the trait under study (Kelly et al., 1998; Simpson et al., 1997). Some researchers attempt to circumvent these issues by developing and testing conditional KO mice, which may lack the gene in a specific brain area (e.g., forebrain-specific KO (Gaveriaux-Ruff and Kieffer, 2007)) or be inducible in adulthood (e.g., tetracycline-inducible KOs (Aiba and Nakao, 2007)), although this approach has not been used in ethanol-induced stimulation research.

RNA interference (RNAi) techniques lack the temporal and spatial issues of KO models. Short-length oligonucleotides enable one to selectively knockdown gene-specific messenger RNA (mRNA) transcripts by post-transcriptional gene silencing. While it is possible to directly inject RNAi transcripts into the brain, they are typically coupled with viral vectors to enhance stability and longevity. The effects of RNAi transcripts coupled with viral vectors can last up to nine months in the brain (AW Lasek, personal communication). Conditions for establishing appropriate RNAi oligonucleotides are now well established in the literature (Lasek et al., 2007; Lesscher et al., 2008; Reynolds et al., 2004). Although this technique has not been used in the study of acute ethanol effects, it has been used successfully to show a reduction in ethanol consumption following targeted knockdown of μ-opioid receptor mRNA in the ventral tegmental area (Lasek et al., 2007). Taken together, genetic tools such as KOs and RNAi add another dimension to the study of ethanol-related phenotypes beyond pharmacological approaches, especially considering that there are not pharmacological tools specific for all gene products.

Selected Lines

The goal of selective breeding, or artificial selection, is to produce animal lines that are divergent in a trait of interest. As selection proceeds, allele frequencies in the divergent lines become different for genes that influence the selection trait (Crabbe, 1999). The more heritable the response, the more potential for divergence in the selected lines, provided that they originate from a

population that differs with regard to genes that impact the selection phenotype and selection pressure is adequate. Bidirectional selection involves mating the most extreme scoring individuals with one another in independent lines (e.g., extreme high scorers are mated with other extreme high scorers, and extreme low scorers are mated with each other). The trait of interest is tested in the founding population, as well as subsequently selected generations (offspring), to determine whether the selective mating had an impact on the magnitude of the phenotype distribution. Also, lines selected for a particular trait can be tested for alternate traits that may share the same or some of the same genetic influences; such traits are termed genetically correlated (Crabbe, 1999; Henderson, 1989).

The FAST and SLOW selected lines are a genetic mouse model of human differential behavioral stimulation to ethanol. They were created by selective breeding over multiple generations for extreme sensitivity (FAST) or insensitivity (SLOW) to the locomotor stimulant effects of ethanol (Crabbe et al., 1987; Phillips et al., 1991; 2002b; Shen et al., 1995b). As would be found in human populations, the FAST mice model a high stimulant response, while the SLOW mice model a low stimulant response. These mice originated from a heterogeneous stock (HS) of mice produced by crossing 8 inbred mouse strains (A, AKR, BALB/c, C3H/2, C57BL, DBA/2, Is/Bi, and RIII; mated with equal frequency). This stock was produced at the Institute for Behavioral Genetics and named HS/Ibg (McClearn et al., 1970). For the founding population, on Day 1, mice were injected with saline, and 2 min later were placed into an open field

arena and activity was measured for 4 min. On Day 2, the same procedure was followed except that mice were injected with 1.5 g/kg ethanol (Crabbe et al., 1987). The selection trait for the FAST and SLOW selection was a difference score: activity after ethanol treatment minus activity after saline treatment. Breeders were paired to establish multiple high scoring pairs (families of FAST line) and multiple low scoring pairs (families of SLOW line), and offspring of these breeders were tested. A within-family selection paradigm was then used, excluding brother-sister mating, whereby two mice with the highest activity scores were chosen from each family to perpetuate the FAST line (highest scoring FAST males and females) and mice with the lowest activity scores to perpetuate the SLOW line (lowest scoring SLOW males and females) (Falconer and Mackay, 1996). The lines were created in replicate, so that half of the founding population was tested to establish the replicate-1 FAST-1 and SLOW-1 lines, and the other half to establish the replicate-2 FAST-2 and SLOW-2 lines. Maintaining independently generated replicate lines is the most rigorous approach for the creation of selected lines, as it helps to mitigate the potential confound of spurious results for the selection trait due to genetic drift (i.e. ascribing the result as a consequence of selection when in fact it results from chance, unlikely to occur to the same extent in two separate replicates) (Henderson, 1997). Also, if common results for an alternate trait are seen in both replicate lines, it provides strong evidence for pleiotropic genetic effects, or the effect of one or more genes on multiple phenotypes (Crabbe et al., 1990; 1999).

Two non-selected control lines were established from the originating population, whose parents were chosen without regard to ethanol stimulation score. They were tested every 3rd generation for their difference score, and provided a measure of genetic drift and environmental effects in this as well as another selective breeding project (Phillips et al., 1991; Shen et al., 1995b). Selection for sensitivity and insensitivity to the stimulant effects of ethanol generated a divergent response between the lines in the initial generation, with FAST mice displaying greater locomotor activation to ethanol than SLOW, but this divergence stalled in subsequent generations (Figure 1.1). The control-1 line mimicked the SLOW-1 response in initial generations, but the control-2 line was intermediate to the FAST-2 and SLOW-2 response (Phillips et al., 1991). Because of the lag in selection response, studies were performed to investigate possible changes in selection procedures that might improve divergence (Crabbe et al., 1988). Based on these results, following the 5th generation of selection, the order of testing was reversed so that the Day 1 test was ethanol and the Day 2 test was saline. In addition, the ethanol dose was increased to 2 g/kg (Phillips et al., 1991). Following these changes, divergence between the FAST and SLOW mice continued to increase, and the difference between the FAST and SLOW lines grew larger across generations (Figure 1.1). During the following mid- to late-selection generations (G_{18-27} (G_{xx} refers to the generation of mice tested)), SLOW mice displayed reduced, or even depressed locomotor responses (Figure 1.1). Selection pressure was maintained for 37 generations, after which it was relaxed and mice have since been maintained as independent

populations, with breeders randomly selected. The difference between the pairs of selected lines has remained stable over generations. These mice are currently in generation 110 ($S_{37}G_{110}$ (S_{xx} refers to the final generation where selection pressure was applied, and G_{xx} refers to the generation of mice tested)), were most recently tested for ethanol-induced locomotor activity at generation 106, and continue to maintain their divergent response to ethanol (Gubner et al., submitted).

Besides displaying differences in locomotor stimulation to ethanol, FAST and SLOW mice differ for other traits thought to measure sensitivity to ethanol reward and aversion. FAST mice consumed greater amounts of ethanol, compared to SLOW mice (Risinger et al., 1994). FAST-2 mice were resistant to the development of ethanol conditioned taste aversion to saccharin compared to SLOW-2 mice, although there was no difference between the replicate-1 lines (Risinger et al., 1994). Furthermore, FAST mice were recently found to have a decreased threshold for intracranial self-stimulation (ICSS) in the medial forebrain bundle following exposure to ethanol, compared to SLOW mice (Fish et al., submitted). ICSS is an operant measure of brain stimulation reward and reinforcement, and indicates subjective response. FAST mice decreased threshold responding compared to SLOW mice that did not decrease threshold responding below baseline response when ethanol was on board, which indicates that FAST mice required less stimulation intensity to feel reward, and found ethanol more rewarding than SLOW mice (Fish et al., submitted).

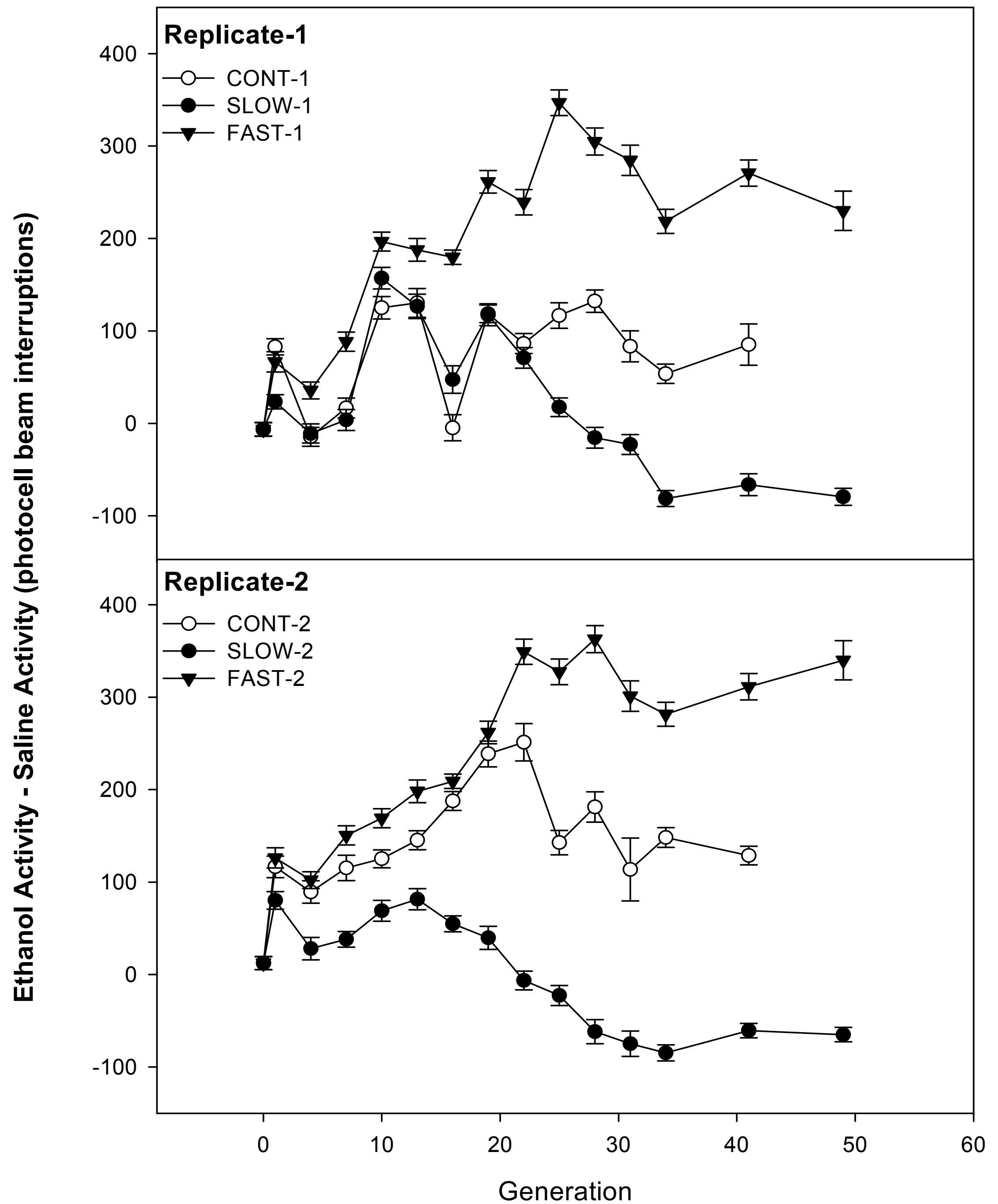

Figure 1.1. The FAST and SLOW lines differ in activation to a 1.5 – 2.0 g/kg dose of ethanol. Shown are mean ± SEM values for response to ethanol minus response to saline. Data are plotted for every 3 generations. Selection was relaxed at G_{37}. The open circles correspond to the non-selected control line (CONT). Data for each replicate set of lines are shown in separate panels. (adapted from Phillips et al., 2002b)

However, FAST and SLOW mice did not display differences in magnitude of preference for cues previously paired with ethanol in a CPP test (Risinger et al., 1994).

The selection has also altered alleles involved in ethanol-induced ataxia, hypothermia and sedation, tests that have been performed across the course of selection, and have diverged along with the selection response. SLOW mice displayed a longer latency to recover to a prone position from a supine one (duration of loss of righting reflex) following a high dose of ethanol, were more sensitive to ethanol-induced hypothermia, and were more sensitive than FAST mice to the depressant effects of many alcohols at varying doses (Palmer et al., 2002; Phillips et al., 1992; Shen et al., 1995b; Shen et al., 1996). In SLOW mice, insensitivity to ethanol's stimulant effects is genetically correlated with the sedative, intoxicating effects of ethanol. However, the data for ataxia-like responses in SLOW mice have been less straightforward. In the grid test, SLOW mice were more sensitive to the ataxic effects of ethanol than FAST mice (Shen et al., 1996). In other measures of ataxia, FAST-1 mice fell off the stationary dowel more quickly than SLOW-1 mice, while SLOW-1 fell from the fixed speed rotarod more quickly than FAST-1 mice (Boehm et al., 2000). There were no differences between replicate-2 mice for the dowel or fixed speed rotarod, nor were there any differences between the lines on the accelerating rotarod (Boehm et al., 2000). However, the majority of data indicate that selection has altered the frequencies of genes that have a pleiotropic influence on acute ethanol-induced stimulant sensitivity and sedation severity.

Interestingly, FAST mice had more severe handling-induced convulsions than SLOW mice following withdrawal from chronic ethanol exposure, indicating that FAST mice may display an overall "hyperexcitability" phenotype (Palmer and Phillips, 2002a; Shen et al., 1996). Generally, FAST mice required a lower dose of convulsant drugs to elicit seizures than did SLOW (Shen et al., 1998). However, this was not true for every convulsant drug tested, and some effects were replicate-dependent (Shen et al., 1998), indicating moderate evidence for genetic correlation between stimulant sensitivity and seizure sensitivity (Crabbe et al., 1990).

The FAST and SLOW selected lines of mice have been tested for differences in neurotransmitter systems in an effort to determine which systems influence acute locomotor stimulation to ethanol. Focusing solely on the stimulant response to ethanol (seen in FAST but not SLOW), a variety of neurotransmitter systems have been implicated as underlying the ethanol stimulant response in FAST mice (Table 1.1). Differences in neurotransmitter systems between FAST and SLOW mice also influence the locomotor response to pharmacological agents, when given alone in the absence of ethanol (Table 1.2). These differences also indicate which neurotransmitter systems have been altered by selection, like any other correlated phenotype. Furthermore, both basal and drug-evoked differences in neurochemistry, gene expression, or peptide levels may indicate predisposition to sensitivity (FAST) or insensitivity (SLOW) to the acute stimulant effects of ethanol (Table 1.3). Differences in neurotransmitter systems between the FAST and SLOW lines of mice in the

absence of ethanol treatment are of interest as they may point to drug-naïve differences between the lines that predict sensitivity to the stimulant effects of ethanol.

Table 1.1. Neurotransmitter system effects on ethanol-induced locomotor stimulation in FAST mice.

Drug	Mechanism	Effect on stimulant response to ethanol in FAST mice	Reference
Acetylcholine (ACh)			
Dihydro-β-erythroidine	Nicotinic α4β2 receptor antagonist	0.5 – 2 mg/kg had no effect	Kamens and Phillips, 2008
Hexamethonium	Peripheral nonselective nicotinic receptor antagonist	2 – 8 mg/kg had no effect	Kamens and Phillips, 2008
Mecamylamine	Nonselective nicotinic receptor antagonist	3 – 6 mg/kg ↓ in FAST-1, 1 – 6 mg/kg ↓ in FAST-2	Kamens and Phillips, 2008
Methyllycaconitine	Nicotinic α7 receptor antagonist	1 – 4 mg/kg had no effect	Kamens and Phillips, 2008
Nicotine	Nicotinic receptor agonist	1 – 2 mg/kg ↑; repeated 1 – 2 mg/kg ↑↑	Gubner et al., submitted
Scopolamine	Nonselective muscarinic receptor antagonist	0.125 – 0.5 mg/kg ↑	Scibelli and Phillips, 2009
Dopamine (DA)			
Haloperidol	D2-like selective DA antagonist	0.16 mg/kg ↓	Shen et al., 1995a
Quinpirole	D2 receptor agonist	0.005 mg/kg had no effect	Phillips and Shen, 1996
Raclopride	D2-selective DA antagonist	0.25 – 0.5 mg/kg ↓	Shen et al., 1995a
SCH-23390	D1-like selective DA antagonist	0.03 mg/kg ↓ in FAST-1	Shen et al., 1995a
SCH-23390 + raclopride	D1 + D2 receptor antagonists	0.03 mg/kg SCH-23390 + 0.25 mg/kg raclopride ↓ in FAST-2	Shen et al., 1995a
SKF-38393	D1 agonist	10 mg/kg ↑ in FAST-1	Phillips and Shen, 1996
SKF-38393 + quinpirole	D1 + D2 receptor agonists	10 mg/kg SKF-38393 + 0.005 mg/kg quinpirole ↑ in FAST-1	Phillips and Shen, 1996
γ-Aminobutyric acid (GABA)			
Baclofen	GABA-B receptor agonist	1.39 – 2.77 μg icv ↓;	Boehm et al.,

		0.01 – 0.02 μg in aVTA ↓; 0.01 – 0.02 μg in pVTA ↑	2002a
Baclofen	GABA-B receptor agonist	5 mg/kg ↓ in FAST-1	Holstein et al., 2009
Muscimol	GABA-A receptor agonist	1 – 2 mg/kg ↓ in FAST-2	Holstein et al., 2009
NO-711	GABA reuptake inhibitor	2.5 – 7.5 mg/kg ↓ in FAST-1, 5 – 7.5 mg/kg in FAST-2	Holstein et al., 2009
Glutamate			
Ketamine	NMDA receptor antagonist	10 – 60 mg/kg shifted ethanol dose-response to the left (10 mg/kg ↑ to 1 g/kg ethanol in FAST-1, 10 – 20 mg/kg ↑ to 1 g/kg ethanol in FAST-2; 5 – 60 mg/kg ↓ to 2 g/kg ethanol in FAST-1, 10 – 60 mg/kg ↓ to 2 g/kg ethanol in FAST-2)	Meyer and Phillips, 2003
MK-801	NMDA receptor antagonist	0.2 mg/kg ↓	Shen et al., 1996
Opioid			
Naloxone	Nonselective opioid receptor antagonist	0.3 – 6 mg/kg had no effect	Holstein et al., 2005

Note: Route of drug administration was intraperitoneally (i.p.) unless otherwise noted. aVTA = anterior ventral tegmental area; icv = intracerebroventricularly; pVTA = posterior ventral tegmental area

Table 1.2. Basal neurotransmitter system effects on behavior in FAST and SLOW.

Drug	Mechanism	Effect on locomotor response between FAST and SLOW mice	Reference
ACh			
Nicotine	Nicotinic receptor agonist	0.5 – 2 mg/kg ↑ in FAST vs. SLOW	Bergstrom et al., 2003
Nicotine	Nicotinic receptor agonist	No difference between FAST and SLOW	Phillips et al., 1992
Scopolamine	Nonselective muscarinic receptor antagonist	0.5 – 1 mg/kg ↑ in FAST vs. SLOW	Bergstrom et al., 2003
DA			
d-Amphetamine	Indirect DA agonist	Shift in dose-response to the left between FAST and SLOW (2.5 mg/kg ↑ in SLOW vs. FAST; 5 – 10 mg/kg ↑ in FAST vs. SLOW G_{11}); no difference between FAST and SLOW G_{14-15}	Phillips et al., 1992
Cocaine	Indirect DA agonist	10 – 40 mg/kg ↑ in FAST-1 vs. SLOW-1	Bergstrom et al., 2003
Methamphetamine	Direct/indirect DA agonist	1 – 8 mg/kg ↑ in FAST-1 vs. SLOW-1	Bergstrom et al., 2003
Raclopride	D2-selective DA antagonist	No difference to 0.03 – 0.24 mg/kg between FAST and SLOW	Shen et al., 1995a
SCH-23390	D1-like selective DA antagonist	No difference to 0.25 – 1.0 mg/kg between FAST and SLOW	Shen et al., 1995a
GABA			
Baclofen	GABA-B receptor agonist	↓ to 2.5 – 5 mg/kg in SLOW-1 vs. FAST-1	Shen et al., 1998
Bicuculline	GABA-A receptor antagonist	No difference between FAST and SLOW	Shen et al., 1998

Diazepam	Benzodiazepine receptor agonist	↑ to 1 – 8 mg/kg in FAST vs. SLOW	Phillips et al., 1992
Diazepam	Benzodiazepine receptor agonist	4, 8, 16 mg/kg ↑ in FAST; 8 mg/kg ↓ in SLOW	Shen et al., 1998
Diazepam	Benzodiazepine receptor agonist	↑ in 1 – 8 mg/kg in FAST-1 vs. SLOW-1; ↑ 2 – 8 mg/kg in FAST-2 vs. SLOW-2	Palmer et al., 2002
Pentobarbital	Benzodiazepine receptor agonist	No difference between FAST and SLOW G_{12}; 10 – 30 mg/kg ↑ in FAST vs. SLOW G_{17-18}	Phillips et al., 1992
Pentobarbital	Benzodiazepine receptor agonist	↑ to 20 – 30 mg/kg in FAST-1 vs. SLOW-1; ↑ 10 – 40 mg/kg in FAST-2 vs. SLOW-2	Palmer et al., 2002
Phenobarbital	Benzodiazepine receptor agonist	60 – 120 mg/kg ↑ in FAST vs. SLOW	Phillips et al., 1992
Picrotoxin	GABA-A receptor antagonist	No difference between FAST and SLOW	Shen et al., 1998
Midazolam	Benzodiazepine receptor agonist	↑ 2.5 – 10 mg/kg in FAST vs. SLOW	Shen et al., 1998
Muscimol	GABA-A receptor agonist	No difference between FAST and SLOW	Shen et al., 1998
NO-711	GABA reuptake inhibitor	2.5 – 7.5 mg/kg ↑ in FAST-1 vs. SLOW-1; ↑ in 5 – 7.5 mg/kg in FAST-2 vs. SLOW-2	Holstein et al., 2009
Glutamate			
Ketamine	NMDA receptor antagonist	↑ to 10 – 60 mg/kg in FAST-1 vs. SLOW-1; ↑ to 10 mg/kg in FAST-2 vs. SLOW-2, ↑ to 60 mg/kg in SLOW-2 vs. FAST-2	Meyer and Phillips, 2003
MK-801	NMDA receptor antagonist	No difference between FAST and	Shen et al., 1996

		SLOW	
Opioid			
Morphine	μ-opioid receptor agonist	No difference between FAST and SLOW in G_{14-15} or G_{20}	Phillips et al., 1992
Morphine	μ-opioid receptor agonist	8 – 32 mg/kg ↑ FAST-1 vs. SLOW-1, 32 mg/kg ↑ FAST-2 vs. SLOW-2	Bergstrom et al., 2003
Other			
Caffeine	A2A receptor antagonist	No difference between FAST and SLOW	Phillips et al., 1992

Note: Route of drug administration was intraperitoneally (i.p.) unless otherwise noted

Table 1.3. Molecular differences in neurotransmitter systems between FAST and SLOW mice.

Drug	Result	Reference
ACh		
Basal measurement	No difference between FAST and SLOW in whole brain gene expression of nicotinic α3 subunit	Kamens and Phillips, 2008
Basal measurement	↑ nicotinic α6 subunit whole brain gene expression in SLOW vs. FAST	Kamens and Phillips, 2008
Basal measurement	No difference between FAST and SLOW in nicotinic *β2* subunit whole brain gene expression	Kamens and Phillips, 2008
Basal measurement	↑ nicotinic *β4* subunit whole brain gene expression in SLOW vs. FAST	Kamens and Phillips, 2008
DA		
Ethanol (2 g/kg)	↑ DA levels in NAc in FAST vs. SLOW by microdialysis	Meyer et al., 2009
Ethanol (50 – 80 mM, in vitro)	↑ DA firing and I_H current in SN cells in FAST-2 vs. SLOW-2 (replicate-1 mice were not tested)	Beckstead and Phillips, 2009
Cocaine (40 mg/kg)	↑ DA levels in NAc in FAST vs. SLOW by microdialysis	Meyer et al., 2009
GABA		
Bipolar-stimulating electrode stimuli	↑ GABA-A receptor input to SN DA neurons in SLOW-2 vs. FAST-2 (replicate-1 mice were not tested)	Beckstead and Phillips, 2009
Glucocorticoid		
Basal measurement	No difference between FAST and SLOW in Ucn levels of EW	Bachtell et al., 2002
Basal measurement	↑ basal corticosterone levels in SLOW-2 vs. FAST-2	Boehm et al., 2002b
Saline	↑ corticosterone levels in SLOW vs. FAST at 15 min. following saline, ↑ corticosterone levels in FAST vs. SLOW at 90 min, no difference at 120 min	Boehm et al., 2002b
Ethanol (2 g/kg)	↑ corticosterone levels in SLOW vs. FAST at 45 and 90 min. following 2 g/kg ethanol	Boehm et al., 2002b
Ethanol (4 g/kg)	↑ corticosterone levels in SLOW vs. FAST	Boehm et al., 2002b
Glutamate		

Basal measurement	No differences between FAST and SLOW in L-glutamate-stimulated intracellular calcium concentration in hippocampal or cortical microsacs	Daniell and Phillips, 1994
Ethanol (50 - 200 mM)	↓ in L-glutamate-stimulated intracellular calcium concentration in hippocampal microsacs following 100 mM ethanol in SLOW vs. FAST; ↓ in L-glutamate-stimulated intracellular calcium concentration in cortical microsacs following 50 – 200 mM ethanol in SLOW vs. FAST	Daniell and Phillips, 1994
Ethanol (2 g/kg)	No effect on glutamate release between FAST and SLOW	Meyer et al., 2009
Cocaine (40 mg/kg)	No effect on glutamate release between FAST and SLOW	Meyer et al., 2009
Other		
Saline-induced cFos expression	↑ cFos expression in SLOW vs. FAST in dorsolateral caudate putamen, subthalamic nucleus, entopeduncular nucleus, SN-pars compacta (SN-pc) and SN-pars reticulata (SN-pr), VTA	Demarest et al., 1999a

EW = Edinger-Westphal nucleus, HIP = hippocampus, I_H = inward hyperpolarizing, NAc = nucleus accumbens, PFC = prefrontal cortex, SN = substantia nigra, Ucn = urocortin, VM = ventral midbrain, VTA = ventral tegmental area

Investigations of neurotransmitter systems in FAST mice indicate which of them influence the stimulant response to ethanol (Table 1.1). Most striking, it is clear that the DA system modulates ethanol's stimulant effects, as pretreatment with a variety of dopaminergic antagonists attenuated the stimulant response to ethanol in FAST mice. However, the FAST-1 line of mice appeared to be more sensitive to dopaminergic agonists than FAST-2, as the dopaminergic agonists enhanced ethanol's stimulant effects in FAST-1 mice but not FAST-2. However,

the extreme stimulant response to ethanol in FAST-2 may have resulted in a ceiling effect, preventing additional stimulation to dopaminergic agonists (Phillips and Shen, 1996). Although the replicate lines differ in sensitivity to DA agonist modulation of ethanol's effects, this does not necessarily mean that there is only weak evidence that DA is involved in the stimulant response to ethanol. It is possible that the FAST-1 and FAST- 2 mice arrived at sensitivity to the stimulant effects of ethanol via different genetic mechanisms, resulting in slightly different neurochemical profiles. Many genes influence the stimulant response to ethanol, as it is a complex trait. However, FAST-2 mice were still sensitive to dopaminergic antagonists, indicating that the DA system does modulate their sensitivity to ethanol. The GABA system also appears to be a key mediator of ethanol's locomotor effects, as virtually all GABA agonists tested decreased the stimulant response to ethanol in FAST mice. Rather than antagonizing the effects of ethanol, GABAergic drugs appear to shift the dose-response curve by acting like ethanol, accentuating ethanol's intoxicating properties. Both FAST lines were also sensitive to the effects of cholinergic and glutamatergic agonists and antagonists on the stimulant response to ethanol.

There are basal differences in response to pharmacological agents in FAST and SLOW mice as well. The difference in response to cholinergic agents alone between FAST and SLOW mice appears to have evolved in later generations, as there was no difference between the lines early in selection, but when tested later, differences emerged (Table 1.2). The FAST and SLOW lines displayed differential sensitivity to pharmacological agents impacting the DA

system, but this appears to be more the case in the replicate-1 lines than the replicate-2. Dopaminergic agonists elicited a larger difference in response between FAST-1 and SLOW-1 mice, compared to the replicate-2 lines, although the dopaminergic antagonists did not appear to differentially affect FAST and SLOW mice. While the GABA agonists tended to reduce locomotor activity in SLOW mice, FAST mice typically displayed locomotor stimulation following these agents. As ethanol has GABAergic properties, the profile of responses to GABAergic drugs in FAST and SLOW mice is similar to their differential locomotor responses to ethanol. Glutamatergic and opioid-related differences between FAST and SLOW mice appeared to arise in later selection generations. Overall, these data indicate that there are differences in neurotransmitter systems between FAST and SLOW mice that may influence the difference between the lines in response to ethanol.

The DA differences between FAST and SLOW mice are evident in molecular preparations as well (Table 1.3). FAST mice had greater DA levels in the NAc following both ethanol and cocaine treatment, and enhanced DA firing in the SN, as compared to SLOW (though DA firing was measured only in the second replicate). There were also differences in GABAergic systems, as SLOW mice had increased GABA-A input to dopaminergic neurons in the SN as compared to FAST. There were also differences in cholinergic gene expression and glucocorticoid levels in FAST and SLOW mice. Taken together, these data indicate that a variety of neurotransmitter systems have been altered as a result of selection for ethanol's stimulant effects. However, the DA and GABA systems

are the most widely implicated in ethanol's stimulant effects in FAST and SLOW mice.

The difference between FAST and SLOW mice in sensitivity to ethanol's stimulant effects is paralleled by differences in DA efflux, DA firing and current, as well as differences to dopaminergic agents when ethanol is not on board that parallel their selection responses. However, the DA system is not solely responsible for the differential stimulant response between FAST and SLOW mice. While FAST mice displayed greater DA efflux from the NAc following both ethanol and cocaine administration than did SLOW, injections of ethanol and cocaine still resulted in an efflux of DA in the NAc of SLOW mice (albeit smaller) (Meyer et al., 2009). Likewise, though FAST mice displayed greater locomotor activity to the indirect DA agonists cocaine and methamphetamine as compared to SLOW, the SLOW-2 line did still display locomotor stimulation to those drugs, although SLOW-1 did not (Bergstrom et al., 2003). While the DA system is involved in ethanol's stimulant effects, the absence of DA effect is not required for a lack of stimulation, as seen in SLOW mice. This may suggest that enhancement of DA is more important for stimulation akin to the responses in FAST, while perhaps enhancement of GABA is more important for locomotor depression as seen in SLOW mice.

The GABA system was one of the first to diverge between the FAST and SLOW lines (Phillips et al., 1992). As previously mentioned, responses to GABA agonists have paralleled the responses to ethanol in FAST and SLOW

mice. Furthermore, SLOW-2 mice had greater GABA-A input in the SN than did FAST-2). These data highlight the key roles of DA and GABA on the differential response to ethanol in FAST and SLOW mice as well as discrepancies that may predispose these mice towards their contrary responses. As this dissertation is focused on the stimulant response to ethanol, the DA system will be a primary focus.

The Mesolimbic DA Pathway

The mesolimbic DA pathway is a key circuit in the behavioral and neurochemical effects of natural rewards (e.g., sugar) and drug rewards, including ethanol. While it is easy to describe the mesolimbic DA pathway simply as the “reward pathway,” the roles of DA and the brain regions on which it acts are much more complex. Briefly, dopaminergic projections from the VTA contact the NAc, which functions as a limbic-motor integrator (Mogenson et al., 1980). These two areas receive a variety of projections, simplified in a diagram below (Figure 1.2). The mesolimbic DA circuitry functions as a motivational and salience prediction unit, as well as playing a role in locomotor activity (Sesack and Grace, 2010).

The basal ganglia, by virtue of their different afferents and efferents, differentially regulate motor control. The striatum is the primary afferent nucleus of the basal ganglia. Its dorsal portion projects to the SN-pr (Grace and Bunney, 1985). GABA-sensitive neurons of the SN-pr inhibit cells of the SN-pc, overall disinhibiting neurons and promoting DA release (Fallon et al., 1978; Grace and

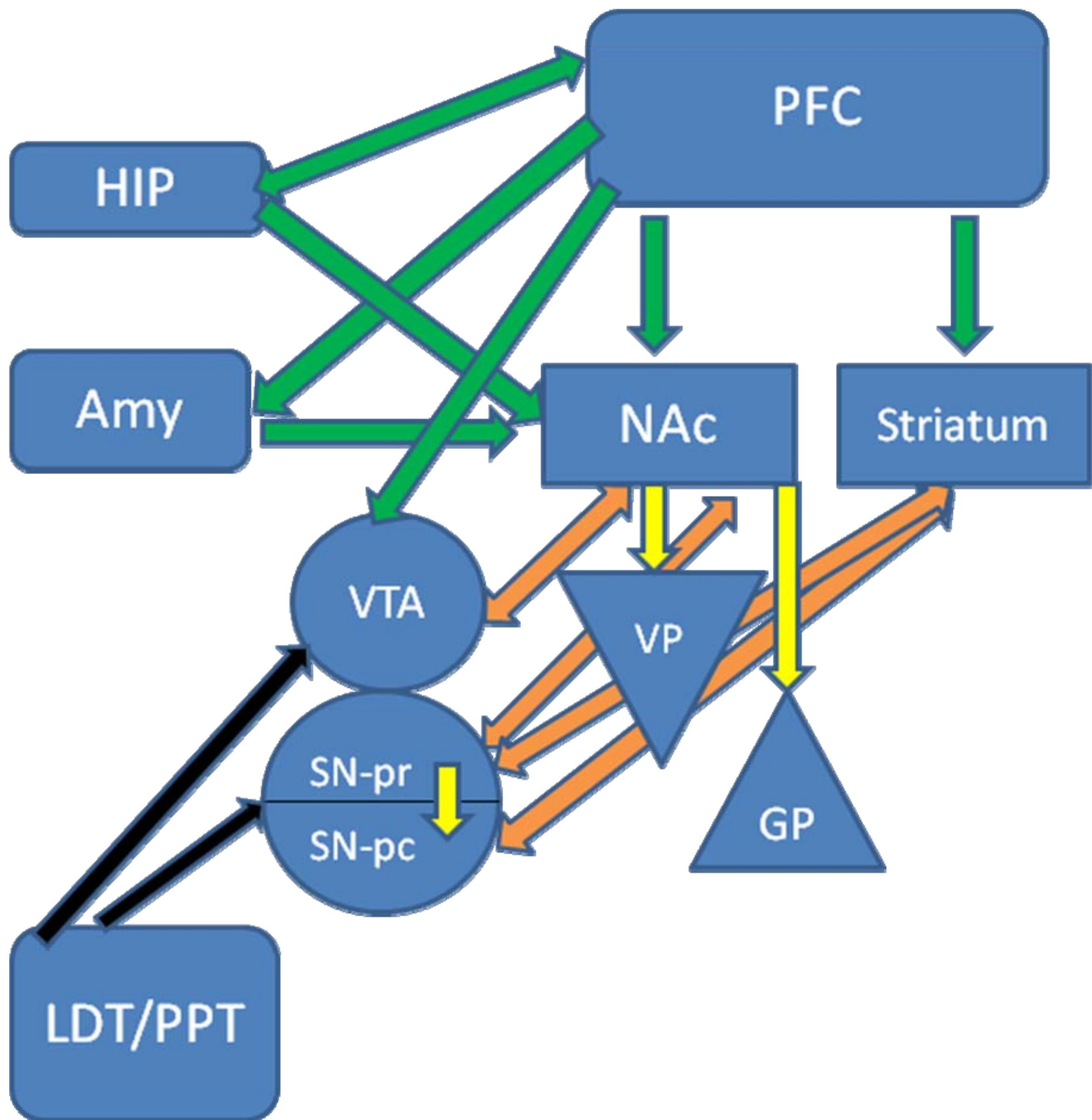

Figure 1.2. A simplified circuitry diagram of the mesolimbic DA pathway and related inputs/outputs. Directional arrows show direction of projection. Major neurotransmitter system relevant to drug reward/stimulation for each projection neuron is indicated by color. Black refers to ACh, green to glutamate, orange to DA, and yellow to GABA. Amy = amygdala; GP = globus pallidus; LDT = laterodorsal tegmentum; HIP = hippocampus; NAc = nucleus accumbens; PFC = prefrontal cortex; PPT = pedunculopontine tegmentum; SN-pc = substantia nigra pars compacta; SN-pr = substantia nigra pars-reticulata; VP = ventral pallidum; VTA = ventral tegmental area. Adapted from Everitt and Robbins, 2005.

Bunney, 1979). The SN-pc receives direct projections from the striatum, but is also innervated by an indirect pathway from the striatum, which projects from the striatum to additional brain areas and then back to the SN-pc. There are also reciprocal connections from the striatum to the SN-pc (Albin et al., 1989). These projection neurons are medium spiny neurons, and are GABAergic in nature (Penney and Young, 1981). The striatum also has a ventral portion, also referred to as the NAc. The nigrostriatal pathway is associated with motor activity, while the mesolimbic VTA projections to the ventral striatum/NAc innervate sites involved in reward and motivation (Oakman et al., 1995; Zhou et al., 2003).

The striatum also possesses tonically active cholinergic interneurons, which fire independent of any synaptic input (Di Chiara et al., 1994; Pickel and Chan, 1991; Pisani et al., 2007). Striatal ACh release from cholinergic interneurons is able to self-modulate, via presynaptic inhibitory m2 autoreceptors (Bernard et al., 1992), but cholinergic neurons also modulate DA transmission in the striatum. Dopaminergic neurons of the nigrostriatal pathway and glutamatergic afferents from the PFC converge on the same medium spiny neurons of the striatum, highlighting the close interactions within this area (Moss and Bolam, 2008). While GABAergic neurons make up the majority of neurons in the striatum (60 - 85%), the less represented DA neurons (5.7%) and cholinergic interneurons (1 - 2%) interact in a lattice framework, making both direct connections as well volume-dependent transmission of DA that impinges on DA receptors expressed on ACh interneurons (Moss and Bolam, 2008; Threlfell and Cragg, 2011). Both the nicotinic (nAChR) and muscarinic acetylcholine receptors

(mAChR) on cholinergic interneurons are able to bidirectionally regulate DA transmission, either promoting or blocking DA release depending on the specific neurochemical inputs (Bonsi et al., 2008; Grilli et al., 2010; Threlfell et al., 2010; Threlfell and Cragg, 2011).

The NAc can be subdivided into a core and a shell region (and possibly others), with slightly different functional circuits (Zahm and Brog, 1992). However, these circuits converge on the same areas, and both the core and shell play a role in activation induced by drugs of abuse (Ikemoto, 2002). NAc motivational inputs to the ventral pallidum (VP) result in movement (Mogenson et al., 1980). The NAc also integrates signals from different inputs. For example, while the NAc receives glutamatergic projections from the ventral subiculum (vSub) of the HIP, concurrent input from other brain regions (such as the amygdala) to the NAc is suppressed (Floresco et al., 2001b). The high frequency stimulus from the vSub to the NAc promotes mesoaccumbal DA release, as well as “priming” the nucleus for excitability in response to stimuli, and highlights the role of DA and the NAc in salience prediction (Floresco et al., 2001b; Salamone et al., 2007; Schultz, 1997). The vSub also modulates DAergic activity in the VTA via both the NAc (glutamatergic projection from vSub to NAc) and the VP in a polysynaptic circuit (Floresco et al., 2001a). The vSub also projects to the medial PFC, which in turn sends glutamatergic projections to the VTA, but only mesocortical DA neurons are innervated (Carr and Sesack, 2000).

The NAc sends GABA projections to the VP, and stimulation of the NAc or its glutamatergic afferents can inhibit VP firing (Jones and Mogenson, 1980). The VP is involved in more than locomotor activity, including reward-related behaviors, and shares many reciprocal connections with virtually all “reward” areas (Smith et al., 2009). For example, VP neurons code for incentive motivation, as they fire in response to cues that have been imbued with salience via conditioning trials, so they are also like a limbic-motor integrator (Tindell et al., 2005). The VP also directly projects (GABAergic) to the VTA (Mogenson et al., 1980), where it promotes tonic firing (Floresco et al., 2003). The VP also projects to the pedunculopontine tegmentum (PPT), which in turn excites DAergic VTA neurons and mesoaccumbal DA efflux by way of burst firing (Floresco et al., 2003). Burst activity of DA neurons is associated with salient stimuli localized to the synapse (that predict delivery of reward, for example) (Schultz, 1997), while tonic firing maintains the cell activity and is much longer lasting and with wider effects. Interestingly, the cholinergic interneurons of the striatum and NAc also possess a timing of burst firing, followed by a pause, and another burst (Fiorillo and Williams, 2000; Threlfell and Cragg, 2011), raising the possibility of synchronicity between these neurotransmitter systems.

The laterodorsal tegmentum (LDT) and PPT nuclei innervate both the VTA and the SN via excitatory cholinergic input (Oakman et al., 1995; Omelchenko and Sesack, 2005). The cholinergic axons of the VTA synapse mostly on mesoaccumbal DA neurons (DA neurons projecting from mesencephalon to accumbens), as opposed to mesofrontal DA neurons (DA neurons projecting

from mesencephalon to prefrontal cortex) (Omelchenko and Sesack, 2005; 2006). There are also glutamatergic projections from the PFC to the VTA, modulating pacemaker firing (Johnson and North, 1992b). DA neurons of the VTA are under tonic GABA-mediated inhibition (Johnson and North, 1992a). Local GABA neurons also regulate the VTA, on other GABA cells as well as DA cells (Johnson and North, 1992a; Omelchenko and Sesack, 2009).

Ethanol and the Mesolimbic DA Pathway

Ethanol directly excites neurons of the VTA both *in vitro* and *in vivo* (Brodie et al., 1999; Gessa et al., 1985). Ethanol also directly causes an increase in extracellular DA in the NAc (Di Chiara and Imperato, 1988; Yim and Gonzales, 2000). Accordingly, dopaminergic antagonists block stimulation to ethanol (Phillips and Shen, 1996; Risinger et al., 1992; Shen et al., 1995a). Recent evidence indicates that mAChRs bidirectionally influence DA transmission in the striatum, in that activity at the receptors can both promote and inhibit release of DA (Threlfell et al., 2010). Similarly, *in vitro* results have presented conflicting evidence with regard to ethanol's effects on cholinergic interneurons. Thus, ethanol has been shown to both enhance (Adermark et al., 2011) and inhibit (Blomeley et al., 2011) cholinergic interneuron activity. Ethanol also has multiple other effects on neurotransmitter systems, including GABA, glycine, and nAChRs (reviewed in Lovinger, 1997; Morikawa and Morrisett, 2010; Spanagel, 2009). However, consistent with the aims of this dissertation, the focus will be on DA and ACh effects of ethanol.

As previously mentioned, dopaminergic antagonists block the stimulant response to ethanol in FAST mice (Shen et al., 1995a), while agonists promote stimulation to ethanol (Phillips and Shen, 1996). However, differences in response to dopaminergic drugs alone in FAST and SLOW mice (without ethanol) have painted a less compelling DA picture. Only replicate-1 mice differed in locomotor response to the indirect DA agonist methamphetamine (FAST-1 were more active following the drug than SLOW-1), but an initial difference in locomotor response to the indirect DA agonist cocaine (found only in replicate-1 mice in G_{41}) was found in both replicates in $G_{65\text{-}68}$ (with FAST more active than SLOW) (Bergstrom et al., 2003). An initial exploration in $G_{14\text{-}15}$ revealed no differences between the lines in sensitivity to amphetamine (Phillips et al., 1992). FAST and SLOW mice did not differ in response to the dopaminergic antagonists raclopride or SCH-23390 (Shen et al., 1995a). However, FAST mice displayed greater extracellular NAc DA levels than SLOW following both a 2 g/kg ethanol dose as well as a 40 mg/kg cocaine dose, suggesting that the DA system of FAST mice is more sensitive to ethanol and to a DA agonist (Meyer et al., 2009). Likewise, FAST-2 mice displayed enhanced basal DA cell firing in the SN as compared to SLOW-2 mice (FAST-1 vs. SLOW-1 was not tested) (Beckstead and Phillips, 2009). In summary, ethanol's stimulant effects are influenced by the DA system, and this system has been altered by selective breeding for sensitivity to ethanol stimulation in FAST and SLOW mice.

Ethanol and ACh

Data from FAST and SLOW mice indicate a genetic correlation between ethanol and nicotine stimulant sensitivity (Bergstrom et al., 2003; Gubner et al., submitted; Phillips et al., 1992). Epidemiological data have indicated that over 70% of alcohol-dependent individuals are also nicotine-dependent (Daeppen et al., 2000). Nicotine acts as an agonist at nicotinic acetylcholine receptors (nAChRs). Ethanol potentiates current through nAChR, where it appears to stabilize the open state of the receptor, like an allosteric modulator (for review, see Dopico and Lovinger, 2009). However, repeated exposure to ethanol may increase receptor desensitization (Dopico and Lovinger, 2009). As previously mentioned, depending on the dose and *in vitro* experimental system, ethanol can both enhance (Adermark et al., 2011) and inhibit (Blomeley et al., 2011) cholinergic interneuron activity. Ethanol action at cholinergic neurons has only been assessed specifically in the HIP. A moderate dose of systemically administered ethanol inhibited hippocampal ACh release, but a low dose of ethanol stimulated hippocampal ACh release, indicating that ethanol has bidirectional modulatory effects on cholinergic hippocampal neurons (Henn et al., 1998). The non-subtype-selective muscarinic receptor antagonist scopolamine, when infused locally, enhanced ACh release in the HIP, though this stimulation was inhibited by subsequent systemic administration of ethanol (Henn et al., 1998). The effect of ethanol on brain ACh levels in other areas besides the HIP remains to be studied.

When administered peripherally, both ethanol and nicotine caused an efflux of DA in the NAc (Benwell and Balfour, 1992; Imperato and Di Chiara, 1986), and when co-administered, resulted in an additively enhanced DA efflux from the NAc shell in rats (Tizabi et al., 2007). Pre-treatment with the non-selective nicotinic antagonist mecamylamine attenuated ethanol-induced DA levels in the NAc (Blomqvist et al., 1993; 1997; Larsson et al., 2002). Mecamylamine infusion into the NAc attenuated ethanol-cue-induced DA efflux in the NAc of ethanol-preferring rats, and into the VTA attenuated conditioned reinforcing properties of ethanol (Lof et al., 2007). Chronic pre-treatment (5 days) with the nicotinic partial agonist varenicline prevented the increase in DA in the NAc following the combination of ethanol and nicotine (Ericson et al., 2009).

The combination of nicotine and ethanol increased locomotor activity as compared to nicotine alone when nicotine was the pre-treatment, but when nicotine was given following a high dose of ethanol, locomotor activity decreased (Blomqvist et al., 1992). Also, ethanol-induced locomotor stimulation was reduced by pre-treatment with mecamylamine (Blomqvist et al., 1992; Kamens and Phillips, 2008; Larsson et al., 2002).

When administered to human subjects in an alcohol rating experiment, mecamylamine increased sedative-like feelings on its own (Chi and de Wit, 2003). Alcohol alone increased subjective stimulant ratings. When combined, mecamylamine decreased the subjective stimulant-like effects of alcohol in these non-smoking, moderate social drinkers (Chi and de Wit, 2003). Chronic

varenicline (7 days) in nicotine-dependent moderate drinkers reduced craving for alcohol and subjective alcohol effects (McKee et al., 2009). McKee and others (2009) have suggested that varenicline works as a partial agonist by increasing DA levels enough to prevent craving, while acting as a competitive antagonist to block drug effects at nAChRs. However, varenicline increased ethanol consumption in C57BL/6J mice (Kamens et al., 2010). These data indicate that nAChR play a role in ethanol reward, at least when studied in humans.

Overall, these data indicate that nAChR influence the stimulant response to ethanol, as nicotinic antagonists attenuated DA levels as well as locomotor activity. Furthermore, FAST mice were more sensitive to the stimulant effects of nicotine than were SLOW mice (Bergstrom et al., 2003). Investigation into the nAChR system has been served by the use of selective pharmacological agents that target specific nicotinic receptor subtypes. However, discussing subtype-specific nicotinic data is beyond the scope of this dissertation, although FAST and SLOW mice do express significantly different levels of some nicotinic subtype messenger RNA (Kamens and Phillips, 2008). Both mAChRs and nAChRs modulate DA release in the NAc, as well as other brain regions involved in reward. However, the lack of subtype-selective pharmacological agents that target the mAChR system have resulted in a lack of data on DA/ACh interactions. Furthermore, the data that do exist are often conflicting. While there is a lack of data on mAChR and ethanol reward, some evidence exists that suggests the mAChR system is a worthwhile research avenue for investigation of ethanol reward behavior, as well as the acute locomotor stimulation endophenotype. As

previously discussed, scopolamine potentiated the stimulant effect of ethanol in FAST mice (Scibelli and Phillips, 2009), and FAST mice were more sensitive to the stimulant effects of scopolamine than SLOW mice (Bergstrom et al., 2003). Other data will be discussed below.

Quantitative Trait Mapping

Behavioral genetics researchers have made use of various techniques to map genes that influence quantitative traits. A quantitative trait is defined as any trait with multiple genetic (and environmental) influences. Genes that influence a quantitative trait can be mapped to a specific chromosome. Given adequate statistical rigor, this region is then known to contain a gene (or multiple genes) that influences the trait, and the region is referred to as a quantitative trait locus (QTL). Briefly, one begins with a genetically diverse population, tests the mice for the phenotype of interest, and genotypes the mice. Genetic markers spaced along the chromosomes at intervals intended to provide full coverage are statistically associated with differences in level of the trait (for a review, see Palmer and Phillips, 2002b). Some researchers have attempted to fine-map QTLs in an effort to find the precise gene (or genes) that influences the behavior, known as a quantitative trait gene (QTG). Unfortunately, this has been challenging as there are most likely many genes, each contributing only a small fraction to the overall phenotype (Mackay et al., 2009), in addition to complicated gene x gene and gene x environment interactions. Furthermore, to generate fine mapping mouse populations requires an immense amount of time and energy,

continually breeding mice to refine the segment one wishes to finely map. Additionally, some genes are identified as good QTG candidates based on convergent evidence from differing approaches (Belknap et al., 2001; Darvasi, 2005; Phillips et al., 2002a). For example, polymerase chain reaction (PCR) follow-up experiments as well as single-gene deletion models such as KO mice, and results from pharmacological manipulations, have been useful for follow-up hypothesis testing about candidate genes.

QTLs for acute locomotor stimulation to ethanol have been mapped in several different mouse populations that originated from the genetically distinct B6 and D2 inbred strains. BXD RI strains (Demarest et al., 1999b), B6D2F2 mice (Demarest et al., 1999b), and Chromosome 2 congenic mice (Palmer et al., 2006) have all supported a QTL for ethanol-induced stimulation on mouse chromosome 2. Other chromosomal regions have also been implicated in ethanol-induced stimulation, most consistently chromosomes 1 and 6, but also 5, 9, and 13 (Demarest et al., 1999b; 2001; Palmer et al., 2006; Kamens et al., 2009). The QTL on mouse chromosome 2 is located between 50 and 148 Mb. Interestingly, this QTL overlaps with another on Chromosome 2 implicated in ethanol consumption and preference (Phillips et al., 1998). Although many genes reside within the relevant chromosome 2 QTL for ethanol-induced locomotion, due to the potential role of ACh in ethanol sensitivity described above, two interesting ones are the m4 (*Chrm4*, 91.6 Mb, 49 cM) and m5 (*Chrm5*, 112.1 Mb, 58 cM) mAChR subtypes (Matsui et al., 1999).

The mAChR System

There are five mAChR subtypes, known as m1 – m5. These G-protein coupled receptors exist as both excitatory (m1, m3, m5) and inhibitory (m2, m4) subtypes. The excitatory m1, m3, and m5 receptor subtypes are positively coupled to phospholipase C (G_q), and the m2 and m4 subtypes are negatively coupled to adenylyl cyclase (G_i) (Eglen, 2005; Smythies, 2005). The m1 receptor subtype is the most widely expressed subtype in the brain, with the highest concentrations in the striatum, cortex, HIP, and nucleus basalis of Meynert. Both the m2 and m4 receptor subtypes are expressed in the cortex, striatum, and NAc, as well as olfactory tubercle and islands of Calleja. The m2 and m4 receptors in the striatum function as inhibitory autoreceptors, able to inhibit cholinergic interneuron firing (Bernard et al., 1992; Grilli et al., 2009). The m4 receptors of the striatum are the most abundant subtype located there (roughly 70%), and are colocalized with DA D1 receptors of the nigrostriatal projection pathway (Chapman et al., 2011; Hersch et al., 1994; Ince et al., 1997; Levey et al., 1991). The m3 receptor subtype is mostly expressed in smooth muscle tissue (Eglen, 2005), although it is expressed in the cortex and HIP, though to a small degree compared to the m1 receptor subtype (Levey et al, 1991; 1993). The m5 receptor subtype is the only known muscarinic receptor subtype expressed in the VTA and SN, the origins of the mesolimbic DA pathway (Weiner et al., 1990), but is also expressed in the HIP. Because of the differential expression of the m1, m2, m4, and m5 receptor subtypes in areas known to play a role in reward, and their expression in regions that impact locomotor activity,

these subtypes are relevant to the study of acute locomotor stimulation to ethanol. Also, as previously mentioned, the m4 and m5 receptor subtype genes reside within a QTL for ethanol-induced activation.

Stimulant Drug Effects on mAChRs of the Reward Pathway

Electrical stimulation of the LDT results in enhanced DA efflux in the NAc, an effect that is attenuated by scopolamine (Lester et al., 2008). Intra-VTA infusion of scopolamine reduced basal accumbal DA efflux, while intra-SN infusion of scopolamine reduced striatal DA efflux (Miller and Blaha, 2005). These results indicate that muscarinic receptors, presumably the m5 subtype, play a key role in facilitation of DA release in the NAc, as their blockade with scopolamine in the VTA attenuates DA efflux. Accordingly, *in vitro* evidence suggests that the non-subtype-selective muscarinic agonist oxotremorine facilitates striatal DA release (Zhang et al., 2002). However, scopolamine has behavioral stimulant effects (Bergstrom et al., 2003; Scibelli and Phillips, 2009; Wang and McGinty, 1996) and peripheral administration of scopolamine at low doses has also been shown to enhance DA release in the NAc (Ichikawa et al., 2002). The dearth of subtype-selective pharmacological agents for the mAChR system make this system notoriously difficult to study, as activity at muscarinic receptors results in different and sometimes opposite effects on molecular and behavioral outcomes (Fiorillo and Williams, 2000).

As selective pharmacological agents are lacking, KO mice for the various mAChR subtypes have been used to try to elucidate their function in drug-related

behaviors, although site-specific administration of agonists and antagonists has also helped this cause. To the best of my knowledge, muscarinic drugs do not act on nicotinic receptors, but it is true that stimulating cholinergic afferents to the midbrain will activate muscarinic and nicotinic responses both pre- and post-synaptically (Zhou et al., 2003).

The m4 mAChR subtype appears to exert an inhibitory influence over dopaminergic neurons of the NAc and striatum. Mice lacking the m4 receptor subtype gene, both globally as well as solely within DA-D1 neurons, displayed elevated extracellular DA levels in the NAc as compared to WT controls, following administration of stimulant drugs (amphetamine, phencyclidine) (Jeon et al., 2010; Tzavara et al., 2004). Also, extracellular NAc ACh levels were reduced in these mice as compared to controls following scopolamine treatment, results which highlight the inhibitory influence of the m4 receptor subtype on DA-D1 neurons. Mice with the m4 receptor specifically knocked out in the dopaminergic projection neurons displayed enhanced locomotor activity to cocaine and amphetamine, as compared to WT controls (Jeon et al., 2010). Oxotremorine, when administered into the m4-rich NAc, reduced cocaine self-administration (Mark et al., 2006). When taken together, these results indicate that the m4 subtype exerts an inhibitory effect on excitatory dopaminergic neurons of the NAc and striatum, areas heavily implicated in drug reward and sensitivity, and suggest that m4 antagonism could potentiate the stimulant effects of ethanol, as similar neurochemical mechanisms may enhance the response to both stimulant drugs and ethanol.

Activation of the m5 mAChR subtype appears to excite DA release in the NAc, which originates from m5 receptors in the VTA and SN. While cocaine enhanced LDT stimulation-evoked NAc DA levels, pre-treatment with scopolamine into the VTA (presumably blocking m5 receptors) attenuated the facilitation of DA efflux (Lester et al., 2010). The m5 KO mice self-administer cocaine at lower rates than WT (Fink-Jensen et al. 2003) and also have lower breakpoints in progressive ratio chronic self-administration paradigms (Thomsen et al. 2005). Additionally, m5 KO mice spend less time in the cocaine-paired side than WT in a CPP paradigm, although these mice do not differ in cocaine-induced locomotor activity (Fink-Jensen et al. 2003). Overall, these results suggest that mice lacking the m5 receptor subtype have reduced reinforcement to stimulant drugs of abuse, suggesting that m5 receptor blockade should decrease reward and reinforcement to these drugs. However, Yamada et al. (2001) found no differences between m5 KO mice and their WT counterparts in open field locomotion following amphetamine treatment. *Chrm5* receptor gene KO mice backcrossed to C57BL/6Tac for 13 generations actually displayed greater acute locomotor activity to amphetamine as compared to WT controls, an increase which was paralleled by enhanced medial forebrain bundle-stimulated DA efflux in the NAc in the KO as compared to WT (Schmidt et al., 2009). However, these mice showed no differences in cocaine-induced locomotor activity or DA levels (Schmidt et al., 2009). Scopolamine pre-treatment into the m5-rich VTA also attenuates morphine-induced DA efflux from the NAc (Miller et al., 2005). *Chrm5* receptor gene KO mice showed a blunted DA response in the

NAc to morphine, a lack of morphine-associated place conditioning, and reduced morphine-induced locomotor activity as compared to WT controls (Basile et al., 2003; Steidl and Yeomans, 2009). Overall, these data generally agree that activity at the m5 receptor subtype directly enhances stimulant sensitivity to drugs of abuse, most likely as a result of its ability to enhance DA efflux in the NAc.

While ethanol-related phenotypes have not been tested in these mice, these data on other stimulant drugs suggest that they may play a role in ethanol-induced locomotor activity. Scopolamine treatment increases locomotor activity on its own (Bergstrom et al., 2003; Chintoh et al., 2003) and it has been shown to counteract the sedative effects of ethanol in rats (Pohorecky et al., 1979). There are conflicting data as to whether or not scopolamine has abuse potential, though it was most recently shown to partially substitute for cocaine in a discriminative stimulus procedure in mice (Ranaldi and Woolverton, 2002; Thomsen et al., 2010). Within the relevant chromosome 2 QTL for ethanol-induced locomotion, among many other genes, lie the genes for the m4 (*Chrm4*, 91.6 Mb, 49 cM) and m5 (*Chrm5*, 112.1 Mb, 58 cM) mAChR subtypes (Matsui et al., 1999). Taken together, these data indicate that the cholinergic system and perhaps more specifically the m4 and m5 mAChR subtypes are worthy candidates for exploration on the acute locomotor stimulation to ethanol.

Dissertation Goals and Hypotheses

The overarching goal of this dissertation project was to investigate the role of the muscarinic acetylcholinergic system in mediating the acute locomotor response to ethanol. As a secondary goal, the m4 and m5 mAChR subtype receptor genes were a focused area of examination, as they were present in a chromosomal region indicated by a QTL study on locomotor stimulation to ethanol, and are expressed in brain areas known to play a role in reward and reinforcement. To accomplish these goals, the FAST and SLOW selected lines of mice, selectively bred for extreme sensitivity and insensitivity to the stimulant effects of ethanol, were used.

The first aim of this dissertation project was to examine the role of cholinergic systems in animals with low, and high, stimulant sensitivity. Previous work in FAST mice revealed that peripheral pre-treatment with the muscarinic non-subtype-selective antagonist scopolamine robustly enhanced the stimulant response to ethanol to a much greater degree than would be predicted from the additive effects of the scopolamine and ethanol groups alone (Scibelli and Phillips, 2009). As a direct extension of this previous work, the response of SLOW mice to the combined effects of scopolamine and ethanol on locomotor activity was explored. SLOW mice show a stimulant response to scopolamine (Bergstrom et al., 2003), and scopolamine has previously been shown to counteract ethanol-induced locomotor depression (Pohorecky et al., 1979). It is possible that this effect is due to an opposing stimulant effect of scopolamine,

rather than antagonism of mAChR-mediated ethanol depressant effects. Regardless, it was hypothesized that scopolamine would attenuate the locomotor depression to ethanol seen in SLOW mice. Additionally, the acute locomotor stimulant response to ethanol in FAST mice following microinjection of scopolamine into the NAc, an area hypothesized to be involved in the robust locomotor stimulant response to the peripherally-administered combination of scopolamine and ethanol in the FAST line (Scibelli and Phillips, 2009), was investigated. I predicted that scopolamine would potentiate ethanol-induced locomotor stimulation via antagonism of inhibitory m4 receptors in the NAc, potentially expressed on cholinergic interneurons (m4 autoreceptors).

The second aim of this dissertation project was to examine basal m4 and m5 gene expression levels in the FAST and SLOW mice. I hypothesized that differences in m4 and m5 gene expression between the lines result in different levels of these receptor subtypes and underlie their innate differences in sensitivity to the stimulant effects of ethanol. Basal gene expression was examined in whole brain as well as the PFC, striatum, HIP, and VM, areas known to express the m4 and/or m5 receptor subtype genes and also to play a role in reward as well as locomotor response to drugs of abuse. I predicted that the FAST mice would display greater m5 gene expression in the VM as compared to SLOW mice, as m5 receptors in the VM potentiate DA release in the NAc, known to promote locomotion to drugs of abuse. I also predicted less m4 gene expression in the striatum of FAST mice as compared to SLOW mice, as m4 receptors in these areas function as inhibitory receptors, exerting an opposing

tone on the excitatory DA D1 neurons. Similarly, I hypothesized that SLOW mice would display greater m4 expression in the PFC than FAST, while FAST mice would display greater m5 expression in the HIP than SLOW, as I predicted that SLOW mice would display greater expression levels of the inhibitory m4 subtype, while FAST mice would display greater levels of the excitatory m5 subtype.

The final aim of this dissertation project was to test the hypothesis that m4 and/or m5 mAChR are involved in the stimulant response to ethanol by examining the acute locomotor stimulant response to ethanol in m4 and m5 KO, and corresponding WT, mice. I predicted that mice lacking the m4 receptor subtype gene would display greater locomotor activity to ethanol as compared to their WT controls, as removing the m4 receptor gene would remove this inhibitory autoreceptor, promoting excitation. Accordingly, mice lacking the m5 receptor subtype gene would display reduced ethanol-induced locomotor stimulation as compared to WT, as activation of the m5 receptor excites DA release in the NAc. I also investigated potential differences between m4 and m5 KO and WT mice in ethanol consumption. I predicted results similar to those for locomotor stimulation for both the m4 and m5 receptor subtype genes as these receptor subtypes presumably play a similar role in both locomotor activation to drugs of abuse and drug consumption.

CHAPTER 2: Effect of scopolamine pre-treatment on locomotor response to ethanol in FAST and SLOW mice

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Abstract

Background: The FAST and SLOW selected lines of mice were selectively bred for extreme sensitivity (FAST) or insensitivity (SLOW) to the stimulant effects of ethanol. Our lab has previously shown that peripheral administration of scopolamine and ethanol resulted in synergistic enhancement of locomotor activity in FAST mice (Scibelli and Phillips, 2009). The current study explored the effects of this drug combination in SLOW mice, as well as testing microinjection of scopolamine into the nucleus accumbens (NAc) of FAST mice followed by ethanol. ***Methods:*** In Experiment 2.1, scopolamine (0, 0.125, 0.5 mg/kg) was administered 10 min prior to ethanol (0, 1 g/kg) in SLOW mice and locomotor activity was measured for 30 min. In Experiments 2.2 – 2.3, Experiment 2.1 results were followed up with rotarod studies testing the ataxic effects of scopolamine (0, 0.125, 0.5 mg/kg) and ethanol (0, 1.2, 1.5 g/kg). Finally, in Experiment 2.4, scopolamine (aCSF, 1, 5 μg/side) was microinjected into the NAc of FAST mice prior to systemic ethanol (0, 1 g/kg) and activity was again measured for 30 min. ***Results:*** While both SLOW-1 and SLOW-2 mice displayed stimulation to scopolamine, only SLOW-1 mice showed sedation to ethanol. The combination of scopolamine and ethanol in SLOW-1 mice resulted in locomotor sedation similar to that of ethanol alone. In SLOW-2 mice, only those treated with the highest dose of ethanol combined with scopolamine showed no stimulant response to scopolamine. The rotarod study in SLOW-1 mice revealed that the combination of 0.5 mg/kg scopolamine and 1.5 g/kg

ethanol significantly enhanced ataxia compared to 1.5 g/kg ethanol alone. Neither intraaccumbal injection of scopolamine nor systemic ethanol resulted in locomotor stimulation in FAST-1 mice. While the combination of intra-NAc scopolamine and peripheral ethanol enhanced locomotor activity in FAST-2 mice, this effect was additive and not synergistic as seen in previous work, when both drugs were given peripherally. ***Conclusions:*** These results suggest that while SLOW mice are sensitive to the stimulant effects of scopolamine, the depressant effects of ethanol override scopolamine stimulation when the drugs are combined. In addition, activity at muscarinic receptors in the NAc did not enhance the synergistic response to the combination of scopolamine and ethanol seen in previous experiments in FAST mice.

Introduction

Level of sensitivity to certain acute effects of ethanol may be a risk factor for susceptibility to alcohol abuse (Holdstock et al., 2000; King et al., 2002; Newlin and Thomson, 1990; Schuckit and Smith, 2001). Acute locomotor stimulation to ethanol in mice is used as a model of human behavioral stimulation, and the FAST and SLOW selected lines of mice were developed as a genetic model of extreme sensitivity (FAST) or insensitivity (SLOW) to the acute stimulant effects of ethanol (Crabbe et al., 1987; Phillips et al., 1991; Shen et al., 1995b). Besides differences in ethanol-induced stimulation between FAST and SLOW mice, FAST mice also demonstrate increased ethanol consumption, as well as reduced sensitivity to the sedative and ataxic effects of ethanol, compared to SLOW mice (Phillips et al., 2002b; Risinger et al., 1994; Shen et al., 1996). These data indicate a genetic correlation between sensitivity to the stimulant effects of ethanol and sensitivity to the rewarding and sedative effects of ethanol in these mice, meaning that common genes mediate both phenotypes.

Several neurotransmitter systems have been implicated in mechanisms underlying the stimulant response to ethanol, but perhaps most consistently implicated have been dopamine (DA) and γ-aminobutyric acid (GABA) (reviewed in Phillips and Shen, 1996; Bergstrom et al., 2003; Holstein et al., 2009; Palmer et al., 2002). A particular emphasis has been placed on the mesolimbic DA pathway, sometimes called the reward pathway. This pathway consists of DA neurons in the ventral tegmental area (VTA), which project to medium spiny

GABA neurons in the nucleus accumbens (NAc). Acute injection of ethanol has been shown to result in increased DA levels in the NAc (Imperato and Di Chiara, 1986; Yim and Gonzales, 2000). In addition, peripherally administered DA antagonists and GABA agonists were shown to attenuate the stimulant response to ethanol in FAST mice (Phillips and Shen, 1996; Shen et al., 1995a; Holstein et al., 2009). FAST mice displayed an enhanced efflux of DA measured within the NAc, compared to SLOW mice, following an acute injection of ethanol (Meyer et al., 2009). FAST mice also exhibited greater DA firing activity in the substantia nigra (SN) following *in vitro* ethanol application than did SLOW mice, an effect potentially due to reduced GABA-A receptor input to DA neurons (Beckstead and Phillips, 2009). Finally, intracerebroventricular administration or microinjection of the GABA-B agonist baclofen into the anterior VTA attenuated the stimulant response to ethanol in FAST mice (Boehm et al., 2002a). These results indicate that selection for sensitivity differences to the stimulant effects of ethanol has altered neurotransmitter systems that modulate the mesolimbic DA pathway, and confirm a role for both DA and GABA in stimulant response to ethanol.

Another neurotransmitter system that modulates the mesolimbic DA pathway, but has been less studied for its role in ethanol stimulation, is the muscarinic acetylcholine receptor (mAChR) system. mAChRs are G-protein coupled, with both excitatory (m1, m3, and m5) and inhibitory (m2 and m4) subtypes (Bymaster et al., 2003). While the m3 receptor subtype is mostly expressed in smooth muscle, the m1, m2, m4, and m5 receptor subtypes are expressed within regions of the mesolimbic DA pathway (Eglen, 2003; Smythies,

2005). The m1 receptor subtype is widely expressed in all major forebrain areas, such as the cortex, hippocampus, striatum, and NAc. The m2 and m4 receptor subtypes function as inhibitory autoreceptors in the striatum and NAc (Eglen, 2006; Hersch et al., 1994; Levey et al., 1991). Finally, the m5 receptor subtype is expressed in the SN and VTA (Weiner et al., 1990).

We have previously shown that peripheral administration of the non-subtype-selective muscarinic antagonist scopolamine resulted in a synergistic enhancement of the stimulant response to ethanol in FAST mice (Scibelli and Phillips, 2009). In the current work, we extended this investigation to the effects of peripheral administration of scopolamine on the acute response to ethanol in SLOW mice. Our lab has previously shown that SLOW mice were sensitive to the stimulant effects of scopolamine (Bergstrom et al., 2003), and others have shown that scopolamine was able to counteract the sedative effects of ethanol in rats (Pohorecky et al., 1979). Therefore, we hypothesized that peripheral administration of scopolamine in SLOW mice would counteract the locomotor depressant effects of ethanol. However, after seeing the absence of an effect of scopolamine on ethanol-induced locomotor depression, but a lack of scopolamine stimulation when scopolamine was given in combination with higher doses of ethanol, we hypothesized that scopolamine might be enhancing the motor incoordinating effects of ethanol, and examined this using a rotarod test (Holstein et al., 2009). The combination of scopolamine and ethanol may initiate competing behaviors that interfere with the animal's ability to display locomotor behavior.

In Scibelli and Phillips (2009), we hypothesized that antagonism of m4 autoreceptors in the striatum and NAc resulted in an enhancement of DA efflux in the NAc, potentiating locomotor activation, although it was the case that peripherally administered DA receptor antagonists did not block the enhanced locomotor stimulant response. It has been shown that mice lacking the m4 receptor subtype gene, and thus this receptor, displayed elevated extracellular DA levels in the NAc, compared to wildtype controls, following administration of other drugs with stimulant properties (amphetamine, phencyclidine, cocaine) (Jeon et al., 2010; Tzavara et al., 2004). Therefore, in the current study, we tested our hypothesis in a more targeted fashion, by microinjecting scopolamine into the m4-rich NAc. Use of a non-receptor subtype selective drug was necessary, as drugs selective for the mAChR subtypes do not exist.

Methods and Materials

Mice

These experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. All procedures were approved by the Portland Veterans Affairs Medical Center (PVAMC) Institutional Animal Care and Use Committee. Animals were maintained on a 12:12 h light:dark cycle, with lights on in the colony room at 0600 h. Standard rodent chow (Purina 5001; Animal Specialties Inc., Hubbard,

OR) and tap water were available *ad libitum*. All behavioral testing was conducted during the light phase between 0800 and 1800 h. All mice were experimentally naïve at the time of testing.

FAST-1, FAST-2, SLOW-1, and SLOW-2 selected line offspring stayed with dam and sire until 21 + 1 days old, at which time they were weaned into same-sex groups, 2 - 5 per cage (28.5 x 17.5 x 12 cm, lined with EcoFRESH bedding (Absorption Corp., Ferndale, WA)). The FAST and SLOW mice used here came from lines that were selectively bred in replicate from independent breeding populations of a genetically heterogeneous stock of mice (HS/Ibg, McClearn et al., 1970). Selection for extreme sensitivity (FAST) or insensitivity (SLOW) to the stimulant effects of ethanol was performed for multiple generations. The details of the selection process have been previously described (Crabbe et al., 1987; Phillips et al., 1991, 2002b). Following generation 37, selection was relaxed and mice were bred randomly within replicate and line (Shen et al., 1995b). The stimulant response difference to ethanol between FAST and SLOW mice had not regressed when tested in ~$S_{37}G_{60}$ (Palmer et al., 2002) or more recently (Gubner et al., submitted). S_{xx} indicates the last generation of selection and G_{xx} indicates the total number of generations of breeding. In the current experiments, FAST-1 mice were from $S_{37}G_{103-108}$, SLOW-1 mice were from $S_{37}G_{93-107}$, FAST-2 mice were from $S_{37}G_{104-108}$, and SLOW-2 mice were from $S_{37}G_{92-98}$.

Drugs

Scopolamine hydrobromide was purchased from Sigma (St. Louis, MO). 100% ethanol was obtained from Decon Labs (King of Prussia, PA). For the microinjection studies, scopolamine was diluted in artificial cerebrospinal fluid (aCSF; 145 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.4 mM glucose, at pH 7.4) on the day of testing. For the locomotor and rotarod experiments, scopolamine was diluted in 0.9% saline (Baxter Healthcare Corp., Deerfield, IL) to the appropriate concentrations on the day of testing and injected intraperitoneally (i.p.) at a volume of 10 ml/kg. Scopolamine was prepared in dark bottles to avoid light-induced degradation. For ethanol injections, ethanol was diluted to 20% (v/v) in saline and delivered i.p. in a volume adjusted by body weight to deliver the appropriate dose.

Locomotor testing

Activity was measured in clear acrylic boxes (40 x 40 x 30 cm) housed inside of automated activity monitors within sound and external light-attenuating chambers (Accuscan Instruments Inc., Columbus, OH). Eight photobeams and detectors, 2 cm above the chamber floor, recorded beam breaks, which was translated into distance traveled (cm). Each chamber was equipped with a 3.3 W incandescent bulb to provide illumination, and a fan provided ventilation and additional noise-masking. The testing procedure followed our previously published 3-day design (Boehm et al., 2002a; Meyer and Phillips, 2003; Scibelli and Phillips, 2009). On test days, mice were moved into the testing room 45 to

60 min prior to behavioral testing to permit acclimation. Day 1 saline data provided a measure of activity in a novel environment. Day 2 saline data provided a measure of baseline activity in the now-familiar environment. Day 3 provided drug response data. All locomotor test activity sessions were 30 min in duration, with data collapsed in 5-min bins. Experimental details for each study are described below.

Rotarod

The rotarod apparatus (AccuRotor Rota Rod, Accuscan Instruments) had a rod diameter of 6.3 cm. The rod was covered in 320 grit wet/dry sandpaper and divided into quarters by 30-cm diameter plastic disks. The rod was 63 cm above 4 equally-spaced 11-cm wide bins filled with sawdust bedding to cushion mice when they fell. Each bin contained two photocell beams, which were blocked when an animal fell off the rod, stopping the timer.

Experiment 2.1: Effect of scopolamine on acute locomotor response to ethanol in SLOW mice

Subjects

SLOW-1 and -2 females were tested in this experiment to parallel the previously published study performed in FAST-1 and -2 female mice (Scibelli and Phillips, 2009). Generations were $S_{37}G_{92\text{-}98}$. All behavioral testing occurred between 0900 and 1600 h.

Behavioral testing procedure

Behavioral testing followed our previously described 3-day procedure (Boehm et al., 2002a; Meyer and Phillips, 2003; Scibelli and Phillips, 2009). On Days 1 and 2, mice were administered saline and returned to their holding cages for 10 min. They were then injected again with saline, and locomotor activity was measured for 30 min in 5-min bins. On Day 3, mice were injected with scopolamine (0, 0.125, or 0.5 mg/kg) and returned to their holding cages for 10 min. Then, ethanol (0 or 1 g/kg) was administered and locomotor activity was measured for 30 min. Based on previously published data, scopolamine doses were chosen to be moderate and high, and the ethanol dose was also considered moderate, in an effort to avoid floor or ceiling effects (Scibelli and Phillips, 2009). Immediately following activity testing on Day 3, 20 µL retroorbital blood samples were taken for BEC determination.

BEC determination

The 20 µl blood samples were aspirated into microcentrifuge tubes containing 50 µl of ice-cold 5% $ZnSO_4$. 50µl of 0.3N $Ba(OH)_2$ was added to each tube, along with 300 µl of double distilled H_2O. The samples were then centrifuged at 12,000 rpm in a cold room for 5 min. Following centrifugation, supernatant was pipetted off into 2 ml glass crimp-top vials, and analyzed for BEC via gas chromatography (Agilent 6890N; Agilent Technologies, Palo Alto, CA) following previously published methods (Boehm et al., 2000).

Statistical analysis

Data were analyzed by factorial ANOVA using Statistica software (StatSoft Version 6.1, Tulsa, OK) for the potential factors of replicate, scopolamine dose, and ethanol dose. Significant complex interactions were followed up using two-way ANOVAs focused on the most relevant factors, followed by simple main effects analyses and Newman Keuls post-hoc tests. The critical dependent variable for locomotor activity was Day 3 (drug response) – Day 2 (baseline) total distance traveled (cm) over the 30 min test. Day 1 and Day 2 distances traveled were also analyzed to check for any group differences not related to drug treatment. Results are expressed as mean ± SEM.

Experiment 2.2 – 2.3: Effect of scopolamine on ataxia induced by ethanol in SLOW-1 mice

Subjects

Experiment 2.2 was designed to pursue questions arising from the locomotor effects seen in Experiment 2.1, to know if the drug combination in SLOW-1 mice resulted only in reduced locomotor activity, or whether there might also be enhancement of ataxia resulting in the absence of scopolamine-induced stimulation competing with forward locomotion. Only SLOW-1 female mice were tested in an initial study using 1.2 g/kg ethanol. Mice in this study were from $S_{37}G_{97}$. After data from the 1.2 g/kg ethanol dose were examined (see

Experimemt 2.2 *Results*), it was determined that examination of a higher ethanol dose would improve interpretation. In a second study (Experiment 2.3), a dose of 1.5 g/kg ethanol was tested, using SLOW-1 male and female mice; an inadequate number of female mice available required our use of both sexes. Mice were from $S_{37}G_{106\text{-}107}$. Testing occurred between 0800 and 1300 h in both studies.

Behavioral testing procedure

The rotarod testing procedure followed our previously published design (Holstein et al., 2009). Mice were moved into the experimental room, weighed, and allowed to acclimate for 45 – 60 min. Each mouse was placed on the dowel, which rotated at a speed of 3 rpm, and given a chance to stay on for 180 sec. Mice were given up to 5 trials to achieve this criterion time (only 1 successful 180 sec trial was required out of 5 potential trials). Once completing the successful 180 sec trial, the criterion segment concluded. The intertrial interval from criterion testing to experimental testing was about 1 hour. Mice not able to remain on the rod for 180 sec were excluded from the study. Following criterion testing, mice were returned to their home cages and tested in squads of 4. Mice were pretreated with scopolamine (0, 0.125, or 0.5 mg/kg) and waited in individual holding cages for 10 min, after which they were injected with ethanol (0 or 1.2 g/kg in Experiment 2.2; 0 or 1.5 g/kg in Experiment 2.3) and immediately placed on the rotating rod. Three trials were conducted at the immediate T_0 timepoint, followed by 3 trials at a T_{10} timepoint (10 min following ethanol

injection). Each trial had a maximum duration of 180 sec. Latency to fall from the dowel in seconds was recorded when a photobeam cell was interrupted by the falling mouse.

Statistical analysis

Because Experiments 2.2 and 2.3 were performed independently, they were analyzed separately. The dependent variable was latency to fall (sec) averaged across 3 trials both immediately following ethanol injection (T_0) and ten minutes following ethanol injection (T_{10}). Because of the 180 sec cut-off time, the data were not normally distributed and were thus analyzed with non-parametric statistics. Individual Kruskal-Wallis tests were performed to examine the effect of scopolamine pre-treatment and ethanol treatment on latency to fall. *A priori* pairwise comparisons were also performed with Mann-Whitney U comparisons. Data were analyzed based on previous procedures in our lab (Holstein et al., 2009). Results are expressed as mean ± SEM in figures.

Experiment 2.4: Effect of scopolamine microinjection into the NAc on acute locomotor response to ethanol in FAST mice

Subjects

FAST-1 and -2 mice were chosen for this experiment based on our previous study (Scibelli and Phillips, 2009) to examine the specific effect of scopolamine microinjection in the NAc on the locomotor response to ethanol in

mice sensitive to the stimulant effects of ethanol. Following surgery, cannulated mice were housed along with their also-cannulated littermates in rat cages (30.8 x 30.8 x 19 cm) lined with ECOFresh bedding. The use of rat cages allowed the cannulated mice room to freely move about without hitting their headmounts on the wire top. All behavioral testing occurred between 0900 and 1800 h.

Bilateral cannulation of the NAc

Mice were anesthetized using a 10 ml/kg cocktail of acepromazine (1.4 mg/kg), ketamine (1.4 mg/kg), and xylazine (7.1 mg/kg) diluted 1 part to 6 parts of saline. The anesthetic cocktail was administered i.p. in a volume of 10 ml/kg according to the following formula [((body weight/100) – 0.08)*2 for males (ml); ((body weight/100) – 0.08)*2 + 0.05 for females (ml)]. Mice were ear punched for identification, a small area of the dorsal scalp was sheared with clippers, eyes were moistened with lubricant (Neo-Poly-Bac, Butler AHS, Dublin, OH), and mice were secured in the stereotaxic apparatus (Kopf Model 1900 with digital manipulator, San Diego, CA). A midline incision was made, extending a few millimeters anterior of bregma to a few millimeters posterior of lambda (about 3 mm wide). Following the incision, the exposed cranium was sterilized with betadine, followed by 100% ethanol.

The distance between bregma and lambda was measured and used to modify the coordinates (the distance was divided by 4.21, the distance between bregma and lambda published in the mouse brain atlas of Paxinos and Franklin (2001). Each predetermined coordinate [x; medial/lateral (ML), y;

anterior/posterior (AP), z; dorsal/ventral (DV)] was multiplied by the resulting quotient to adjust the set of stereotaxic coordinates. Original coordinates for the NAc were +1.0 AP, ±1.35 ML, and -2.88 DV, but were adjusted during the experiment to +1.2 AP, ±1.35 ML, and -2.65 DV to improve targeting of the NAc (Paxinos and Franklin, 2001). A hole was drilled with a 65-gauge drill bit mid-way between bregma and lambda for insertion of an anchor screw (1/8 inch, Small Parts Inc., Miami Lakes, FL), used to secure the mounting cement. Two cannula holes were drilled with a 72-gauge drill bit at the appropriate AP/ML coordinates. The cannulae were simultaneously lowered into the brain using a dual cannula holder (Kopf model 1973) to the appropriate depth. Durelon carboxylate cement (Norristown, PA) was applied to the exposed cranium, securing the cannulae and covering the anchor screw. Mice were placed in heated rat cages to recover from the effects of the anesthesia. After about 2.5 hours, they were moved to the colony room, and allowed to recover for 7 ± 2 days before subsequent behavioral testing.

Guide cannulae, stylets, and microinjectors

Guide cannulae, and tubing to make stylets and microinjectors were obtained from Small Parts Inc. Guide cannulae were made from 26-guage stainless-steel tubing, deburred, and precut to 10-mm. Stainless-steel wire (0.0095 inch) was used to make 10-mm stylets, which functioned to keep the cannulae free of debris when microinjections were not occurring. Microinjectors were made from two sections of stainless-steel tubing. A 30-mm section of 33-

gauge tubing was inserted into a 30-mm section of 26-gauge tubing so that 15-mm protruded. Super glue held the sections in place. When assembled and inserted into a 10-mm cannula, 2 mm of the microinjector extended out from the bottom of the cannula.

Intra-NAc microinjections

Two 0.5-m segments of PE-20 tubing were each attached to microinjectors and loaded with either aCSF or scopolamine. The other ends of the tubing were fitted over the needles of two aCSF- or scopolamine-filled 10-μL glass Hamilton syringes (Hamilton Co., Reno, NV). The glass syringes were fitted into a CMA-400 (Stockholm, Sweden) quad infusion pump. For microinjection, the mouse was lightly grasped by the scruff of the neck and the stylets were removed with a pair of forceps. Two microinjectors were then inserted into and through the guide cannulae, and secured with dental wax. Once fully inserted, the tips of the microinjectors extended 2-mm beyond the ends of the guide cannulae, reaching the NAc. The microinjectors were inserted 5 min prior to the beginning of infusion to allow mice to acclimate. The microinjection took approximately 1 min, in a volume of 0.1 μL/side, and the microinjectors were left in place for an additional minute to allow for diffusion away from the injection site. Microinjectors and dental wax were then slowly removed from the cannulae.

Behavioral testing procedure

Testing followed our 3-day procedure. Days 1 and 2 served to habituate the mice to the microinjection and testing procedures, while Day 3 was the measure of drug response. Pilot testing in our laboratory has indicated that slowly introducing the mice to new features of the microinjection study each day (timing and handling on Day 1, sham insertion of microinjectors on Day 2, drug infusion on Day 3) produces reliable locomotor data in our laboratory and minimizes tissue damage. On all days, mice were moved into the experimental room 45 – 60 min for acclimation prior to testing. Table 2.1 details the time course of experimental events for each day. On Day 1, stylets were removed and reinserted, but no microinjectors were inserted. Mice were then placed in a standard rat holding cage, and 7 min later, stylets were again removed and reinserted to mimic microinjector removal. Eight min following, saline was injected, and locomotor activity was measured. On Day 2, microinjectors were inserted, but no infusion took place. Seven min later, microinjectors were removed. Eight min following, saline was injected, and locomotor activity was measured. On Day 3, microinjectors were inserted, and 5 min later, aCSF or scopolamine (0.1μL per side) was microinjected at a rate of 0.1μL/min. Scopolamine doses (0, 1, 5 μg per side) were chosen based on piloted doses that matched locomotor stimulation equivalent to peripheral administration from previously published data (Scibelli and Phillips, 2009). After the minute-long injection and the minute allowed for diffusion, microinjectors were removed. Scopolamine microinjection occurred 10 min prior to ethanol administration, to

match the previously published time course of treatments (Scibelli and Phillips, 2009). The 1 g/kg dose of ethanol was chosen to allow for potential synergistic enhancement of locomotor activity and to avoid ceiling effects (Scibelli and

Table 2.1. Timeline of events for Experiment 2.4 by day.

	Experimental Day Event		
Time (min)	**Day 1**	**Day 2**	**Day 3**
0	Animals lightly handled to remove and then reinsert stylets	Animals lightly handled to remove stylets and insert microinjectors	Animal lightly handled to remove stylets and insert microinjectors
5			Drug infusion began (0, 1, or 5 µg/side scopolamine)
6			Time allowed for diffusion
7	Stylets removed/reinserted	Microinjectors removed and stylets inserted	Microinjectors removed and stylets inserted
15	Saline injection i.p.; animal placed in locomotor apparatus for 30 min	Saline injection i.p.; animal placed in locomotor apparatus for 30 min	Ethanol (0 or 1 g/kg) injection i.p.; animal placed in locomotor apparatus for 30 min

Phillips, 2009). Saline or ethanol was injected i.p. after the 10 min wait, and locomotor activity was measured. Immediately following activity testing, 20 µL retroorbital blood samples were taken for BEC determination and processed as in Experiment 2.1.

Histological verification of injection sites

Within 3 days of scopolamine infusion, animals were bilaterally microinjected with 0.1 – 0.2 μl per side of 10 mg/ml methyl blue dye. The brain of each mouse was removed and rapidly frozen in an isopentane/dry ice slurry and stored at -80°C. Each brain was sliced into 40-μm coronal sections using a cryostat (Leica CM1850; Nussloch, Germany), and thaw-mounted onto Superfrost Plus slides (VWR, West Chester, PA). The slides were alternately plated, and half were stained using thionin (Aldrich Chemical Co., Milwaukie, WI). Dye injections were visualized and photographed with a Leica DM/LB microscope. Data from mice with microinjection sites located outside the NAc were excluded from the analysis. The locations of injection sites were verified by an independent evaluator.

Statistical analysis

Data were analyzed as in Experiment 2.1.

Results

Experiment 2.1: Combined effects of scopolamine and ethanol on locomotor activity in SLOW-1 and -2 mice

Initial analyses of Day 1, Day 2 and Day 3 – Day 2 data identified significant main and interaction effects involving replicate. Therefore, SLOW-1 and SLOW-2 data were analyzed separately.

Analysis of Day 1 locomotor activity data in SLOW-1 mice revealed that the mice were not perfectly matched for initial levels of activity prior to drug treatment. There were main effects of both assigned scopolamine pre-treatment group [$F_{(2, 124)} = 7.05$, $p < 0.01$] and ethanol dose group [$F_{(3, 124)} = 3.64$, $p < 0.05$]. Likewise, for Day 2 baseline activity levels, there were main effects of scopolamine pre-treatment group [$F_{(2, 124)} = 4.6$, $p < 0.05$] and ethanol treatment group [$F_{(2, 124)} = 3.73$, $p < 0.05$] (Table 2.2). Variation in basal activity level is not surprising, as these mice are genetically heterogeneous. To control for these differences when assessing drug effects, Day 2 baseline scores were subtracted from Day 3 drug scores, and the Day 3 minus Day 2 difference scores were the dependent measure used in subsequent analyses.

In SLOW-1, mice treated with ethanol did not show scopolamine-induced stimulation as seen in non-ethanol treated mice (Figure 2.1A). A factorial ANOVA with data grouped on scopolamine dose and ethanol dose revealed a main effect of scopolamine [$F_{(2, 124)} = 5.89$, $p < 0.01$] and a main effect of ethanol [$F_{(3, 124)} = 34.1$, $p < 0.001$], but no significant interaction. Newman-Keuls post-hoc tests revealed that the 0.5 mg/kg dose of scopolamine resulted in

Table 2.2. Experiment 2.1 SLOW-1 and SLOW-2 Day 2 mean (± SEM) activity (cm) and group number (n).

Sco Dose (mg/kg)	Ethanol Dose (g/kg)							
	0		0.75		1.5		2.25	
	SLOW-1	SLOW-2	SLOW-1	SLOW-2	SLOW-1	SLOW-2	SLOW-1	SLOW-2
0	6854.3 ± 578 n = 10	2361.2 ± 245 n = 12	4820.1 ± 464 n = 10	2573.8 ± 279 n = 12	6188.4 ± 593 n = 11	2398.5 ± 272 n = 12	8517.9 ± 857 n = 12	2497.5 ± 194 n = 12
0.125	10342.3 ± 1486 n = 9	2410.8 ± 211 n = 11	7401.8 ± 1122 n = 12	2241.4 ± 147 n = 12	8188.2 ± 1100 n = 13	2893.7 ± 271 n = 14	8319.8 ± 830 n = 13	2974.6 ± 296 n = 12
0.5	7874.5 ± 736 n = 10	2975 ± 315 n = 12	6768.8 ± 1317 n = 12	2691.6 ± 278 n = 12	6225.9 ± 719 n = 12	2590.8 ± 215 n = 12	8224.8 ± 613 n = 12	3008.2 ± 338 n = 12

Sco = scopolamine

significantly greater locomotor activity [4932.8 ± 1722 cm] compared to saline [425.0 ± 786 cm] and the 0.125 mg/kg scopolamine dose [744.3 ± 854 cm]. Also, ethanol dose-dependently decreased locomotor activity in SLOW-1 mice, regardless of scopolamine treatment. Thus, in the presence of ethanol, scopolamine did not have stimulant effects.

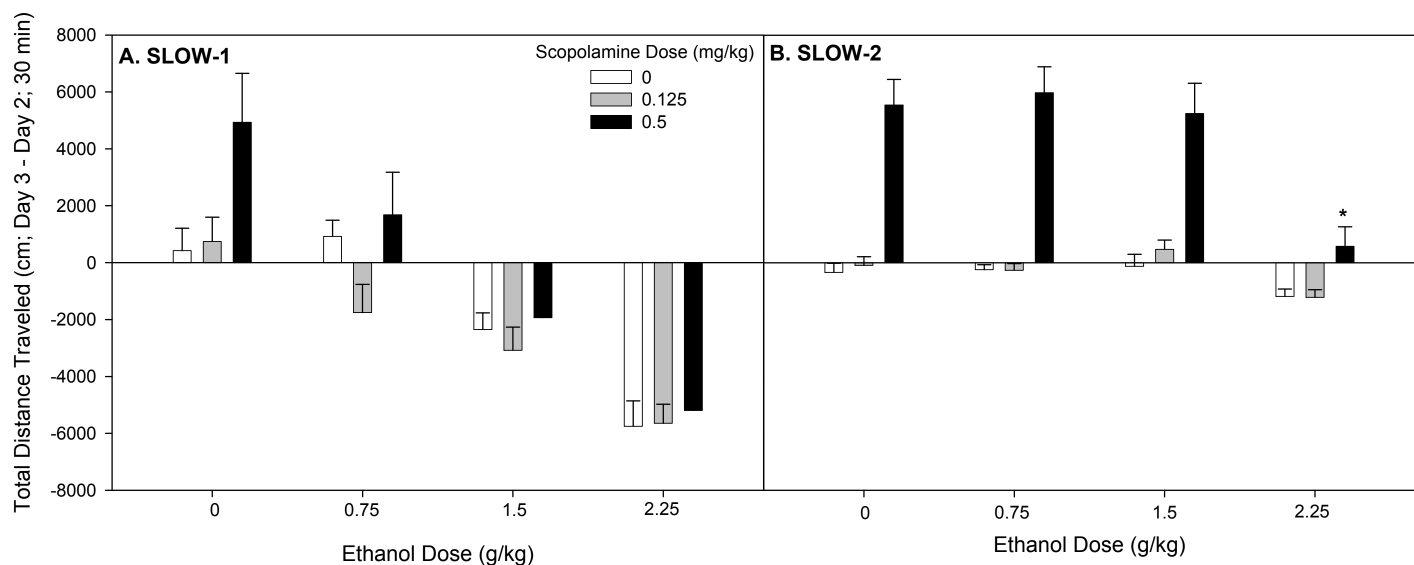

Figure 2.1. Effect of scopolamine alone or in combination with ethanol treatment on locomotor activity in SLOW-1 (A) and SLOW-2 (B) mice. Shown are means ± SEM for total distance traveled (cm) on Day 3 corrected for Day 2 baseline activity (subtraction) for data accumulated for 30 min. Means above the solid line indicates stimulation, while means below the solid line reflect sedation. SLOW-1 female mice n = 9 – 13 mice per scopolamine per ethanol dose; N = 136. Mice were 76 ± 1 d old. SLOW-2 female mice n = 11 – 14 mice per scopolamine per ethanol dose; N = 145. Mice were 74 ± 1 d old. * $p < 0.05$ compared to (0.5 mg/kg scopolamine + all other ethanol doses).

For SLOW-2 mice, there were no significant differences among groups slated to receive different doses of scopolamine or ethanol on Day 1 or Day 2 (Table 2.2). However, for consistency with SLOW-1 data, Day 2 baseline data were subtracted from Day 3 drug data as the measure of drug effect.

SLOW-2 mice did not exhibit a locomotor depressant response to ethanol alone, and the stimulant response to scopolamine was attenuated only when combined with the highest dose of ethanol. A factorial ANOVA (scopolamine dose by ethanol dose) revealed a significant two-way interaction [$F_{(6, 133)} = 4.15$, $p < 0.001$]. Simple main effects analyses indicated that SLOW-2 females were significantly more stimulated by the 0.5 mg/kg scopolamine dose, with or without ethanol, compared to mice treated with saline or the 0.125 mg/kg dose of

scopolamine, with or without ethanol (Figure 2.1B). However, the combination of 0.5 mg/kg scopolamine plus 2.25 g/kg ethanol resulted in significantly less locomotor activity than 0.5 mg/kg scopolamine plus saline or any other dose of ethanol.

There was a main effect of ethanol dose on BEC values in SLOW-1 mice [$F_{(2, 96)} = 125$, $p < 0.001$] (Table 2.3), but no significant effect of scopolamine pretreatment or interactions. Mean BEC value for the 0.75 g/kg ethanol dose was significantly lower than for 1.5 or 2.25 g/kg ethanol. Results were similar for SLOW-2 mice. There was only a main effect of ethanol dose [$F_{(2, 91)} = 60.7$, $p < 0.001$] (Table 2.3), and BEC values dose-dependently increased at increasing ethanol doses.

Table 2.3. Experiment 2.1 mean (± SEM) BEC values (mg/ml) 30 min after ethanol injection in SLOW-1 and SLOW-2 females.

	Ethanol Dose (g/kg)					
Scopolamine Dose (mg/kg)	**0.75**		**1.5**		**2.25**	
	SLOW-1	**SLOW-2**	**SLOW-1**	**SLOW-2**	**SLOW-1**	**SLOW-2**
0	0.6 ± 0.1	0.5 ± 0.1	1.7 ± 0.1	1.3 ± 0.1	1.9 ± 0.1	2.1 ± 0.2
0.125	0.7 ± 0.1	1.0 ± 0.2	1.9 ± 0.1	0.96 ± 0.2	1.8 ± 0.1	2.0 ± 0.2
0.5	0.56 ± 0.1	0.56 ± 0.1	1.7 ± 0.1	1.4 ± 0.1	1.9 ± 0.1	2.2 ± 0.1

Experiment 2.2 – 2.3: Combination of scopolamine and two different doses of ethanol on ataxia-like behavior in female (Experiment 2.2) and female and male (Experiment 2.3) SLOW-1 mice

All mice in Experiment 2.2 (the 1.2 g/kg ethanol dose experiment) were able to satisfy the criterion requirements on the rotarod. There were no differences among the scopolamine pre-treatment groups at T_0 or T_{10} (Figure 2.2). However, Kruskal-Wallis analyses indicted that ethanol-treated mice fell off the rotarod more quickly than saline-treated mice at T_0 [$H_{1, N = 31} = 5.84$, $p < 0.05$]. This effect of ethanol had dissipated by the T_{10} timepoint.

In Experiment 2.3, sex was not included as a statistical factor in this study, because there were too few mice (n = 3 – 6) of each sex in each experimental group (see Table 2.4 for means). Three mice (0.05% of total number tested) in the 1.5 g/kg ethanol dose experiment were unable to satisfy the criterion requirements and were therefore excluded from the study. Kruskal-Wallis analyses indicated that there was no effect of scopolamine group on latency to fall at either T_0 or T_{10}, but ethanol significantly affected latency to fall at both T_0 [$H_{1, N = 58} = 18.2$, $p < 0.001$] and T_{10} [$H_{1, N = 58} = 13.0$, $p < 0.001$] (Figure 2.3). Therefore, the 1.5 g/kg dose of ethanol produced ataxia as compared to saline at both time points. The main *a priori* comparisons we wished to make were among mice treated with 1.5 g/kg ethanol at the 3 doses of scopolamine (0, 0.125, 0.5 mg/kg). These *a priori* comparisons between the scopolamine doses and 1.5

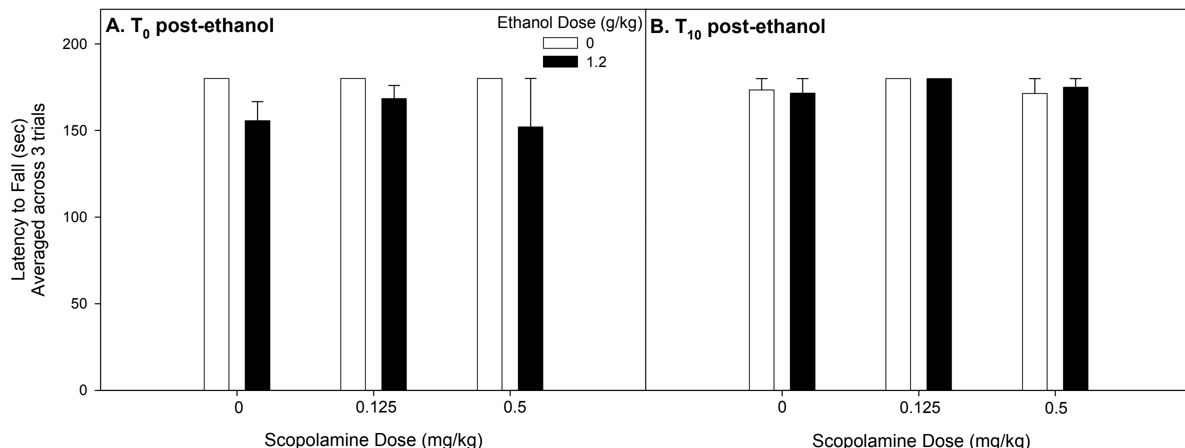

Figure 2.2. Scopolamine did not enhance ataxia to a 1.2 g/kg ethanol dose in SLOW-1 female mice. Shown are data for latency to fall (sec) averaged across 3 trials immediately after ethanol injection (T_0, A) and 10 min post ethanol injection (T_{10}, B). n = 4 – 6 mice per scopolamine per ethanol dose; N = 31. Mice were 72 ± 3 d old.

g/kg ethanol groups showed that while there was no difference at T_0 between ethanol mice treated with 0 mg/kg scopolamine and 0.125 mg/kg scopolamine, mice treated with 0.5 mg/kg scopolamine fell off the rotarod significantly faster than mice treated with 0 mg/kg scopolamine [Mann-Whitney U = 15.5, n_1 = 8, n_2 = 10, p = 0.027 two-tailed]. Therefore, the highest dose of scopolamine plus ethanol significantly enhanced ataxia as compared to ethanol alone at T_0. At T_{10}, while the same *a priori* comparisons were made, the sole significant result was between the 0 mg/kg scopolamine + 0 g/kg ethanol group and 0 mg/kg scopolamine + 1.5 g/kg ethanol [Mann-Whitney U = 22.5, n_1 = 9, n_2 = 11, p

= 0.038 two-tailed]; the mice treated with 0 mg/kg scopolamine + 1.5 g/kg ethanol fell off the rotarod faster than those treated with 0 mg/kg scopolamine + 0 g/kg ethanol. Scopolamine had no effect on latency to fall off the rotarod at T_{10} in any group.

Table 2.4. Experiment 2.3 SLOW-1 latency to fall mean (± SEM) values (sec) and group number (n).

	T_0		T_{10}	
	female	**male**	**female**	**male**
0 mg/kg sco – 0 g/kg etoh	180 n = 4	180 n = 5	180 n = 4	180 n = 5
0 mg/kg sco – 1.5 g/kg etoh	149 ±13.8 n = 5	142 ± 18.9 n = 3	105 ± 21.9 n = 5	180 n = 3
0.125 mg/kg sco – 0 g/kg etoh	157 ± 15.3 n = 5	169 ± 10.9 n = 5	180 n = 5	173 ± 6.91 n = 5
0.125 mg/kg sco – 1.5 g/kg etoh	135 ± 28.5 n = 5	124 ± 25.3 n = 6	156 ± 22.8 n = 5	148 ± 12.3 n = 6
0.5 mg/kg sco – 0 g/kg etoh	165 ± 15.3 n = 5	180 n = 5	151 ± 28.7 n = 5	180 n = 5
0.5 mg/kg sco – 1.5 g/kg etoh	107 ± 21.6 n = 5	94.3 ± 27.4 n = 5	123 ± 26.8 n = 5	150 ± 21.7 n = 5

etoh = ethanol; sco = scopolamine

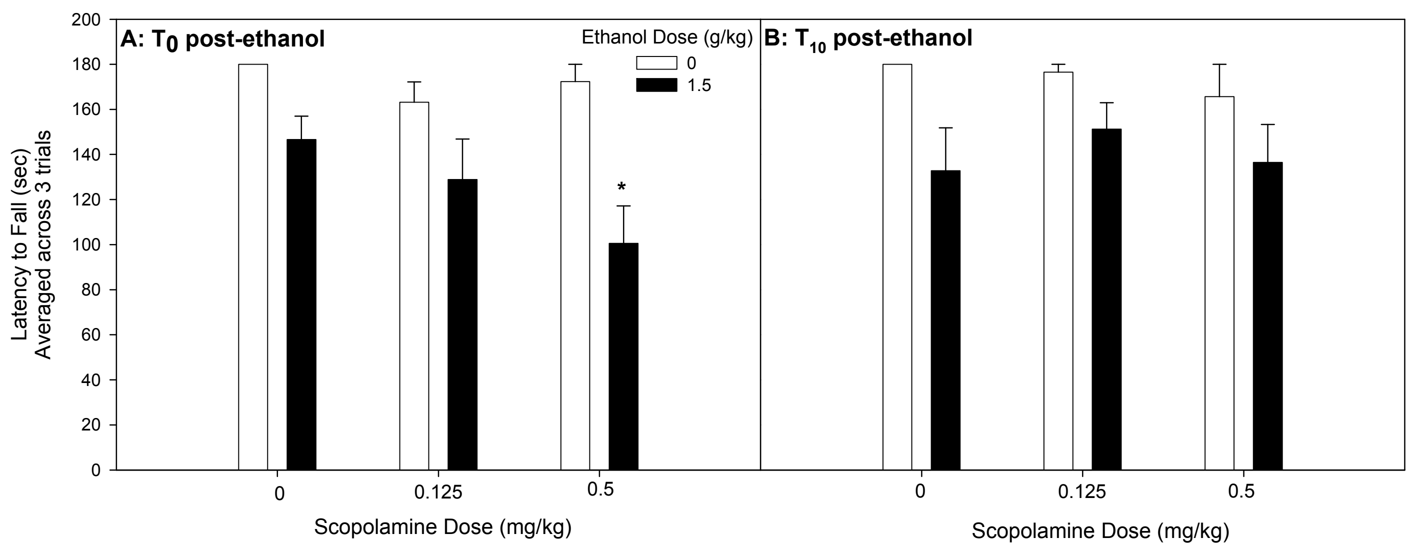

Figure 2.3. The highest dose of scopolamine enhanced the ataxic effects of ethanol in SLOW-1 mice. Shown are data for latency to fall (sec) averaged across 3 trials immediately after ethanol injection (T_0, A) and 10 min post ethanol injection (T_{10}, B). n = 8 – 10 mice per scopolamine per ethanol dose; N = 58. Mice were 68 ± 2 d old. * $p < 0.05$ compared to (0 mg/kg scopolamine + 1.5 g/kg ethanol).

Experiment 2.4: Combination of NAc microinjection of scopolamine and ethanol in FAST mice

Initial analyses of Day 1, Day 2 and Day 3 – Day 2 data identified significant main and interaction effects involving replicate. Therefore, FAST-1 and FAST-2 data were analyzed separately. Sex did not interact with any factors in the analyses; therefore, data are presented collapsed on sex. However, Day 3 – Day 2 activity group means and sizes for each dose group, replicate line, and sex are presented in Table 2.5.

In FAST-1 mice, there were no group effects or interactions on Day 1 or Day 2 activity. In general, locomotor activity decreased from Day 1 [11340.7 ± 1007.4 cm] to Day 2 [5444.9 ± 772.2 cm], indicating habituation.

Table 2.5. Experiment 2.5 Day 3 – Day 2 mean (± SEM) activity and group number (n).

	FAST-1				FAST-2			
	Ethanol Dose (g/kg)				Ethanol Dose (g/kg)			
	0		1		0		1	
Sco Dose (μg/side)	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
0	3788 ± 3899 n = 6	3336 ± 2202 n = 6	7157 ± 4752 n = 4	6556 ± 3860 n = 5	-916 ± 641 n = 4	-601 ± 507 n = 4	4345 ± 2983 n = 6	5013± 1789 n = 7
1	7171 ± 4031 n = 5	10250 ± 4471 n = 7	4887 ± 2622 n = 4	7623 ± 4048 n = 4	1251 ± 280 n = 7	1321 ± 689 n = 6	8811 ± 1986 n = 6	10386 ± 2190 n = 5
5	7886 ± 1949 n = 5	4361 ± 3148 n = 4	8573 ± 9585 n = 4	5583 ± 5045 n = 4	3020± 1700 n = 5	6450 ± 3820 n = 6	15228 ± 4615 n = 6	12971 ± 5044 n = 6

Sco = scopolamine

A factorial ANOVA on Day 3 minus Day 2 scores, with data grouped on scopolamine dose, ethanol dose, and sex did not reveal any significant interactions or main effects (Figure 2.4A). Mice were not significantly stimulated to scopolamine doses nor to the 1 g/kg dose of ethanol. There was no significant effect of scopolamine on BEC values (0 µg scopolamine = 0.54 ± 0.1 mg/ml; 1 µg scopolamine = 0.63 ± 0.1 mg/ml; 5 µg scopolamine = 0.62 ± 0.1 mg/ml). Microinjector placements in FAST-1 mice are shown in Figure 2.5.

In FAST-2 mice, there were no group effects on Day 1 or Day 2 activity. In general, locomotor activity decreased from Day 1 [6421.3 ± 468.5 cm] to Day 2 [2117.7 ± 187.5 cm], indicating habituation.

A factorial ANOVA on Day 3 minus Day 2 scores, with data grouped on scopolamine dose, ethanol dose, and sex revealed main effects of both scopolamine dose [$F_{(2, 56)} = 6.5$, $p < 0.01$] and ethanol dose [$F_{(1, 56)} = 21.6$, $p < 0.01$], but no significant interactions. Newman-Keuls post-hoc analyses revealed that FAST-2 mice were significantly more stimulated by the 5 µg dose of scopolamine than 0 or 1 µg (Figure 2.4B). FAST-2 mice were also stimulated by the 1 g/kg dose of ethanol as compared to saline. Mice treated with both the highest dose of scopolamine and ethanol, exhibited greater stimulation than other groups, but a synergistic locomotor response was not seen. There was no significant effect of scopolamine on BEC values (0 µg scopolamine = 0.74 ± 0.1 mg/ml; 1 µg scopolamine = 0.75 ± 0.1 mg/ml; 5 µg scopolamine = 0.67 ± 0.1 mg/ml). Microinjector placements in FAST-2 mice are shown in Figure 2.6.

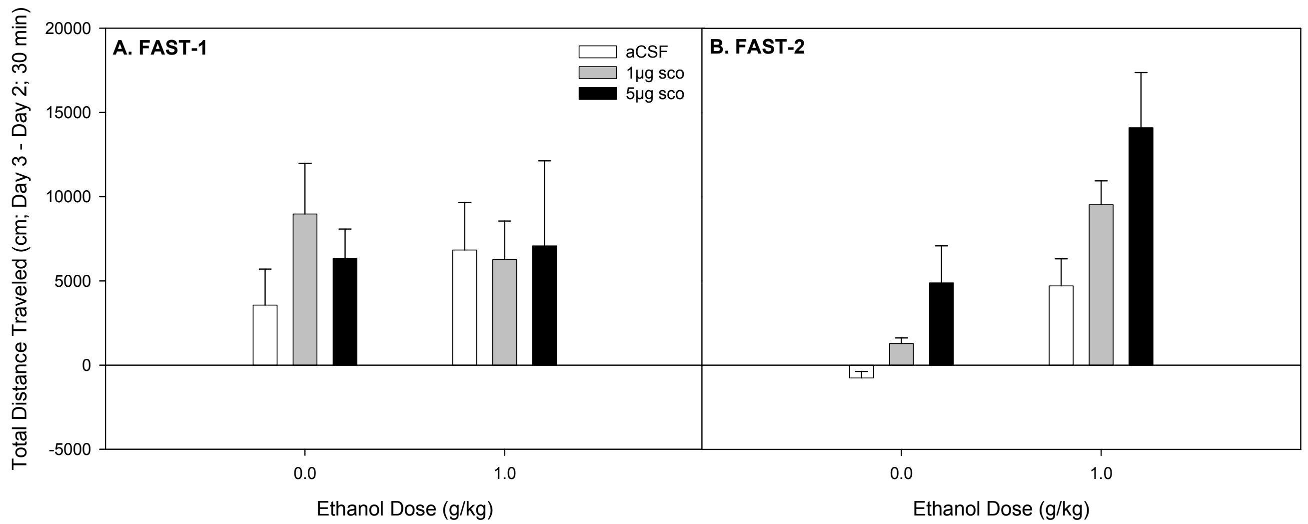

Figure 2.4. Effect of scopolamine microinjection into the NAc alone or in combination with ethanol treatment on locomotor activity in FAST-1 (A) and FAST-2 (B) mice. Shown are means ± SEM for total distance traveled (cm) on Day 3 corrected for Day 2 baseline activity (by subtraction) for data accumulated for 30 min. Means above the solid line indicates stimulation, while means below the solid line reflect sedation. FAST-1 mice n = 8 – 12 mice per scopolamine per ethanol dose; N = 58. Mice were 92 ± 1 d old. FAST-2 mice n = 8 – 13 mice per scopolamine per ethanol dose; N = 68. Mice were 92 ± 1 d old.

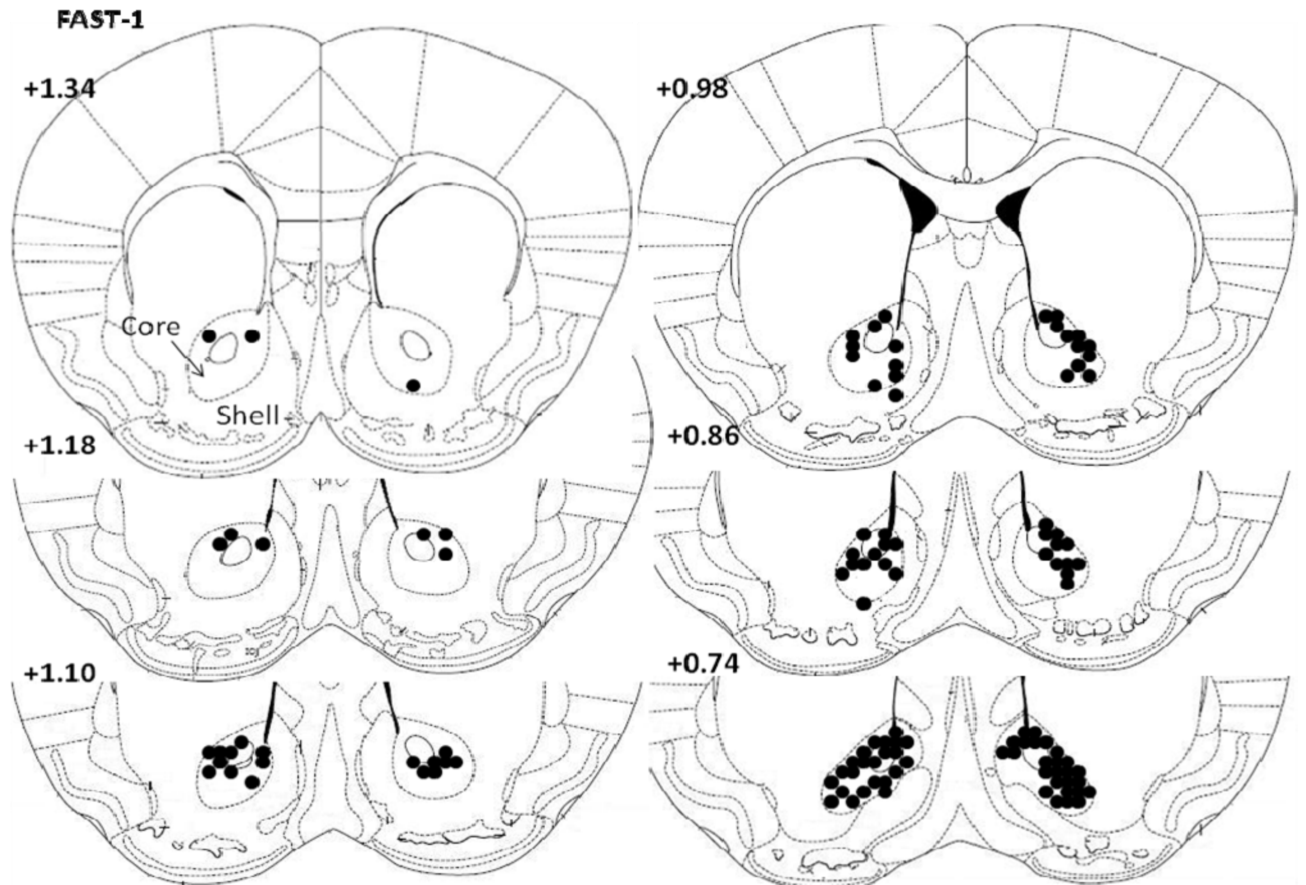

Figure 2.5. Schematic representation of microinjector placements in the NAc of FAST-1 mice. Placements are represented on atlas drawings taken from Paxinos and Franklin (2001). Dots indicate the centered location of each injector. Numbers on the left side of each coronal section indicate millimeters anterior from bregma. The NAc core (indicated in layer +1.34 with an arrow) is denoted within the marked circle. The NAc shell (marked in layer +1.34) is located ventral to the NAc core.

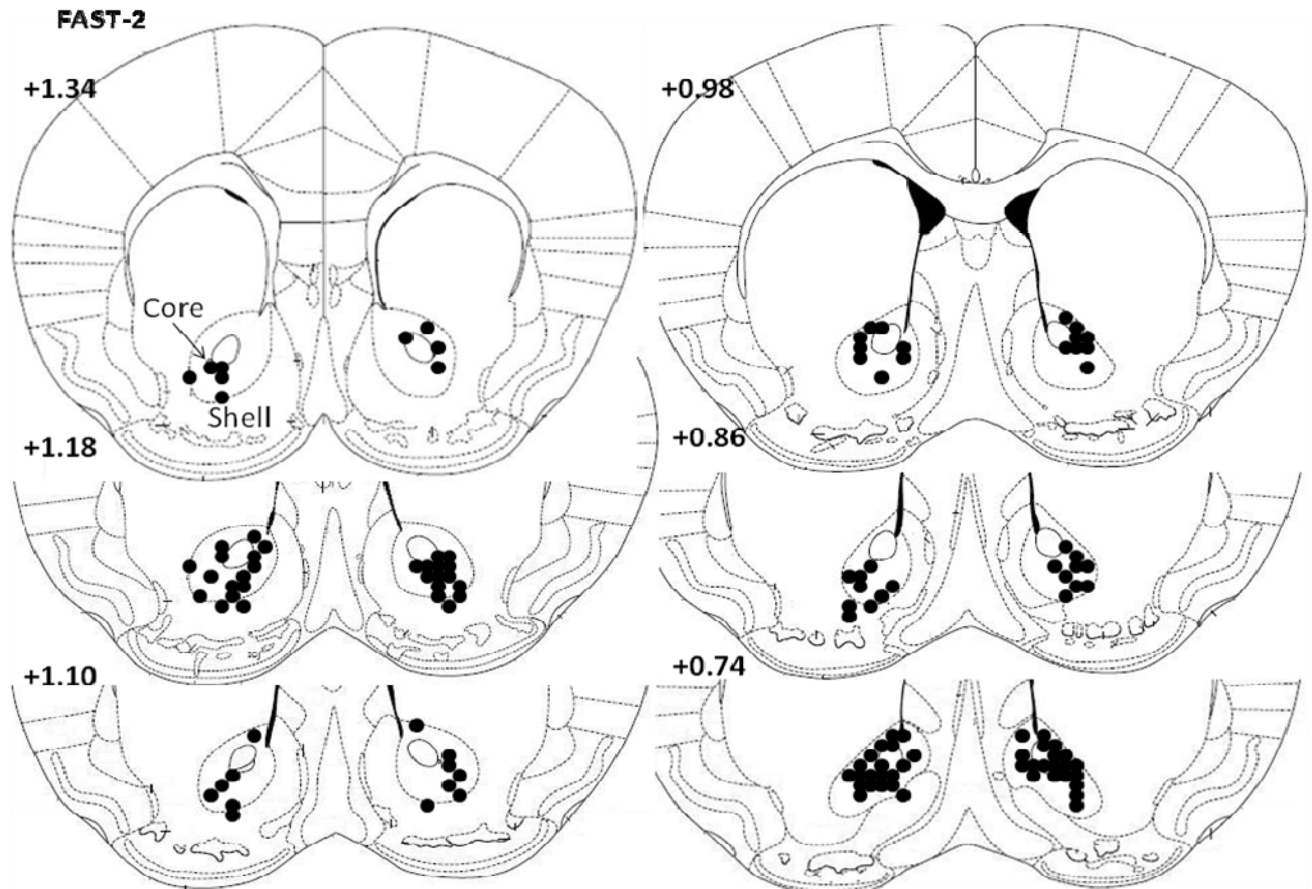

Figure 2.6. Schematic representation of microinjector placements in the NAc of FAST-2 mice. Placements are represented on atlas drawings taken from Paxinos and Franklin (2001). Dots indicate the centered location of each injector. Numbers on the left side of each coronal section indicate millimeters anterior from bregma. The NAc core (indicated in layer +1.34 with an arrow) is denoted within the marked circle. The NAc shell (marked in layer +1.34) is located ventral to the NAc core.

Discussion

In the current experiment, we found that the SLOW replicate lines were differentially sensitive to the combination of scopolamine and ethanol. SLOW-1 mice exhibited locomotor depression in response to increasing doses of ethanol, while SLOW-2 mice did not display locomotor depression to any ethanol dose in the study. While the stimulant effects of scopolamine were dose-dependently attenuated by increasing doses of ethanol in SLOW-1 mice, stimulation to scopolamine was reduced by only the highest dose of ethanol (2.25 g/kg) in SLOW-2 mice. Rotarod studies with two different ethanol doses revealed that the drug combination of 0.5 mg/kg scopolamine and 1.5 g/kg ethanol induced ataxia in SLOW-1 mice, relative to 0 mg/kg scopolamine and 1.5 g/kg ethanol alone. Scopolamine microinjected into the NAc of FAST-1 mice did not significantly enhance locomotor stimulation, and neither did a peripheral injection of 1 g/kg ethanol. In FAST-2 mice, though the highest dose of scopolamine significantly enhanced stimulation both alone and in combination with ethanol, there was no interaction, indicating that the effects of the two drugs were additive. Overall, this indicates that m4 antagonism in the NAc is not responsible for the synergistic enhancement of locomotor activity previously seen with peripheral administration of these two drugs in FAST mice (Scibelli and Phillips, 2009). The findings reported here also reproduce previous results showing that both SLOW and FAST mice are sensitive to the stimulant effects of scopolamine (Bergstrom et al., 2003).

The SLOW replicate lines appeared to be differentially sensitive to the sedative effects of ethanol. While their stimulant sensitivity to 0.5 mg/kg scopolamine alone without ethanol was similar (4932.8 ± 1722.2 cm in SLOW-1 vs. 5544.3 ± 897.2 cm in SLOW-2), their response to the sedative effects of ethanol was not. Although data in some previous studies were not analyzed to statistically examine differences between SLOW-1 and SLOW-2 mice, SLOW-1 mice have appeared to be more sensitive to the sedative effects of ethanol than SLOW-2 (Holstein et al., 2009; Palmer et al., 2002).

Experiment 2.2 was designed to pursue questions arising from the locomotor effects seen in Experiment 2.1, to see whether the drug combination in SLOW-1 mice resulted only in reduced locomotor activity, or whether there might also be enhancement of ataxia competing with forward locomotion resulting in the absence of scopolamine-induced stimulation. Treatment with a drug following pre-treatment with another drug may obscure the effects of the previous drug or result in a competing behavior. Overall, this may manifest as a reduction in the effect of the previous drug. For example, a drug with ataxic effects, such as ethanol, may obscure the stimulant effects of a drug such as scopolamine. Likewise, the pre-treatment with scopolamine may shift the dose-response of ethanol towards greater sedation and intoxication. The FAST and SLOW selected lines of mice model human differences in magnitude of stimulant and sedation sensitivity, with SLOW mice specifically modeling a higher degree of intoxicating response to ethanol, which has been associated in humans with low risk for developing an alcohol-use disorder (Schuckit, 1994; Schuckit and Smith,

2001). There was no effect of scopolamine alone on latency to fall from the rotarod, but the combination of the highest dose of scopolamine and ethanol at a dose that caused sedation on its own (1.5 g/kg) accentuated the ataxic effects of ethanol, and produced a greater response than did the 1.5 g/kg dose of ethanol alone. However, the locomotor response to ethanol alone, and ethanol combined with scopolamine was similar in Experiment 2.1 in SLOW-1 mice.

In a previous study, scopolamine, when combined with ethanol, counteracted the sedative effects of ethanol (Pohorecky et al., 1979). We did not see this result in the current work. In SLOW mice, scopolamine did not block mAChR-mediated ethanol sedative effects. However, SLOW mice were sensitive to the stimulant effects of scopolamine alone. Muscarinic receptors are able to both inhibit and enhance DA transmission in the brain, partly via interaction with nicotinic acetylcholine receptors (nAChRs) (Exley and Cragg, 2008; Threlfell et al., 2010; Threlfell and Cragg, 2011). SLOW mice had higher gene expression levels of the nAChR α6 subtype than FAST mice (Kamens and Phillips, 2008). The α6 subtype acts as a filter for NAc DA transmission (Exley and Cragg, 2008). The higher expression level of this receptor subtype in the sedative-sensitive SLOW mice, as compared to the stimulant-sensitive FAST mice, may indicate that mAChR activation in SLOW mice is met with a greater level of filter than in FAST, preventing DA transmission, and potentially locomotor stimulation.

The lack of subtype selective pharmacological agents targeting the mAChR has made investigation of these subtypes challenging. We wished to determine whether m4 antagonism in the NAc would synergistically enhance the locomotor response to ethanol in FAST mice (Scibelli and Phillips, 2009). Because of the lack of selective drugs, we opted to inject scopolamine into the m4-rich NAc. The m1 and m2 receptor subtypes are also expressed in the NAc and striatum; one would expect similar results at the inhibitory receptor subtype m2, but antagonism of the excitatory m1 receptor subtype should ostensibly reduce locomotor activity. However, we did not see this in our results.

We mainly targeted the NAc core, but also areas intermediate to the core and shell (the “shore”, GP Mark, personal communication) (Figures 2.4 and 2.5). Previous studies have indicated that both the NAc core and shell are involved in locomotor responses to drugs of abuse (Ikemoto and Sharpe, 2001; Ikemoto, 2002). Both FAST-1 and FAST-2 had similar levels of activity following microinjection of the 5μg dose of scopolamine in the NAc [6319 ± 1758 cm in FAST-1 vs. 4891 ± 2190 cm in FAST-2], although the high activity to the 0μg dose of scopolamine prevented a significant stimulant response to scopolamine in FAST-1 animals.

In the previous study (Scibelli and Phillips, 2009), both FAST replicate lines responded similarly to the combination of scopolamine and ethanol. As FAST-1 mice did not respond at all to the intraaccumbal administration, and FAST-2 mice only displayed an additive response to the combination of

scopolamine and ethanol, this indicates that the m4 receptors in the NAc did not influence the synergistic locomotor response seen in the previous study. It is possible that when scopolamine was injected into the periphery in our previous study, it acted on both inhibitory and excitatory receptor subtypes, of which the combined effect produced synergism of locomotor activity (Scibelli and Phillips, 2009). For example, scopolamine injected in the periphery may have acted to block the inhibitory m4 receptor subtypes in the NAc, as well as block mAChR located on GABA interneurons of the VTA, leading to a disinhibition of DAergic neurons of the VTA and promoting DA release in the NAc and enhancing locomotor activity (Omelchenko and Sesack, 2006; Steidl and Yeomans, 2009). Perhaps targeting these other mAChR-expressing regions is required for the synergistic response. As previously mentioned, there are also m1 and m2 receptors in the NAc, although the majority of mAChR in the NAc are m4 (Chapman et al., 2011; Vilaro et al., 1991). We were unable to block the synergistic response seen in the peripheral study with dopaminergic antagonists, which may suggest that this response was sponsored by glutamatergic excitatory inputs instead (Scibelli and Phillips, 2009; Zhou et al., 2003).

In general, both SLOW and FAST mice were sensitive to the stimulant effects of scopolamine. However, while mAChR appeared to partially influence locomotor responses to ethanol in both FAST and SLOW mice, action at mAChR did not appear to strongly modulate the effects of ethanol in the current study.

CHAPTER 3: Differences in *Chrm5* sequence and expression between FAST and SLOW mice

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Abstract

Background: The muscarinic acetylcholine receptor (mAChR) subtype genes *Chrm4* and *Chrm5* are potential candidate genes for magnitude of acute locomotor stimulation to ethanol. To test this possibility, we sequenced the *Chrm4* and *Chrm5* genes in mice selectively bred for extreme sensitivity (FAST) and insensitivity (SLOW) to the stimulant effects of ethanol. We also examined gene expression levels of *Chrm4* and *Chrm5* in drug-naïve FAST and SLOW mice in whole brain as well as brain regions known to express these receptor subtypes. ***Methods:*** Genomic DNA was obtained from FAST and SLOW tails, amplified with PCR, purified, and sequenced with dye terminator chemistry with custom primers. Gene sequences were compared to the reference inbred strain C57BL/6J (B6). In separate sets of mice, brain regions (prefrontal cortex, striatum, hippocampus (HIP), and ventral midbrain (VM)) were dissected for gene expression analysis. Once dissected, brain tissue was processed for RNA extraction, and quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine gene expression levels. ***Results:*** There was one synonymous single nucleotide polymorphism (SNP) between B6 and FAST/SLOW for the *Chrm4* gene. There were 3 coding region differences between B6 and FAST/SLOW for the *Chrm5* gene; one was a synonymous SNP, while the other 2 were non-synonymous SNPs. The 2 non-synonymous SNPs were represented differentially between FAST-2 and SLOW-2 mice. FAST mice displayed greater relative expression levels of *Chrm5* than SLOW mice in the VM. Also, the SLOW-1 replicate line possessed greater *Chrm5* levels in the HIP

than the FAST-1 line. ***Conclusions:*** Unexpectedly, SLOW-1 mice had higher gene expression of *Chrm5* in the HIP than FAST-1 mice. The *Chrm5* gene expression difference in VM may underlie stimulant sensitivity between FAST and SLOW, but this cannot be fully explained by a polymorphic *Chrm5* sequence difference between the lines.

Introduction

Future alcohol intake may be predicted by one's initial experience with alcohol. It has been suggested that those with a family history of alcoholism are more at risk to develop alcohol abuse behaviors because they experience the stimulating, euphoric effects of alcohol more strongly than the sedative, intoxicating effects (Newlin and Thomson, 1990; Holdstock et al., 2000; King et al., 2002). Accordingly, a low level of response to the putatively negative, intoxicating effects of alcohol is associated with a greater risk of alcoholism, and is a heritable response (Schuckit et al., 2005; Wilhelmsen et al., 2003). Locomotor stimulation to ethanol in mice is a model of human behavioral stimulation. Understanding the genetic underpinnings of this trait may help to predict and treat future alcohol use disorders in humans.

The FAST and SLOW lines of mice were selectively bred for extreme sensitivity (FAST) or insensitivity (SLOW) to the stimulant effects of ethanol in our and the Crabbe lab over multiple generations (Crabbe et al., 1987; Phillips et al., 1991; Shen et al., 1995b). They originated from a heterogenous stock of mice (HS/Ibg), which contained 8 inbred strains mated with equal frequency (A, AKR, BALB/c, C3H/2, C57BL, DBA/2, Is/Bi, and RIII) (McClearn et al., 1970). The selection trait was a difference score for ethanol-induced activity. Mice with extreme high activity scores were mated together to create the FAST line, and SLOW mice were selected for low acute activity scores. The lines were bred in replicate, maintained as independent breeding populations (FAST-1, SLOW-1,

FAST-2, SLOW-2). Selection pressure was maintained for 37 generations, and future generations were randomly mated. These mice are currently in generation 110. Naïve mice were most recently tested at generation 106 and have still maintained their divergent response to ethanol (Gubner et al., unpublished data). It is likely that selection has led to genetic changes reflected in differential sequence and expression of some genes involved in the acute locomotor response to ethanol.

Data from our lab and others have indicated the presence of a quantitative trait locus (QTL) on mouse Chromosome 2 for acute locomotor stimulation to ethanol (Demarest et al., 1999; 2001; Hitzemann et al., 1998; Palmer et al., 2006). Although there are many genes included in this QTL region on Chromosome 2, the m4 and m5 muscarinic acetylcholine receptor (mAChR) subtypes (*Chrm4*, 91.6 Mb, 49 cM; *Chrm5*, 112.1 Mb, 58 cM) (Matsui et al., 1999) are attractive candidate genes for investigation. The inhibitory m4 receptor subtype is detected in the striatum, colocalized with dopamine (DA) D1R-containing projection neurons of the striatonigral pathway, which influences motor behavior (Hersch et al., 1994; Ince et al., 1997). Immunoreactivity for the m4 receptor subtype is also detected in the nucleus accumbens (NAc), olfactory tubercle, islands of Calleja, substantia nigra (SN), and cortex (Hersch et al., 1994; Levey et al., 1991; Vilaro et al., 1991). The excitatory m5 receptor's mRNA is expressed in the ventral tegmental area (VTA) and the SN, the origins of the mesolimbic DA pathway (Weiner et al., 1990). Mice with the m4 receptor specifically knocked out in the dopaminergic-D1R projection neurons of the

striatum displayed enhanced locomotor activation to cocaine and amphetamine as compared to wildtype (WT) controls (Jeon et al., 2010), indicating this receptor subtype gene exerts an inhibitory influence on D1R neurons. Also, m5 knockout (KO) mice display reduced morphine-induced locomotor activation as compared to WT controls (Steidl and Yeomans, 2009), self-administer cocaine at lower rates than WT (Fink-Jensen et al. 2003), and have lower breakpoints in progressive ratio chronic self-administration paradigms (Thomsen et al. 2005). This suggests m5 is required for drug reward-related behavior. Thus, these receptor subtypes are involved in responses to other drugs with stimulant properties, and may also play a role in the stimulant effects of ethanol.

The locomotor stimulant response to scopolamine, a non-selective muscarinic antagonist, was tested in a previous study in FAST and SLOW mice (Bergstrom et al., 2003). FAST-1 and SLOW-1 mice exhibited a similar stimulant response following scopolamine injection, but FAST-2 mice displayed significantly greater locomotor stimulation than SLOW-2. Because the activity difference was only seen in one set of replicate lines, the difference could be mediated by genes relevant to ethanol sensitivity, or it could be a result of chance fixation of alleles relevant to the scopolamine response but not ethanol (Crabbe et al., 1990). While only tested in FAST mice, the combination of scopolamine and ethanol in this line robustly enhanced locomotor activation in both replicate lines in a synergistic fashion (Scibelli and Phillips, 2009). Gene expression or sequence could possibly underlie the selection response. To this aim, we examined gene expression and sequence differences in drug-naïve

FAST and SLOW mice. Basal differences between the lines in the absence of drug may indicate predisposition to differential stimulant responses to ethanol in these mice. We hypothesized that SLOW mice would display greater expression levels of *Chrm4* than FAST mice in the regions that we tested (striatum, prefrontal cortex (PFC)), as the inhibitory nature of this gene's product may underlie the SLOW's lack of stimulant response to ethanol. Likewise, we hypothesized that FAST mice would display greater *Chrm5* expression levels in the hippocampus (HIP), and ventral midbrain (VM), as this receptor promotes excitation of the reward pathway.

Methods and Materials

Husbandry

These experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. All procedures were approved by the Portland Veterans Affairs Medical Center (PVAMC) Institutional Animal Care and Use Committee.

FAST and SLOW

FAST and SLOW mice for the sequencing experiment were from $S_{37}G_{107\text{-}108}$. Tail snips were taken from FAST and SLOW male breeder mice for extraction of genomic DNA (gDNA) for the sequencing project and ranged from 87 – 115 days

of age. FAST and SLOW mice for brain dissections were from $G_{96-100,}$ and were 83 ± 2 days of age at time of dissection.

Sequencing

Tail samples from 4 male breeder mice per replicate line (n = 16) for each pass representing all FAST-1 and -2 and SLOW-1 and -2 breeder pairs were taken and gDNA was extracted from the samples. Briefly, tails were collected into 300 µL of Qiagen Cell Lysis Solution. 1.7 µl of 20 mg/ml Proteinase K was added to the tube and nutated in an incubator overnight at 60°C. The next morning, 1.7 µL of RNase was added to the samples, which were then incubated for 45 min while gently rocking. 100 µl of Qiagen Protein Precipitation Solution was added, the samples were vortexed, and were put in the freezer for 10 min to chill. Samples were then centrifuged at 14000 x g for 7 min to collect the protein pellet at the base of the tube. Supernatant was poured off into a fresh tube containing 300 µL of isopropanol, and samples were then mixed gently and left at room temperature for 10 min. The tubes were centrifuged for 10 sec, the supernatant was poured off, and the DNA pellet was washed with 300 µL of 70% ethanol. Samples were again centrifuged for 2 min, ethanol was poured off, and the pellet was dried for 1 hr. The pellet was rehydrated in an oven in 300 µl of TE buffer at 45°C. DNA was stored at 4°C.

Primers intended to amplify PCR products of 500 – 1000 bp were designed using Primer3 (Rozen and Skaletsky, 2000), confirmed for specificity using BLASTN (NCBI), and ordered from Integrated DNA Technologies, Inc.

(Coralville, IA). The primers for *Chrm4* (NM_007699.2) spanned 91.76 – 91.77 Mb on Chromosome 2, and the primers for *Chrm5* (NM_205783.2) spanned 112.31 – 112.32 Mb on Chromosome 2, designed to cover the annotated exon(s) for each gene. After gDNA was amplified, it was run on a gel, and bands were excised and cleaned using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Purified PCR products were sent either to Macrogen (Rockville, MD) or the OHSU Sequencing Core for direct sequencing of PCR products via dye terminator chemistry using custom primers. The FAST and SLOW sequence results were compared to the C57BL/6J (B6) mouse inbred strain sequence noted in ENSEMBL.

Brain dissections

Experimentally naïve FAST and SLOW mice were cervically dislocated and decapitated. Each brain was dissected to obtain PFC (olfactory bulbs were removed from the ventral side of the brain and a slice was made at +1.75 bregma), striatum (sliced between +1.75 and +0.25 bregma), HIP (the cerebral cortices were peeled back to reveal the hippocampi, which were removed with forceps), and VM (containing the VTA, SN, and interpeduncular nuclei) (sliced between -3.25 to -4.25 bregma, and horizontally sliced below the periaqueductal gray). Each region was placed into individual RNase-free tubes and stored in the -80°C freezer until extraction could occur.

RNA extraction

RNA extraction was performed using our previously published methods (Kamens and Phillips, 2008; Kamens et al., 2009). For specific brain regions, RNA was extracted with a Stratagene Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA). Briefly, brain regions were lysed with β-mercapoethanol/lysis buffer (250 – 600 μL, depending on weight of brain region) and homogenized with RNase-free Pellet Pestles (Kontes, VWR, Batavia, IL). 100 μL of 70% ethanol was added to the homogenate, briefly vortexed, and added to a RNA-binding spin cup. Following centrifugation (14000 x g, 1 min), 600 μL of low-salt wash buffer was added to the spin cup, and again centrifuged. The filtrate was removed, and the column was dried by centrifugation for 2 min. RNase-free DNase I (50 μL) and DNase digestion buffer were added to the spin cup matrix, and incubated for 15 min at 37°C. Samples were then washed with 500 μL of high-salt wash buffer, followed by 2 rounds of washes and centrifugation for 2 min with low-salt wash buffer (600 μL, followed by 300 μL). 30 μL of elution buffer, warmed to 60°C, was added to the matrix, and the samples were centrifuged at 14000 x g to elute the RNA. RNA quality and quantity was assessed with a spectrophotometer (NanoDrop ND-1000, NanoDrop, Wilmington, DE).

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

RNA was reverse-transcribed to cDNA using an Applied Biosystems High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA).

Messenger RNA gene expression of the *Chrm4* and *Chrm5* mAChR genes and of the control gene *Hprt1* (hypoxanthine guanine phosphoribosyltransferase 1) was performed using predesigned TaqMan gene expression assays (Applied Biosystems). The control gene *Hprt1* was used as it has been validated as a reference gene that remains stable for mouse neuronal gene expression assays (Meldgaard et al., 2006). Quantitative PCR reactions were run using an iCycler (Bio-Rad, Hercules, CA). Each sample was run in triplicate, and the average crossing threshold (C_t) for the *Chrm4*, *Chrm5*, and *Hprt1* genes was determined. For each sample, the C_t for *Hprt1* was subtracted from the expression of the mAChR subunits. Relative expression based on the $\Delta\Delta C_t$ method (calculated as 2 to the power of the average expression of FAST-1 mice minus each individual value) (Livak and Schmittgen, 2001) was used as the dependent variable. All data was normalized to FAST-1, which was arbitrarily chosen but allows for statistical comparisons between both line and replicate.

Statistics

Gene expression data for each region were analyzed with a factorial ANOVA with the potential factors line, replicate, and sex. Interactions were followed up with simple main effects analyses and Newman-Keuls post-hoc tests when appropriate. Statistica (StatSoft, Tulsa, OK) was used for all statistical analyses. α was set at 0.05. Sequencing results were analyzed using Sequencher (www.genecodes.com).

Results

Experiment 3.1: Sequence differences between FAST and SLOW mice

The FAST and SLOW selected lines of mice were sequenced for *Chrm4* and *Chrm5* for two reasons. First, we wished to know whether FAST and/or SLOW lines differed from the C57BL/6J (B6) reference strain, which would make the use of B6 as a reference strain problematic for creation of oligonucleotides for an RNA interference project. Secondly, it was important to know whether or not these lines differed from each other in *Chrm4* or *Chrm5* sequence for interpretation of results associated with the RNA interference project and to draw conclusions about the impact of selection on genetic sequence differences for these genes. Therefore, SNPs could be designated as both synonymous/non-synonymous between the B6 reference strain and FAST/SLOW, or as synonymous/non-synonymous between the FAST line and the SLOW line.

The data are represented in Tables 3.1 and 3.2, similar to Downing et al. (2010), as this was not a haplotype analysis nor an analysis of minor allele frequencies. For the *Chrm4* gene, all FAST and SLOW replicates were largely similar in sequence to B6, with a few exceptions noted below. There were no differences from B6 in the 5' untranslated region (UTR). In the coding region, there were no differences from B6 with the exception of amino acid (AA) 355 (91.768470 Mb). At this location, the B6 sequence corresponds to ACT, which codes for threonine. In 15 of the FAST and SLOW samples (one SLOW-1 sample did not have any results in this location), the sequence was ACG, which

also codes for threonine, making this a synonymous single nucleotide polymorphism (SNP) (Table 3.1). Coverage of the 3' UTR ranged from 60 – 75%, due to technical difficulties of sequencing through poly-N regions. There were 3 nucleotides that varied from the B6 sequence. For two of these, the variation was the same in all 15 – 16 FAST and SLOW samples. For the third, one sample was heterozygous, while the other 14 matched the B6 allele.

Table 3.1. Sequence information for amino acid (AA) 355 and 3 base pairs (bp) in the *Chrm4* gene containing SNPs between the B6 sequence and FAST/SLOW mice.

	Accession number (B6) or family (FAST/SLOW)	AA 355, bp 1252	3' UTR, bp 1917	3' UTR, bp 2034	3' UTR, bp 2066
B6	NM_007699.2	ACT[a]	T	A	T
SLOW-1	211	ACG[a]	C[a]	A	C[a]
SLOW-1	215	ACG[a]	C[a]	A	C[a]
SLOW-1	216	no data	C[a]	A	C[a]
SLOW-1	218	ACG[a]	C[a]	A	C[a]
FAST-1	222	ACG[a]	C[a]	A	C[a]
FAST-1	225	ACG[a]	C[a]	A/G[a,b]	C[a]
FAST-1	228	ACG[a]	C[a]	no data	no data
FAST-1	229	ACG[a]	C[a]	A	C[a]
SLOW-2	264	ACG[a]	C[a]	A	C[a]
SLOW-2	266	ACG[a]	C[a]	A	C[a]
SLOW-2	268	ACG[a]	C[a]	A	C[a]
SLOW-2	269	ACG[a]	C[a]	A	C[a]
FAST-2	271	ACG[a]	C[a]	A	C[a]
FAST-2	272	ACG[a]	C[a]	A	C[a]
FAST-2	276	ACG[a]	C[a]	A	C[a]
FAST-2	279	ACG[a]	C[a]	A	C[a]

[a]: different from B6 sequence. [b]: heterogenous

The *Chrm5* gene sequencing experiment was performed twice, as we wished to confirm results seen in the first pass. There were no differences between the B6 sequence and FAST/SLOW mice in the 3' or 5' UTR. There were, however, 3 SNPs between B6 and FAST/SLOW in the coding region (Table 3.2). At AA 265, the B6 codon of GTA codes for a valine. The 15 out of the 16 FAST and SLOW mice with data at that location possessed a GAA, which codes for glutamic acid. In the second round of sequencing, the one SLOW-1 mouse without data now also read as a GAA. At AA 305, the B6 sequence of AGC (serine) was similar for 9 FAST and SLOW samples (1 SLOW-1, 4 FAST-1, 4 SLOW-2), but 2 SLOW-1s weren't read, and 1 SLOW-1 and 4 FAST-2 samples read as AGT, also coding for serine (a synonymous change). In this case, the replicate-2 lines differed in SNPs at AA 305. In the second round of sequencing, the 2 SLOW-1 samples with no data now read as heterogenous for the final letter of the serine codon (AG(T/C)). Finally, the sequence read at AA 325 was TGC for the B6 inbred strain, which codes for cysteine. Again, 9 of the FAST/SLOW mice matched the B6 sequence (1 SLOW-1, 4 FAST-1, 4 SLOW-2), 2 mice lacked data (1 SLOW-1, 1 FAST-2), and 5 mice (2 SLOW-1, 3 FAST-2) had the TAC codon, representing tyrosine. In the second round of sequencing, the SLOW-1 mouse without sequencing data now contained a non-synonymous SNP (T(A/G)C), 1 SLOW-1 that previously read as TAC now also contained the non-synonymous SNP, and the FAST-2 mouse without data now read as TAC. Again, FAST-2 and SLOW-2 mice displayed sequence differences at AA 325, resulting in a non-synonymous SNP.

Table 3.2. Sequence information for amino acid (AA) 265, 305, and 325 in the *Chrm5* gene containing SNPs between the B6 sequence and FAST/SLOW mice.

		Round 1				Round 2		
	Accession number (B6) or family (FAST/SLOW)	**AA 265**	**AA 305**	**AA 325**		**AA 265**	**AA 305**	**AA 325**
B6	NM_007699.2	GTA	AGC	TGC		GTA	AGC	TGC
SLOW-1	211	GAA[a]	no data	no data		GAA[a]	AG(T/C)[b]	T(A/G)C[b]
SLOW-1	215	GAA[a]	AGT[a]	TAC[a]		GAA[a]	AGT[a]	TAC[a]
SLOW-1	216	no data	no data	TAC[a]		GAA[a]	AG(T/C)[b]	T(A/G)C[b]
SLOW-1	218	GAA[a]	AGC	TGC		GAA[a]	AGC	TGC
FAST-1	222	GAA[a]	AGC	TGC		GAA[a]	AGC	TGC
FAST-1	225	GAA[a]	AGC	TGC		GAA[a]	AGC	TGC
FAST-1	228	GAA[a]	AGC	TGC		GAA[a]	AGC	TGC
FAST-1	229	GAA[a]	AGC	TGC		GAA[a]	AGC	TGC
SLOW-2	264	GAA[a]	AGC	TGC		GAA[a]	AGC	TGC
SLOW-2	266	GAA[a]	AGC	TGC		GAA[a]	AGC	TGC
SLOW-2	268	GAA[a]	AGC	TGC		GAA[a]	AGC	TGC
SLOW-2	269	GAA[a]	AGC	TGC		GAA[a]	AGC	TGC
FAST-2	271	GAA[a]	AGT[a]	no data		GAA[a]	AGT[a]	TAC[a]
FAST-2	272	GAA[a]	AGT[a]	TAC[a]		GAA[a]	AGT[a]	TAC[a]
FAST-2	276	GAA[a]	AGT[a]	TAC[a]		GAA[a]	AGT[a]	TAC[a]
FAST-2	279	GAA[a]	AGT[a]	TAC[a]		GAA[a]	AGT[a]	TAC[a]

[a]: different from B6 sequence. [b]: heterogenous

While there was only a non-synonymous SNP in the FAST/SLOW mice compared to the B6 reference strain, 3 SNPs were discovered for the *Chrm5* gene. Furthermore, 2 of them were non-synonymous, coding SNPs that were differentially present between FAST-2 and SLOW-2. These SNPs may underlie the differential response to ethanol seen in these mice.

Experiment 3.2: FAST and SLOW Chrm4 and Chrm5 gene expression levels in chosen brain regions

Group sizes are listed in Tables 3.3 – 3.6. There were no differences among line, replicate, or sex for expression levels of the housekeeping gene *Hprt1* in the PFC, striatum, HIP, or VM. There were no interactions or main effects of sex for either *Chrm4* or *Chrm5* in any region. Therefore, data are collapsed on sex, but are provided separated by sex in Tables 3.3 – 3.6. Only significant results (either main effects or interactions) are discussed.

Table 3.3. Values for PFC *Hprt1* cycle threshold (C_t) and *Chrm4* (relative expression) by line, replicate, and sex.

	PFC			
	Hprt1 **C_t**		***Chrm4*** **relative expression**	
	N	**Mean ± SEM**	**N**	**Mean ± SEM**
SLOW-1 female	6	23.3 ± 0.4	6	0.92 ± 0.1
SLOW-1 male	6	23.0 ± 0.1	6	0.92 ± 0.1
FAST-1 female	6	23.1 ± 0.3	6	1.1 ± 0.3
FAST-1 male	3	22.7 ± 0.1	3	0.8 ± 0.07
SLOW-2 female	6	23.2 ± 0.3	6	0.91 ± 0.18
SLOW-2 male	5	23.1 ± 0.2	5	0.99 ± 0.2
FAST-2 female	6	23.1 ± 0.2	6	0.89 ± 0.07
FAST-2 male	6	23.3 ± 0.4	6	1.6 ± 0.43

Table 3.4. Values for Striatum *Hprt1* cycle threshold (C_t) and *Chrm4* (relative expression) by line, replicate, and sex.

	Striatum			
	Hprt1 C_t		*Chrm4* relative expression	
	N	**Mean ± SEM**	**N**	**Mean ± SEM**
SLOW-1 female	6	23.7 ± 0.1	6	1.07 ± 0.07
SLOW-1 male	6	23.5 ± 0.08	6	1.14 ± 0.07
FAST-1 female	6	23.7 ± 0.2	6	1.78± 0.25
FAST-1 male	4	23.7 ± 0.11	4	1.16 ± 0.12
SLOW-2 female	6	23.6 ± 0.14	6	1.0 ± 0.03
SLOW-2 male	6	23.5 ± 0.12	6	1.03 ± 0.1
FAST-2 female	6	23.5 ± 0.1	6	1.24 ± 0.2
FAST-2 male	6	23.6 ± 0.2	6	1.4 ± 0.3

Table 3.5. Values for HIP *Hprt1* cycle threshold (C_t) and *Chrm5* (relative expression) by line, replicate, and sex.

	HIP			
	Hprt1 C_t		*Chrm5* relative expression	
	N	Mean ± SEM	N	Mean ± SEM
SLOW-1 female	4	21.8 ± 0.33	4	1.23 ± 0.14
SLOW-1 male	5	21.9 ± 0.17	5	1.39 ± 0.15
FAST-1 female	4	21.4 ± 0.09	4	1.15± 0.05
FAST-1 male	4	21.6 ± 0.12	4	1.23 ± 0.17
SLOW-2 female	3	21.8 ± 0.07	3	1.0 ± 0.09
SLOW-2 male	3	21.7 ± 0.29	3	1.00 ± 0.16
FAST-2 female	4	21.6 ± 0.13	4	1.18 ± 0.14
FAST-2 male	3	21.9 ± 0.2	3	1.65 ± 0.09

Table 3.6. Values for VM *Hprt1* cycle threshold (C_t) and *Chrm5* (relative expression) by line, replicate, and sex.

	VM			
	Hprt1 C_t		*Chrm5* relative expression	
	N	Mean ± SEM	N	Mean ± SEM
SLOW-1 female	6	24.7 ± 0.26	6	0.64 ± 0.07
SLOW-1 male	4	24.8 ± 0.3	4	0.5 ± 0.15
FAST-1 female	4	24.8 ± 0.55	4	1.01± 0.26
FAST-1 male	4	24.7 ± 0.17	4	0.69 ± 0.06
SLOW-2 female	4	26.1 ± 1.77	4	1.11 ± 0.3
SLOW-2 male	6	25.3 ± 0.47	6	1.02 ± 0.11
FAST-2 female	8	24.9 ± 0.18	8	0.94 ± 0.06
FAST-2 male	4	24.7 ± 0.15	4	0.84 ± 0.24

Separate factorial ANOVAs (line x replicate x sex) were performed on relative expression data for each region and gene. For consistency, relative expression data are shown for each replicate line for each region, as there were no main effects or interactions of sex for any gene or region (Table 3.7). In the striatum (n = 4 – 6 per line per replicate per sex), there was only a main effect of replicate [$F_{(1, 42)} = 9.26$, $p < 0.01$]; replicate-2 mice possessed significantly higher *Chrm4* levels than replicate-1. *Chrm5* was examined in the HIP (n = 3 – 5 per line per replicate per sex). Analysis revealed an interaction of replicate and line [$F_{(1, 22)} = 6.88$, $p < 0.05$]. SLOW-1 mice displayed significantly greater levels of *Chrm5* in the HIP than FAST-1. For *Chrm5* expression in the VM (n = 4 – 8 mice

per line per replicate per sex), there was a main effect of line [$F_{(1,36)} = 6.35$, $p < 0.05$]. FAST mice had greater expression of *Chrm5* in the VM than did SLOW mice. In the PFC (n = 3 – 6 per line per replicate per sex), there were no differences between FAST and SLOW in *Chrm4* expression.

The only consistent gene expression difference in the FAST and SLOW lines was for *Chrm5* expression in the VM; FAST displayed higher levels than did SLOW. Contrary to what we hypothesized, SLOW-1 had higher levels of *Chrm5* in the HIP than did FAST-1.

Table 3.7. Mean (± SEM) relative expression of *Chrm4* and *Chrm5* in FAST and SLOW mice.

	Chrm4				***Chrm5***			
Region	**FAST -1**	**SLOW -1**	**FAST -2**	**SLOW -2**	**FAST -1**	**SLOW -1**	**FAST-2**	**SLOW -2**
Striatum	1.02 ± 0.06	1.10 ± 0.05	1.33 ± 0.16	1.53 ± 0.18				
@HIP					1.02 ± 0.08	1.32 ± 0.10	1.38 ± 0.12	1.19 ± 0.08
*VM					1.06 ± 0.13	0.59 ± 0.07	0.91 ± 0.08	0.85 ± 0.13
PFC	0.92 ± 0.08	0.95 ± 0.13	0.97 ± 0.2	1.25 ± 0.23				

* $p < 0.05$ significant difference between FAST and SLOW; @ $p < 0.05$ significant difference between FAST-1 vs. SLOW-1;! $p < 0.05$ significant difference between SLOW-1 vs. SLOW-2

Discussion

SNPs are single nucleotide substitution variations in DNA that result in genetic and phenotypic variation. They are found throughout the genome,

occurring at a rate of 1 in every 1000 bp (Brookes, 1999). It is estimated that 50% of SNPs occur in noncoding gene regions, 25% cause missense mutations, and 25% are silent (Brookes, 1999). Silent SNPs are also referred to as synonymous SNPs, as they do not result in amino acid substitutions. Polymorphisms in protein coding regions of genes indicate potential changes in protein function (non-synonymous SNPs), while polymorphisms in the noncoding regions indicate potential changes in gene expression or other post-transcriptional modifications that may alter mRNA levels (Flint et al., 2005). However, non-synonymous SNPs are not always revealed as changes in gene expression, as differences in gene expression can change over time and result from different mechanisms (Flint et al., 2005).

This study revealed a SNP between B6 and FAST/SLOW for the *Chrm4* gene, but it was synonymous (resulting in the same protein product). There were 3 amino acid differences between B6 and FAST/SLOW for the *Chrm5* gene; one was a synonymous SNP, while the other 2 were non-synonymous SNPs (coding for alternative amino acids). So, FAST/SLOW were largely similar to B6 mice, but did differ in a few locations for the *Chrm4* and *Chrm5* genes. The more crucial comparison is between FAST and SLOW mice. Interestingly, there were clear differences between FAST-2 and SLOW-2 for both non-synonymous SNPs at AA 305 and AA 325 for the m5 gene.

We detected 3 SNPs within the FAST and SLOW lines that differed from the B6 reference strain. Interestingly, the inbred strains known to possess these

SNPs include AKR, BALB/c, C3H/2, and DBA/2, all strains that helped to comprise the HS/Ibg stock. We do not know if the non-synonymous SNPs result in functional changes or gene expression differences, though they are more likely to result in gene expression differences or some sort of post-transcriptional modification rather than function, as non-synonymous SNPs do not affect amino acids. However, there is clearly a difference between the replicate-2 lines for these two SNPs. Furthermore, the replicate lines are not identical in behavior. For example, we saw a significant difference in stimulant response to the muscarinic antagonist scopolamine in replicate-2, but not -1 (Bergstrom et al., 2003).

The gene expression assay measures levels of messenger RNA that may indicate differential amounts of receptor protein levels. There were no significant differences between the lines in *Chrm4* expression in any region, but this study revealed two differences in regional gene expression between the FAST and SLOW selected lines of mice for *Chrm5*. It is intriguing that FAST mice displayed greater expression levels of *Chrm5* than SLOW mice. This was in the direction of our hypothesis, as m5 receptors of the VM promote DA release in the NAc and striatum, effects associated with both ethanol reward and locomotor behavior (Imperato and Di Chiara, 1986; Lester et al., 2008; Miller and Blaha, 2005; Miller et al., 2005). Furthermore, we have previously shown that FAST mice displayed greater pacemaker firing than SLOW mice in DA cells of the SN (Beckstead and Phillips, 2009). We did not, however, expect to see greater expression of *Chrm5* in the HIP of SLOW-1 than FAST-1. This effect was only seen in one set of

replicate lines, providing weak to moderate evidence that this difference somehow underlies stimulant sensitivity in these mice (Crabbe et al., 1990). It is very likely that the replicate lines possess differential amounts of mAChR protein, though as this difference was only seen in one set, it may be merely due to chance or genetic drift.

There is a lack of concordance between the FAST and SLOW regarding the *Chrm5* SNPs and the gene expression differences in the VM. The expression differences appear to be driven by replicate 1 and not replicate 2, which suggests that the SNPs are not having a functional effect on gene expression. We are currently sequencing the promoter region (2.5 Mb upstream of the gene), as data exists suggesting that sequence polymorphisms in the promoter region may have functional consequences (Barr et al., 2009; Hansson et al., 2006; Mexal et al., 2007).

Overall, these data provide evidence that basal expression differences in the *Chrm5* gene may underlie differences in stimulant sensitivity to ethanol. However, the SNP pattern of results in replicate-2 does not fully parallel the gene expression results or behavioral data.

CHAPTER 4: The stimulant response to ethanol is attenuated in *Chrm5* knockout mice on a DBA/2J background

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Abstract

Background: Muscarinic acetylcholine receptors (mAChR) may influence the stimulant response to ethanol. To investigate this possibility, we tested the response to several ethanol doses in knockout (KO) and wildtype (WT) mice lacking the m4 or m5 mAChR subtypes. Since some data suggest that similar mechanisms influence drug reward and locomotor effects, we also tested these mice for ethanol consumption in a two-bottle choice experiment. ***Methods:*** In Experiments 4.1 and 4.2, saline or ethanol (1, 1.5, 2, 2.75 g/kg) was given to *Chrm4* and *Chrm5* KO and WT mice on a C57BL/6 strain background and locomotor activity was tested for 15 min. In Experiments 4.3 and 4.4, *Chrm4* and *Chrm5* mice were tested for potential differences in ethanol consumption. Tastant solutions (saccharin, quinine, potassium chloride) were offered following ethanol to check for potential differences in taste sensitivity between the genotypes. Finally, in Experiments 4.5 and 4.6, the effect of ethanol (1, 1.5, 2 g/kg) on locomotor behavior was tested in *Chrm4* and *Chrm5* KO and WT mice that had been backcrossed for 2 generations onto a DBA/2J background. ***Results:*** Only locomotor depressant effects of ethanol were seen in *Chrm4* and *Chrm5* mice on the C57BL/6 background, and there were no significant differences between KO or WT mice for either gene. There were no differences between *Chrm4* or *Chrm5* KO and their WT in ethanol or tastant consumption. Finally, there were no differences between *Chrm4* KO and WT on the partial DBA/2J background, however, the *Chrm5* KO lacked the stimulant response to ethanol seen in their matched WT mice. ***Conclusions:*** These results suggest

that the *Chrm5*, but not *Chrm4*, gene is involved in the stimulant response to ethanol, and that neither gene has a role in ethanol consumption.

Introduction

Behavioral stimulation to ethanol is a sensitivity trait for the development of alcoholism (Erblich and Earleywine, 2003; King et al., 2002; Newlin and Thomsen, 1990). Those with a family history of alcoholism experience the pleasurable, stimulant-like effects of ethanol more significantly than they do the sedative/intoxicating effects (Newlin and Thomsen, 1990). In addition, moderate to heavy drinkers are more sensitive to the pleasurable effects of alcohol than light drinkers (Holdstock et al., 2000; King et al., 2002). This human sensitivity trait may be modeled in mice as acute locomotor stimulation to ethanol. Understanding this trait in mice may provide helpful clues to prevent and treat human alcoholics

A genomic region that influences a complex trait, such as acute locomotor stimulation to ethanol, can be mapped to a specific chromosome. Such regions are called quantitative trait loci (or locus, singular) (QTL). A QTL is comprised of a region on a chromosome that contains a gene (or genes) that is statistically associated with and influences the magnitude of the trait. Data from our lab and others have indicated the presence of a QTL on mouse Chromosome 2 for acute locomotor stimulation to ethanol (Demarest et al., 1999b; 2001; Hitzemann et al., 1998; Palmer et al., 2006). Within this region lie the genes that code for the m4 and m5 muscarinic acetylcholine receptor (mAChR) subtypes (*Chrm4*, 91.6 Mb, 49 cM; *Chrm5*, 112.1 Mb, 58 cM) (Matsui et al., 1999). The m4 and m5 mAChR subtypes may influence acute locomotor stimulation to ethanol, as they are

expressed in brain regions known to influence drug reward and reinforcement. The m4 receptor subtype is detected in the striatum, nucleus accumbens (NAc), and cortex, as well as the olfactory tubercle and islands of Calleja (Hersch et al., 1994; Ince et al., 1997; Levey et al., 1991; Vilaro et al., 1991). The m5 receptor mRNA is expressed in the ventral tegmental area (VTA) and the substantia nigra (SN), the origins of the mesolimbic dopamine (DA) pathway (Weiner et al., 1990).

Because selective pharmacological agents targeting the individual mAChR subtypes are lacking, genetic tools have been an invaluable resource in determining the effects of the individual mAChR subtypes on drug-related behaviors. Knockout (KO) mice for the m4 (Gomeza et al., 1999) and m5 (Yamada et al., 2001) mAChR subtypes have been generated and tested for a variety of drug-related behavioral traits. Mice with the m4 receptor specifically knocked out in the dopaminergic-D1 projection neurons of the striatum displayed enhanced locomotor activation to cocaine and amphetamine as compared to wildtype (WT) controls, as well as enhanced DA efflux from the NAc following amphetamine treatment (Jeon et al., 2010). It has been shown that m5 KO mice self-administer cocaine at lower rates than WT (Fink-Jensen et al. 2003) and also have lower breakpoints in progressive ratio chronic self-administration paradigms (Thomsen et al. 2005). Additionally, m5 KO mice spend less time in the cocaine-paired side than WT in a CPP paradigm, although these mice do not differ in cocaine-induced locomotor activation (Fink-Jensen et al. 2003). Morphine treatment in m5 receptor KO mice resulted in a blunted DA response in the NAc in these mice, a lack of morphine-associated place conditioning, and

reduced morphine-induced locomotor activation as compared to WT controls (Basile et al., 2003; Steidl and Yeomans, 2009). To the best of our knowledge, the m4 and m5 KO mice have never been tested for ethanol sensitivity traits. When taken together, these results indicate that the m4 subtype exerts an opposing effect on dopaminergic neurons of the NAc and striatum, areas heavily implicated in drug reward and sensitivity. This suggests that m4 antagonism may potentiate the stimulant effects of ethanol. Accordingly, existing results for m5 KO mice suggest that mice lacking the m5 receptor subtype have reduced reinforcement to stimulant drugs of abuse, suggesting that m5 receptor blockade should decrease reward and reinforcement (however, see Schmidt et al., 2009).

The current experiments were designed to test m4 and m5 KO and WT mice for potential differences in acute locomotor stimulation to ethanol. We hypothesized that mice lacking the inhibitory m4 receptor subtype would display enhanced stimulant sensitivity to ethanol as compared to WT, while mice lacking the excitatory m5 receptor subtype would display attenuated stimulation to ethanol. Because these mice originally existed on a C57BL/6 background, which does not show stimulation to ethanol (e.g., Dudek et al., 1991), we also backcrossed these mice for 2 generations onto the ethanol stimulation-sensitive DBA/2J background. Our lab has also identified an ethanol preference QTL in a region of Chromosome 2 similar to that of the QTL for acute locomotor stimulation to ethanol, with a peak at 49 cM, which corresponds precisely with the location of *Chrm4* (Matsui et al., 1999; Phillips et al., 1994; 1998). Since some data suggest that similar mechanisms influence drug reward and locomotor

effects (Risinger et al., 1994), we also tested the *Chrm4* and *Chrm5* KO and WT mice for ethanol consumption. We predicted that, compared to WT, m4 KO mice would consume more ethanol as these mice lack the inhibitory m4 receptor, potentially promoting drinking behavior and increased reward as compared to the WT. We hypothesized that m5 KO mice would consume less ethanol than their WT counterparts, as the excitatory m5 receptor subtype has been implicated in a variety of drug responses.

Methods and Materials

Animals

These experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. All procedures were approved by the Portland Veterans Affairs Medical Center (PVAMC) Institutional Animal Care and Use Committee. Initial breeder pairs, consisting of *Chrm4* and/or *Chrm5* KO mice and C57BL/6NTac (Taconic Farms, Germantown, NY) WT mice, were obtained from Dr. Jurgen Wess (Molecular Signaling Section, National Institute of Diabetes and Digestive and Kidney Diseases). These mice were mated, and then resulting heterozygote offspring were set up as breeder pairs for subsequent generations, following convention in our lab (Palmer et al., 2003). Tails were taken at approximately 45 d for *Chrm4* (Gomeza et al., 1999) and *Chrm5* (Yamada et al., 2001) genotyping, and KO and

WT littermates were used for behavioral testing in Experiments 4.1 – 4.4. For Experiments 4.5 – 4.6, *Chrm4* and *Chrm5* KO mice were backcrossed for two generations to DBA/2J mice (Jackson Laboratories West, Sacramento, CA), after which mice were genotyped and heterozygotes were set up as breeder pairs to generate subsequent offspring. Littermates were again used for these studies. Animals were weaned at 21 + 1 d into same-sex groups, 2 - 5 per cage (28.5 x 17.5 x 12 cm, lined with EcoFRESH bedding (Absorption Corp., Ferndale, WA)). Animals were maintained on a 12:12 h light:dark cycle, with lights on in the colony room at 0600 h. Standard rodent chow (Purina 5001; Animal Specialties Inc., Hubbard, OR) and tap water were available *ad libitum*. All behavioral testing was conducted during the light phase between 0800 and 1800 h, except for the drinking studies, as detailed below.

Drugs

100% ethanol was obtained from Decon Labs (King of Prussia, PA). Saccharin sodium salt, quinine hemisulfate salt, and potassium chloride salt were obtained from Sigma (St. Louis, MO). For injections, ethanol was diluted to 20% (v/v) in physiological saline (0.9% NaCl; Baxter Healthcare Corp., Deerfield, IL) and delivered intraperitoneally (i.p.) in a volume adjusted for body weight. For drinking solutions, 100% ethanol and tastants were constituted in tap water to the concentrations given below.

Activity apparatus

Activity was measured in clear acrylic boxes (40 x 40 x 30 cm) housed inside of automated activity monitors within sound and external light-attenuating chambers (Accuscan Instruments Inc., Columbus, OH). Eight photobeams and detectors, 2 cm above the chamber floor, recorded beam breaks, which were translated into distance traveled (cm). Each chamber was equipped with a 3.3 W incandescent bulb to provide illumination, and a fan provided ventilation and additional noise-masking.

Dose-response analysis of the acute locomotor response to ethanol

The testing procedure followed our previously published 3-day design (Boehm et al., 2002a; Meyer and Phillips, 2003; Scibelli and Phillips, 2009). On test days, mice were moved into the testing room 45 to 60 min prior to behavioral testing to permit acclimation, weighed, and then locomotor activity was monitored for 15 min in 5-min periods. Testing began immediately following i.p. injection. Day 1 saline data provided a measure of activity in a novel environment. Day 2 saline data provided a measure of baseline activity in the now-familiar environment. Day 3 provided ethanol (0, 1.0, 1.5, 2, or 2.75 g/kg in Experiments 4.1 – 4.2; 1.0, 1.5, and 2 g/kg in Experiments 4.5 – 4.6) response data. Ethanol doses were chosen to allow for a dose-response profile, capturing the biphasic effects of ethanol (Dudek et al., 1991; Palmer and Phillips, 2002a). Immediately following the test on Day 3, 20 µl retro-orbital blood samples were taken for determination of blood ethanol concentration (BEC).

Two-bottle choice ethanol drinking

The procedure for measuring 24-h ethanol consumption followed previously published protocols in our lab (Phillips et al., 1994; Sharpe et al., 2005). The cages were equipped with two inverted 25-ml glass graduated cylindrical vials fitted with rubber stoppers. Food was arranged around the two drinking vials. On experimental days -1 and 0, mice were individually housed and offered these novel drinking tubes for the first time. Tap water was presented in both tubes, and consumption was measured as a baseline measure of fluid consumption. Beginning on experimental day 1, one water tube was replaced with a tube containing 3% ethanol. This concentration of ethanol was offered for 4 consecutive days, followed by 6%, 10%, and 20% ethanol. Tubes were alternated every other day to control for potential side biases. Mice were weighed on the first day of each new concentration presentation, which was used to calculate g/kg ethanol consumed. The volume in each tube was recorded daily, and levels were corrected for evaporation or spillage by subtraction of fluid lost from tubes in control cages with no mice. Drinking volumes were measured with 0.2 ml accuracy. The g/kg ethanol consumed was calculated by averaging the g/kg consumption on the second and fourth day of each concentration presentation, which allowed animals adequate time to identify the new location of the ethanol tube, providing a stable measurement. Total volume was calculated by combining the fluid levels from both the ethanol and the water tubes, and preference ratios were calculated by dividing total volume by ethanol volume.

Tastant consumption

Following testing for ethanol consumption, animals were returned to tap water for four days and were then tested for tastant consumption and preference. The tastants were bitter quinine (QUIN) (0.015 followed by 0.03 mM), sweet saccharin (SACC) (0.033 followed by 0.066%) and salty potassium chloride (KCl) (100 followed by 200 mM). Each tastant was offered for eight days, with 4-day access to the lower concentration, followed by 4 days at the higher concentration. The order of tastant presentation was counterbalanced to control for order effects. The dependent variables were mg/kg tastant consumed, in addition to total volume and preference ratio.

BEC determination

The 20 µl blood samples were aspirated into microcentrifuge tubes containing 50 µl of ice-cold 5% $ZnSO_4$. 50µl of 0.3N $Ba(OH)_2$ was added to each tube, along with 300 µl of double distilled H_2O. The samples were then centrifuged at 12,000 rpm in a cold room for 5 min. Following centrifugation, supernatant was pipetted off into 2 ml glass crimp-top vials, and analyzed for BEC via gas chromatography (Agilent 6890N; Agilent Technologies, Palo Alto, CA) following previously published methods (Boehm et al., 2000).

Data analysis

Data were analyzed by factorial ANOVA, with repeated measures when appropriate, using Statistica software (StatSoft Version 6.1, Tulsa, OK).

Significant complex interactions were followed up using two-way ANOVAs focused on the most relevant factors, followed by simple main effects analyses and Newman Keuls post-hoc tests. The critical dependent variable for locomotor activity was Day 3 – Day 2 total distance traveled (cm) over the 15 min test, which provides a measure of drug activation (Day 3) corrected for baseline activity (Day 2). Day 1 and Day 2 distances traveled were also analyzed to check for any group differences not related to drug treatment. For the drinking studies, the main dependent variable was g/kg ethanol or mg/kg tastant consumed. Preference ratios and total volume of consumption were also analyzed. α was set at ≤ 0.05. Results are expressed as mean ± SEM.

Results

Experiment 4.1: Chrm4-B6 KO and WT mice are equally sensitive to the locomotor depressant effects of ethanol.

After saline injection on Day 1, females had higher levels of locomotor activity than males ($F_{(1, 73)} = 14.5$, $p < 0.001$), and *Chrm4*-B6 KO mice had higher levels of locomotor activity than did WT ($F_{(1, 73)} = 11.7$, $p < 0.01$) (4573.6 ± 222 cm in KO vs. 4129.7 ± 136 cm in WT). On Day 2, females still had higher locomotor activity levels than males ($F_{(1, 73)} = 8.38$, $p < 0.01$), but there was no longer a difference between the genotypes. There were no differences among assigned dose groups on either Day 1 or Day 2. Mice showed reduced levels of

activity from Day 1 to Day 2 (mean ± SEM: 4411.5 ± 128.3 cm vs. 3530.6 ± 152.1 cm), suggesting habituation.

A factorial ANOVA (genotype x dose x sex) performed using difference score data (Day 3 – Day 2) derived from data accumulated during the 15-min test revealed only a main effect of dose [$F_{(4, 73)} = 6.68$, $p < 0.001$]. Newman-Keuls post-hoc analyses indicated that locomotor activity was significantly depressed by the 2.75 g/kg dose of ethanol, compared to all other doses, regardless of genotype (Figure 4.1). There was no effect of genotype on BEC, although BEC values significantly increased in a dose-dependent fashion ($F_{(3, 64)} = 66.7$, $p < 0.001$); Table 4.1).

Experiment 4.2: Chrm5-B6 KO and WT mice are equally sensitive to the locomotor depressant effects of ethanol.

There were no differences between genotypes or among the pre-assigned groups after saline injection on Day 1. On Day 2, females had higher levels of locomotor activity than males ($F_{(1, 85)} = 14.9$, $p < 0.001$), but there were no differences between genotypes or among assigned dose groups. Mice showed reduced levels of activity from Day 1 to Day 2 (3989.7 ± 114.2 cm vs. 3413.6 ± 134.5 cm), indicating habituation.

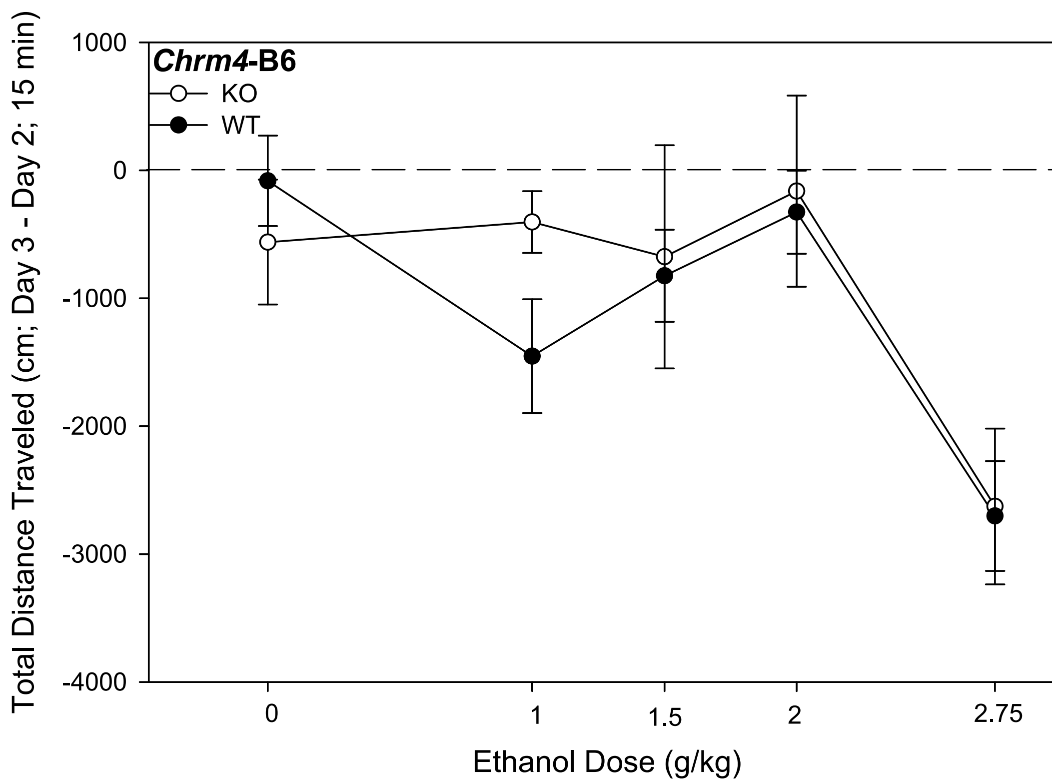

Figure 4.1. *Chrm4*-B6 mice of both genotypes were sensitive to the depressant effects of ethanol at the highest dose. Shown are data for total distance traveled (cm) on Day 3 corrected for Day 2 baseline activity for data accumulated for 15 min. Data above the dotted line indicate a stimulant response after correction for basal activity, while data below the solid line indicate net sedation. n = 8 – 11 mice/genotype/dose, N = 93. Mice were 69 ± 1 d old.

Table 4.1. Experiment 4.1 and 4.2 mean (± SEM) BEC values (mg/ml) 15 min after ethanol injection.

	Ethanol Dose (g/kg)			
	1.0	**1.5**	**2.0**	**2.75**
***Chrm4*-B6 KO**	0.86 ± 0.06	1.18 ± 0.09	1.71 ± 0.13	2.21 ± 0.11
***Chrm4*-B6 WT**	0.77 ± 0.1	1.20 ± 0.09	1.83 ± 0.09	2.12 ± 0.14
***Chrm5*-B6 KO**	0.75 ± 0.05	1.12 ± 0.1	1.79 ± 0.11	2.13 ± 0.13
***Chrm5*-B6 WT**	0.61 ± 0.09	1.1 ± 0.14	1.19 ± 0.16	2.02 ± 0.12

A factorial ANOVA (genotype x dose x sex) performed using difference score data (Day 3 – Day 2) accumulated during the 15-min test revealed only a main effect of dose [$F_{(4, 85)} = 5.61$, $p < 0.001$]. Newman-Keuls post-hoc analyses indicated that locomotor activity was significantly depressed by the 1.5, 2, and 2.75 g/kg doses of ethanol compared to the 0 and 1 g/kg doses, regardless of genotype (Figure 4.2). BEC values significantly increased in a dose-dependent fashion ($F_{(3, 76)} = 51.0$, $p < 0.001$), and *Chrm5*-B6 KO mice displayed significantly greater BEC values, overall, than did *Chrm5*-B6 WT mice [$F_{(1, 76)} = 6.96$, $p < 0.05$] (Table 4.1). However, although there was not a significant interaction of genotype and dose ($p = 0.08$), the effect of genotype appeared to be driven by a low BEC value for the 2 g/kg WT dose group.

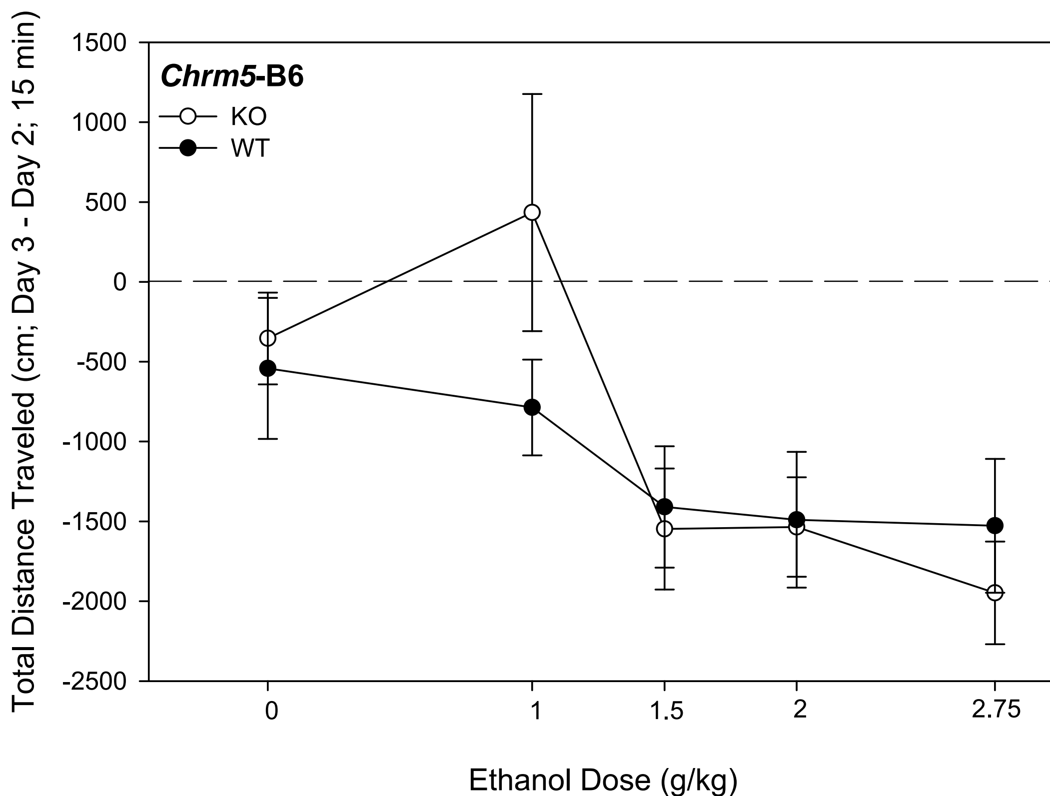

Figure 4.2. *Chrm5*-B6 mice of both genotypes are sensitive to the depressant effects of moderate to high doses of ethanol. Shown are data for total distance traveled (cm) on Day 3 corrected for Day 2 baseline activity for data accumulated for 15 min. Data above the dotted line indicate a stimulant response after correction for basal activity, while data below the solid line indicate net sedation. 9 – 12 mice/genotype/dose, N = 105. Mice were 68 ± 1 days old.

Experiment 4.3: Chrm4-B6 KO and WT mice do not differ in ethanol consumption or preference.

A repeated measures ANOVA (day x genotype x sex) for data from baseline water consumption days (days -1 and 0) revealed a main effect of day

[$F_{(1, 36)} = 11.9$, $p < 0.01$] and sex [$F_{(1, 36)} = 8.5$, $p < 0.01$]. Animals consumed more fluid on Day 0 (mean ± SEM = 4.34 ± 0.2 ml) than Day -1 (mean ± SEM = 3.75 ± 0.2 ml), possibly indicating their increasing familiarity with the drinking apparatus. Also, female mice consumed more water than males.

There were no differences between *Chrm4*-B6 KO and WT for any measure. Repeated measures ANOVA (concentration x genotype x sex) for g/kg ethanol, preference ratio, and total volume consumed all revealed interactions of concentration and sex for all 3 measures (g/kg ethanol: [$F_{(3, 102)} = 6.37$, $p < 0.001$]; preference ratio: [$F_{(3, 102)} = 3.43$, $p < 0.05$]; total volume: [$F_{(3, 102)} = 7.95$, $p < 0.001$]). Simple main effects analyses indicated that females consumed more ethanol than males (Figure 4.3) and showed greater ethanol preference (Table 4.2) at all ethanol concentrations except 3%. Furthermore, females consumed significantly more ethanol at the 20% ethanol concentration than any other concentration, and they also consumed less ethanol at the 3% concentration, compared to 6 and 10% ethanol. Males did not significantly differ in the amount of ethanol consumed across concentrations. For preference, females significantly preferred the 6% ethanol concentration over 20%, while males preferred the 3% ethanol concentration over all other concentrations. However, preference ratios did not exceed 0.5, indicating that neither males nor females preferred ethanol over water at any concentration. Simple main effects analyses for total volume indicated that females consumed more total volume than males at all ethanol concentrations (Table 4.2). Also, females consumed

significantly more total volume of fluid during the 20% ethanol phase than all other concentration phases.

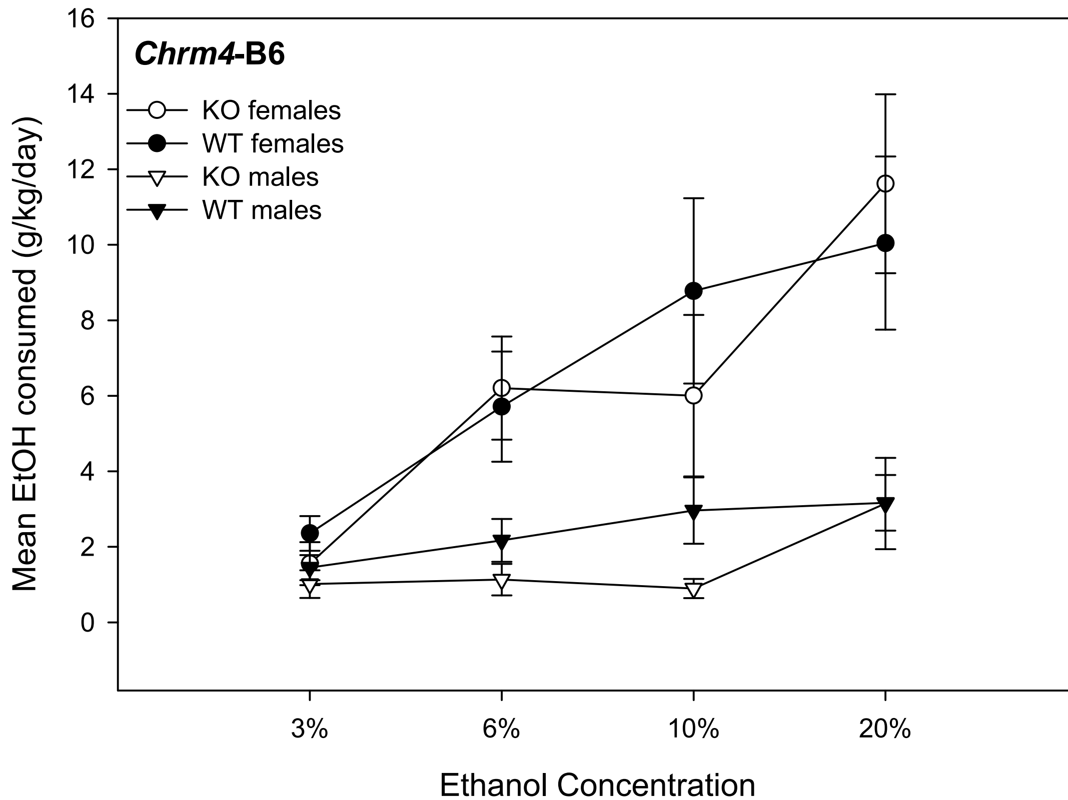

Figure 4.3. Greater ethanol consumption in female mice, but no difference between *Chrm4*-B6 and WT mice. Shown are mean (± SEM) g/kg/day ethanol consumed at each ethanol concentration. n = 10 mice/genotype/sex, N = 40. Mice were 79 ± 2 days old when testing began.

Consumption, preference, and total volume for each tastant were considered in separate ANOVA analyses. There were no significant effects

Table 4.2. Preference ratio and total volume values for ethanol concentration in *Chrm4*-B6 (Experiment 4.3) and *Chrm5*-B6 (Experiment 4.4) KO and WT mice.

	Ethanol Concentration			
	3%	**6%**	**10%**	**20%**
	Preference Ratio ((mls EtOH)/(mls EtOH + mls H2O))			
Chrm4-B6 KO females	0.24 ± 0.09	0.45 ± 0.10	0.23 ± 0.10	0.21 ± 0.04
Chrm4-B6 WT females	0.41 ± 0.07	0.48 ± 0.12	0.46 ± 0.13	0.20 ± 0.04
Chrm4-B6 KO males	0.27 ± 0.10	0.13 ± 0.05	0.09 ± 0.03	0.10 ± 0.04
Chrm4-B6 WT males	0.37 ± 0.09	0.26 ± 0.07	0.10 ± 0.02	0.13 ± 0.04
Chrm5-B6 KO females	0.27 ± 0.07	0.41 ± 0.10	0.37 ± 0.09	0.25 ± 0.06
Chrm5-B6 WT females	0.49 ± 0.08	0.56 ± 0.09	0.62 ± 0.12	0.32 ± 0.04
Chrm5-B6 KO males	0.36 ± 0.06	0.28 ± 0.08	0.18 ± 0.05	0.13 ± 0.05
Chrm5-B6 WT males	0.20 ± 0.08	0.14 ± 0.06	0.19 ± 0.07	0.10 ± 0.03
	Total Volume (mls EtOH + mls H2O)			
Chrm4-B6 KO females	5.87 ± 0.14	6.50 ± 0.18	6.31 ± 0.31	7.48 ± 0.34
Chrm4-B6 WT females	5.44 ± 0.30	5.63 ± 0.17	5.72 ± 0.18	6.62 ± 0.35
Chrm4-B6 KO males	4.39 ± 0.25	4.88 ± 0.23	4.97 ± 0.23	4.81 ± 0.17
Chrm4-B6 WT males	4.38 ± 0.15	4.47 ± 0.18	4.77 ± 0.36	4.66 ± 0.32
Chrm5-B6 KO females	5.48 ± 0.32	5.83 ± 0.78	5.36 ± 0.30	5.90 ± 0.21
Chrm5-B6 WT females	5.65 ± 0.33	5.63 ± 0.45	5.51 ± 0.43	6.15 ± 0.57
Chrm5-B6 KO males	4.35 ± 0.33	5.17 ± 0.40	4.68 ± 0.21	4.76 ± 0.33
Chrm5-B6 WT males	4.21 ± 0.19	4.14 ± 0.26	4.26 ± 0.17	4.47 ± 0.19

associated with genotype. For mean SACC consumed by *Chrm4*-B6 mice, there were no significant interactions, but there was a main effect of SACC concentration [$F_{(1, 35)} = 66.0$, $p < 0.001$] and of sex [$F_{(1, 35)} = 5.21$, $p < 0.05$]. Males consumed significantly more SACC than females. Mice consumed significantly more SACC at the 0.066% concentration than 0.033% (Figure 4.4A). For preference ratio, there was a significant concentration by sex interaction [F $_{(1,}$

$_{35)} = 10.2$, $p < 0.01$]; females preferred the 0.033% concentration over 0.066%, while males preferred the higher SACC concentration over the lower (Table 4.3). SACC was strongly preferred over water, with preference ratios ranging from 84 to 98%. Mice consumed more total volume at the 0.066% than the 0.033% concentration (Table 4.3), and females consumed more total volume than males.

For QUIN, there was no significant difference between the *Chrm4*-B6 and WT genotypes in mg/kg consumed (Figure 4.4B), although a main effect of sex [$F_{(1, 35)} = 26.7$, $p < 0.01$] revealed that females consumed more QUIN than males. A difference in mg/kg consumed at the different QUIN concentrations only reached a statistical trend for significance [$F_{(1, 35)} = 3.22$, $p = 0.081$], with less QUIN consumed at the 0.03 mM concentration. For preference ratio, there was a significant concentration by sex interaction [$F_{(1, 35)} = 4.88$, $p < 0.05$]; females preferred the 0.015 mM concentration over 0.03 mM (Table 4.3). There was no difference between the genotypes in QUIN preference, or total volume consumed (Table 4.3), although females consumed significantly more total volume than males ($F_{(1, 35)} = 25.7$, $p < 0.001$).

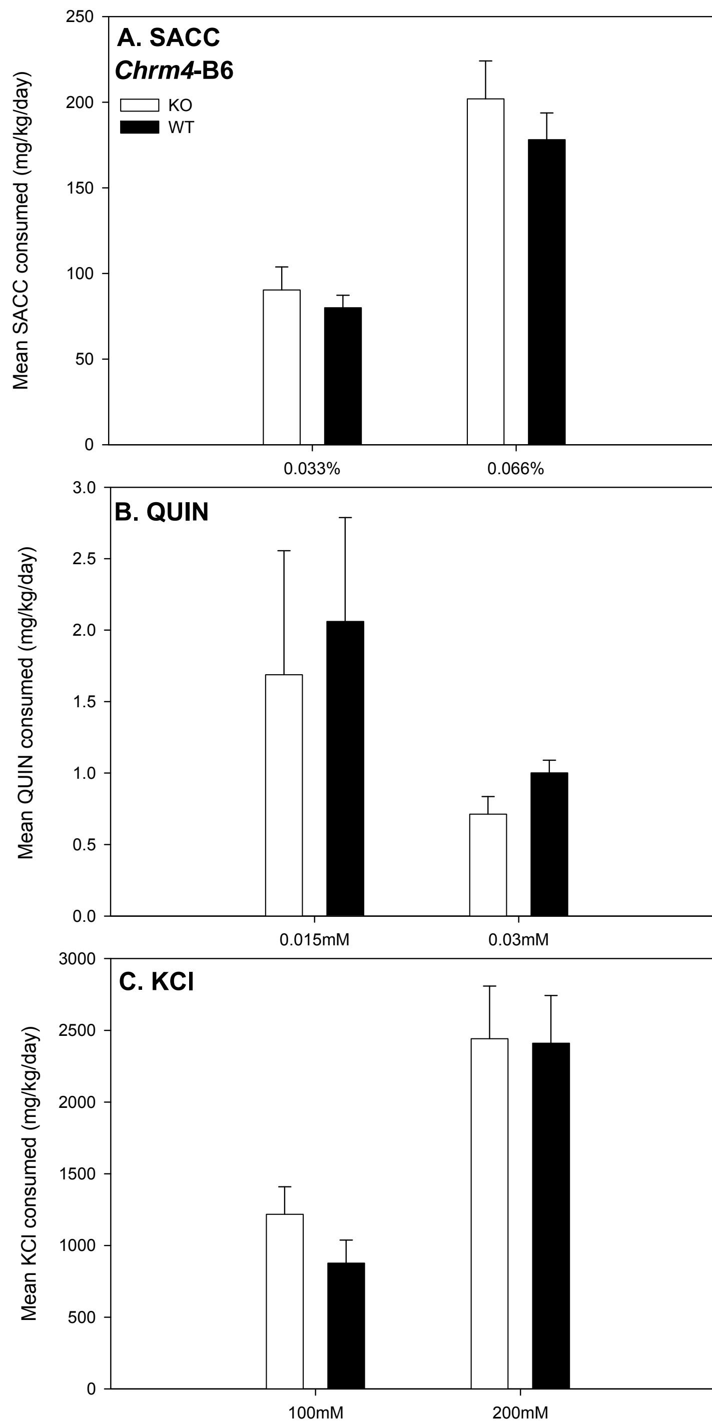

Figure 4.4: *Chrm4*-B6 genotypes do not differ in tastant consumption. Shown is mean mg/kg/day A; saccharin (SACC) B; quinine (QUIN) C; potassium chloride (KCl).

Table 4.3. Preference ratio and total volume values for tastants in *Chrm4*-B6 (Experiment 4.3) KO and WT mice.

	Preference Ratio ((mls tastant)/(mls tastant + mls H2O))	
	SACC	
	0.033%	**0.066%**
Chrm4-B6 KO females	0.94 ± 0.04	0.92 ± 0.04
Chrm4-B6 WT females	0.95 ± 0.02	0.84 ± 0.06
Chrm4-B6 KO males	0.85 ± 0.06	0.97 ± 0.001
Chrm4-B6 WT males	0.93 ± 0.02	0.98 ± 0.003
	QUIN	
	0.015 mM	**0.03 mM**
Chrm4-B6 KO females	0.42 ± 0.07	0.27 ± 0.07
Chrm4-B6 WT females	0.43 ± 0.04	0.38 ± 0.04
Chrm4-B6 KO males	0.24 ± 0.06	0.26 ± 0.05
Chrm4-B6 WT males	0.35 ± 0.03	0.44 ± 0.06
	KCl	
	100 mM	**200 mM**
Chrm4-B6 KO females	0.67 ± 0.08	0.49 ± 0.09
Chrm4-B6 WT females	0.60 ± 0.06	0.56 ± 0.07
Chrm4-B6 KO males	0.66 ± 0.07	0.57 ± 0.09
Chrm4-B6 WT males	0.50 ± 0.07	0.52 ± 0.05
	Total Volume (mls tastant + mls H2O)	
	SACC	
	0.033%	**0.066%**
Chrm4-B6 KO females	8.71 ± 0.40	10.5 ± 0.83
Chrm4-B6 WT females	8.78 ± 0.42	9.46 ± 0.53
Chrm4-B6 KO males	5.87 ± 0.28	7.34 ± 0.44
Chrm4-B6 WT males	5.81 ± 0.26	7.14 ± 0.54
	QUIN	
	0.015 mM	**0.03 mM**
Chrm4-B6 KO females	6.71 ± 0.19	7.19 ± 0.70
Chrm4-B6 WT females	6.31 ± 0.23	6.42± 0.32
Chrm4-B6 KO males	4.97 ± 0.25	5.37 ± 0.47
Chrm4-B6 WT males	4.94 ± 0.23	4.91 ± 0.31
	KCl	
	100 mM	**200 mM**
Chrm4-B6 KO females	7.97 ± 0.38	8.25 ± 0.44
Chrm4-B6 WT females	7.43 ± 0.53	8.29 ± 0.55
Chrm4-B6 KO males	5.88 ± 0.52	6.58 ± 0.50
Chrm4-B6 WT males	5.98 ± 0.44	6.11 ± 0.39

For KCl, there was a main effect of concentration [$F_{(1, 35)} = 34.1$, $p < 0.001$] as well as sex [$F_{(1,35)} = 8.73$, $p < 0.01$]. Mice consumed significantly more KCl at the 200 mM concentration than 100 mM (Figure 4.4C). Also, females consumed significantly more KCl than males. There were no differences between the genotypes in preference ratio (Table 4.3) or total volume (Table 4.3), although females consumed significantly more total volume than males. The mice slightly preferred the KCl over water, with moderate preference ratio values ranging from 53 to 67% (Table 4.3).

Experiment 4.4: Chrm5-B6 KO and WT mice do not differ in ethanol consumption or preference.

A repeated measures ANOVA (day x genotype x sex) for data from baseline water consumption days (days -1 and 0) revealed a main effect of sex [$F_{(1, 34)} = 13.2$, $p < 0.001$]; female mice consumed more water than males. However, mice increased their water consumption from Day -1 (3.88 ± 0.24 ml) to Day 0 (4.01 ± 0.20 ml), possibly indicating familiarity with the drinking apparatus.

Repeated measures ANOVA (concentration x genotype x sex) for g/kg ethanol revealed a two-way interaction of concentration and sex [$F_{(3, 102)} = 11.8$, $p < 0.001$], and a trend towards an interaction of genotype and sex [$F_{(1, 34)} = 3.87$, $p = 0.057$]. Because the interaction was not statistically significant, we did not examine data for lines within each sex separately. There were no drinking

differences between the genotypes, although *Chrm5*-B6 females of both genotypes consumed more ethanol compared to males at every concentration (Figure 4.5). Females consumed less ethanol at 3% than at any other concentration, and less at 6% than at 10 or 20%. A repeated measures ANOVA (concentration x genotype x sex) for preference ratio revealed an interaction of genotype and sex [$F_{(1, 34)} = 5.36$ $p < 0.05$] as well as concentration and sex [$F_{(3, 102)} = 3.07$, $p < 0.05$]. Simple main effects analyses indicated that WT females significantly preferred ethanol over WT males, as well as KO females (Table 4.2). There were no differences in preference ratio between *Chrm5*-B6 KO males and females. Indeed, the preference ratio of WT females exceeded 0.5 for 6% and 10%, indicating some preference for ethanol over water. In general, females preferred the 6%, 10%, and 20% ethanol concentrations over males, although they preferred the 20% concentration less than 6 or 10%. A repeated measures ANOVA (concentration x genotype x sex) for total volume revealed only a main effect of sex [$F_{(1, 34)} = 17.5$, $p < 0.001$]; females consumed more total volume than males (Table 4.2).

During the *Chrm5*-B6 tastant phase, a repeated measures ANOVA for mean SACC consumption (concentration x genotype x sex) revealed a two-way interaction of genotype and sex [$F_{(1, 33)} = 5.36$, $p < 0.05$] and concentration and sex [$F_{(1, 33)} = 5.06$, $p < 0.05$]. Simple main effects analyses indicated that WT females consumed more SACC than WT males, although there were no differences between the sexes in *Chrm5*-B6 KO mice (Figure 4.6A). Both females and males consumed more SACC at the 0.066% concentration

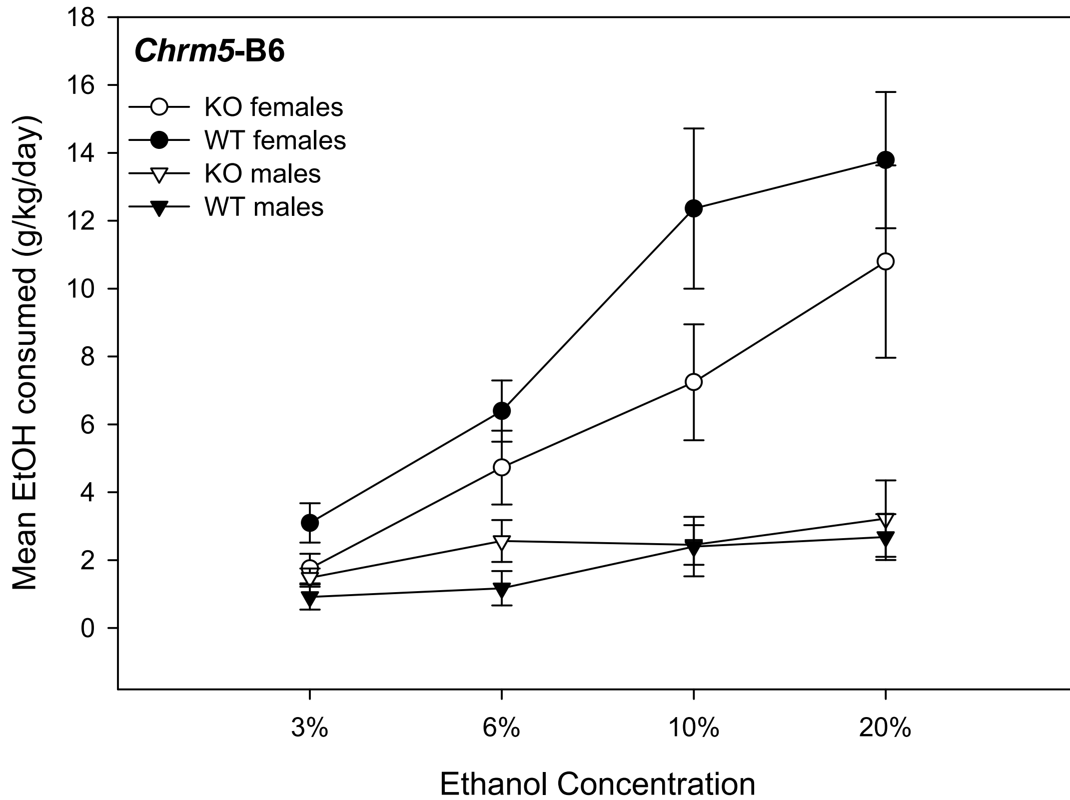

Figure 4.5. Female mice of both *Chrm5*-B6 genotypes consume more g/kg ethanol than males. Shown are mean data for g/kg ethanol consumed at each respective ethanol concentration (g/kg/day). n = 8-10 mice/genotype/sex, N = 38. Mice were 83 ± 2 days old when testing began.

than the 0.033%, though females consumed more SACC than males at both the 0.033% and 0.066% concentrations. There were no significant differences in SACC preference ratio between the *Chrm5*-B6 genotypes or sexes (Table 4.4).

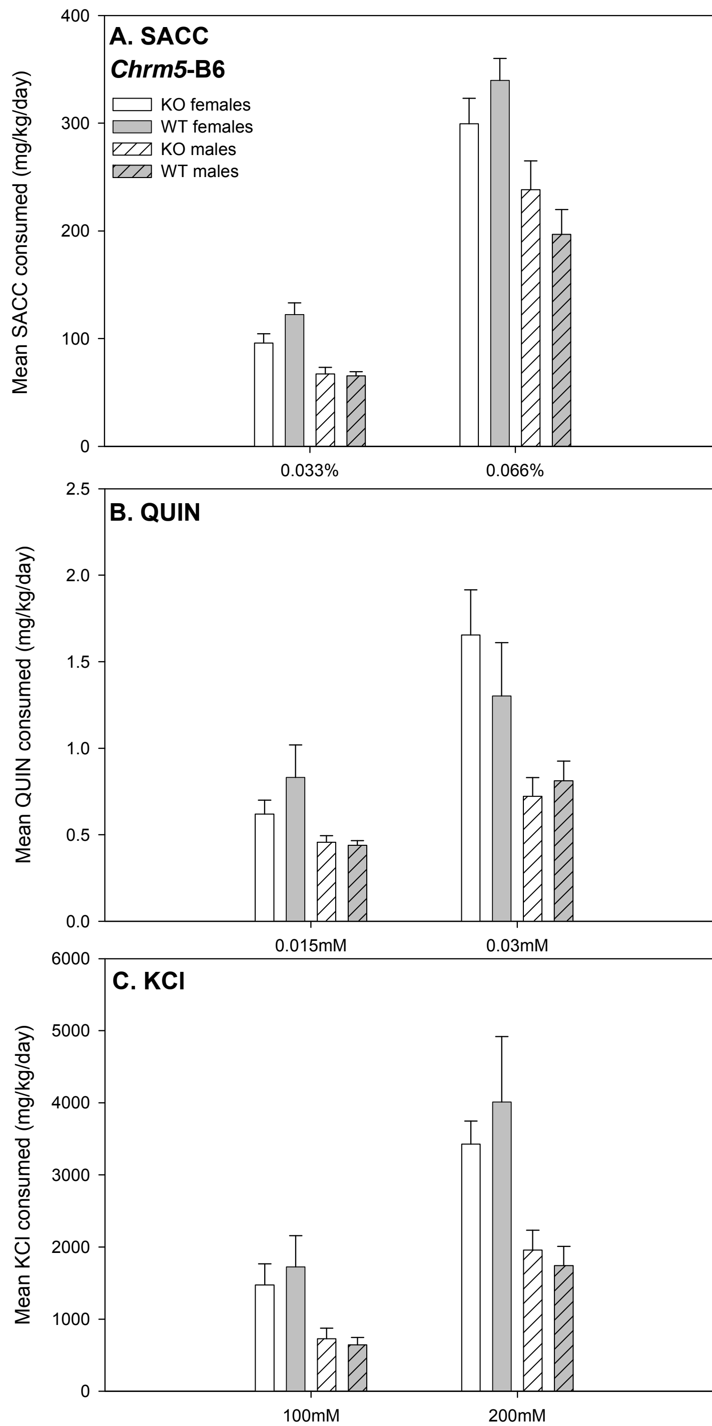

Figure 4.6. *Chrm5*-B6 genotypes do not differ in tastant consumption. Shown is mean mg/kg/day A; saccharin (SACC) B; quinine (QUIN) C; potassium chloride (KCl).

Again, *Chrm5*-B6 mice strongly preferred SACC, having preference ratios that ranged from 88 to 93%. For total volume, there was an interaction of concentration and sex [$F_{(1, 33)} = 13.0$, $p < 0.01$]; both females and males consumed more total volume at 0.066% than 0.033%, though females consumed more total volume at both concentrations than males (Table 4.4).

While there were no differences between the *Chrm5*-B6 genotypes in mean QUIN consumed, there was an interaction of concentration and sex [$F_{(1, 34)} = 5.65$, $p < 0.05$]; both males and females consumed more QUIN at the 0.03 mM than at 0.015 mM, though females consumed more QUIN than males at both concentrations (Figure 4.6B). For QUIN preference ratio, there was a three-way interaction of concentration, genotype, and sex [$F_{(1, 34)} = 4.85$, $p < 0.05$], which was broken down by looking at *Chrm5*-B6 genotype x concentration within each sex. Simple main effects analyses revealed only a main effect of concentration in male mice, where males of both genotypes preferred the 0.015 mM concentration of QUIN over 0.03 mM (Table 4.4). A repeated measures ANOVA for total volume (concentration x genotype x sex) revealed main effects of sex [$F_{(1, 34)} = 28.2$, $p < 0.001$] and concentration [$F_{(1, 34)} = 9.7$, $p < 0.01$]. Animals consumed more total volume at the 0.03 mM than the 0.015 mM, and females consumed more total volume than males, although there were no differences between the *Chrm5*-B6 genotypes (Table 4.4).

Table 4.4. Preference ratio and total volume values for tastants in *Chrm5*-B6 (Experiment 4.4) KO and WT mice.

	Preference Ratio ((mls tastant)/(mls tastant + mls H2O))	
	SACC	
	0.033%	**0.066%**
Chrm5-B6 KO females	0.87 ± 0.05	0.90 ± 0.05
Chrm5-B6 KO males	0.90 ± 0.04	0.93 ± 0.04
Chrm5-B6 WT females	0.91 ± 0.05	0.94 ± 0.04
Chrm5-B6 WT males	0.96 ± 0.01	0.89 ± 0.09
	QUIN	
	0.015 mM	**0.03 mM**
Chrm5-B6 KO females	0.41 ± 0.05	0.49 ± 0.05
Chrm5-B6 KO males	0.46 ± 0.04	0.35 ± 0.05
Chrm5-B6 WT females	0.54 ± 0.12	0.40 ± 0.09
Chrm5-B6 WT males	0.46 ± 0.03	0.40 ± 0.06
	KCl	
	100 mM	**200 mM**
Chrm5-B6 KO females	0.75 ± 0.05	0.54 ± 0.05
Chrm5-B6 KO males	0.63 ± 0.04	0.40 ± 0.05
Chrm5-B6 WT females	0.66 ± 0.10	0.51 ± 0.08
Chrm5-B6 WT males	0.53 ± 0.05	0.38 ± 0.05
	Total Volume (mls tastant + mls H2O)	
	SACC	
	0.033%	**0.066%**
Chrm5-B6 KO females	7.64 ± 0.41	10.0 ± 0.73
Chrm5-B6 KO males	5.87 ± 0.39	6.97 ± 0.55
Chrm5-B6 WT females	8.69 ± 0.32	11.2 ± 0.53
Chrm5-B6 WT males	5.38 ± 0.27	6.18 ± 0.36
	QUIN	
	0.015 mM	**0.03 mM**
Chrm5-B6 KO females	6.06 ± 0.25	6.68 ± 0.52
Chrm5-B6 KO males	4.91 ± 0.24	5.12 ± 0.35
Chrm5-B6 WT females	5.93 ± 0.49	6.73 ± 0.31
Chrm5-B6 WT males	4.45 ± 0.17	4.76 ± 0.16
	KCl	
	100 mM	**200 mM**
Chrm5-B6 KO females	6.90 ± 0.44	10.0 ± 0.80
Chrm5-B6 KO males	5.57 ± 0.30	9.16 ± 0.77
Chrm5-B6 WT females	7.75 ± 0.63	11.5 ± 1.27
Chrm5-B6 WT males	4.86 ± 0.20	8.29 ± 0.69

For KCl, a repeated measures ANOVA (concentration x genotype x sex) revealed a two-way interaction of concentration and sex [$F_{(1, 33)} = 4.77$, $p < 0.05$]. Both females and males consumed more KCl at the 200 mM concentration than 100 mM (Figure 4.6C). Furthermore, females consumed more KCl than males at both concentrations. There were no differences, however, between the *Chrm5*-B6 genotypes in KCl consumption. For preference ratio, there were main effects of both sex [$F_{(1, 33)} = 7.45$, $p < 0.01$] and concentration [$F_{(1, 33)} = 25.2$, $p < 0.001$]. Animals preferred the 100 mM concentration of KCl over 200 mM, and females had higher preference than males (Table 4.4). *Chrm5*-B6 mice slightly preferred the 100 mM KCl concentration, with preference ratios ranging from 59 – 69%. For KCl total volume, there were main effects of both sex [$F_{(1, 33)} = 13.1$, $p < 0.001$] and concentration [$F_{(1, 33)} = 77.6$, $p < 0.001$]. Animals consumed more total volume at the 200 mM concentration than 100 mM, and females consumed more total volume than males (Table 4.4).

Experiment 4.5: Chrm4-D2 KO and WT mice exhibit similar sensitivity to the stimulant effects of ethanol.

After saline injection on Days 1 and 2, males had higher levels of locomotor activity than females. However, there were no differences among preassigned dose groups on either Day 1 or Day 2. Activity levels were higher on Day 1 than Day 2 (3880.0 ± 105.6 cm vs. 3498.9 ± 121.5 cm), indicating habituation.

A factorial ANOVA (genotype x dose x sex) on Day 3 – Day 2 difference score data compiled over the 15 min test revealed only a significant main effect of dose [$F_{(2, 123)} = 5.65$, $p < 0.01$]. Newman-Keuls post-hoc analysis for data collapsed on genotype and sex indicated that locomotor activity was stimulated by the 1.5 g/kg dose of ethanol as compared to both the 1 and 2 g/kg doses (Figure 4.7). There were no significant effects of genotype or of sex on BEC, although BEC values significantly increased in a dose-dependent fashion ($F_{(2, 119)} = 60.8$, $p < 0.001$) (Table 4.5).

Experiment 4.6: Chrm5-D2 KO and WT mice show different locomotor responses to ethanol.

After saline injection on Day 1, there was a significant main effect of sex [$F_{(1, 143)} = 4.78$, $p < 0.05$]; males were more active than females, but there was no difference between the genotypes. On Day 2, there was a main effect of pre-assigned dose [$F_{(2, 143)} = 4.27$, $p < 0.05$]; animals scheduled to receive 1.5 g/kg ethanol were slightly more active (4082.1 ± 185.8 cm) than animals slated to receive the other ethanol doses (3271.0 ± 198.8 cm and 3636.4 ± 185.8 cm for the 1.0 and 2.0 g/kg dose groups, respectively). Locomotor values decreased

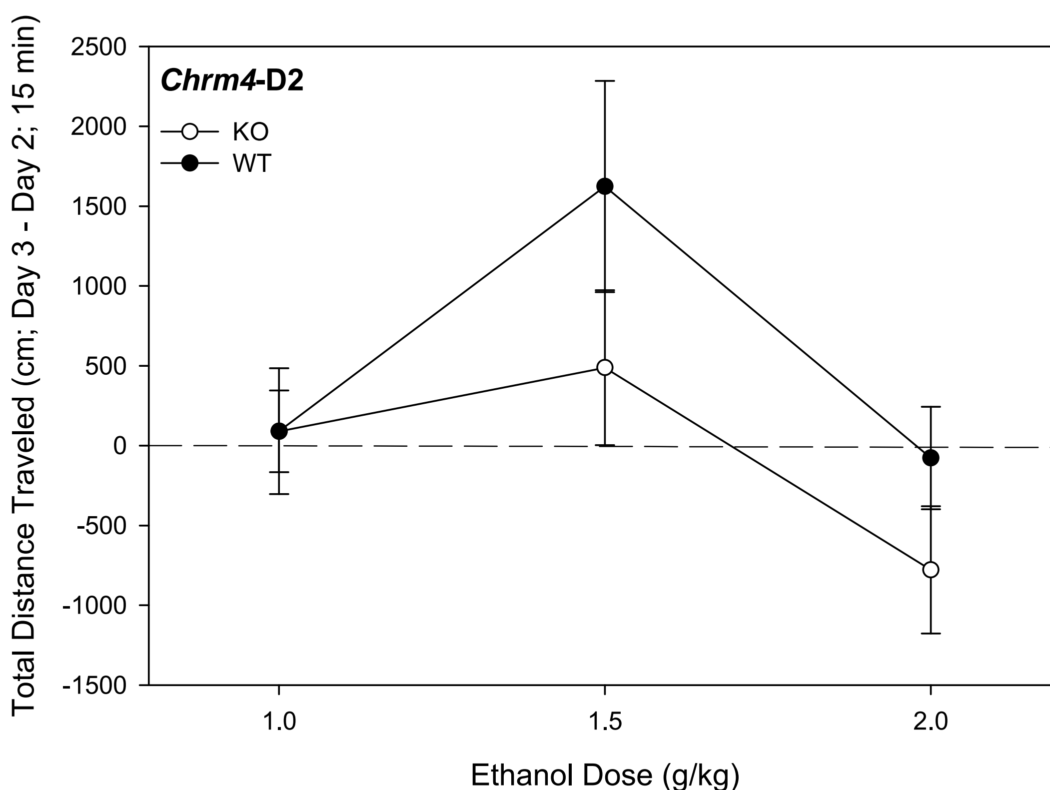

Figure 4.7. *Chrm4*-D2 mice of both genotypes are stimulated to a moderate dose of ethanol. Shown are data for total distance traveled (cm) on Day 3 corrected for Day 2 baseline activity for 15 min. Data above the dotted line indicate a stimulant response after correction for basal activity, while data below the solid line indicate net sedation. n = 21 – 24 mice/genotype/dose, N = 135. Mice were 78 ± 1 days old.

Table 4.5. Experiment 4.5 and 4.6 mean (± SEM) BEC values (mg/ml) 15 min after ethanol injection.

	Ethanol Dose (g/kg)		
	1.0	**1.5**	**2.0**
***Chrm4*-D2 KO**	1.22 ± 0.1	1.59 ± 0.1	2.18 ± 0.1
***Chrm4*-D2 WT**	1.21 ± 0.1	1.56 ± 0.1	2.31 ± 0.1
***Chrm5*-D2 KO**	1.17 ± 0.1	1.70 ± 0.1	2.30 ± 0.1
***Chrm5*-D2 WT**	1.18 ± 0.1	1.66 ± 0.1	2.17 ± 0.1

from Day 1 to Day 2 (4100.1 ± 115.7 cm vs. 3655.2 ± 112.5 cm), indicating habituation.

A factorial ANOVA (genotype x dose x sex) on Day 3 – Day 2 data over the total 15 min test revealed a main effect of genotype [$F_{(1, 143)} = 16.7$, $p < 0.001$] and sex [$F_{(1, 143)} = 6.55$, $p < 0.05$]. The *Chrm5*-D2 KO mice exhibited locomotor depression after ethanol treatment, while the *Chrm5*-D2 WT mice were stimulated by ethanol (Figure 4.8). Also, females displayed more activation than males ($F_{(1, 143)} = 6.55$, $p < 0.05$). There was no effect of genotype on BEC, although BEC values significantly increased in a dose-dependent fashion ($F_{(2, 142)} = 66.8$, $p < 0.001$) (Table 4.5).

Discussion

We originally hypothesized that mice lacking the *Chrm4* receptor gene would display an enhanced stimulant response to ethanol as compared to WT controls, while *Chrm5* KO mice would show a reduced stimulant response as compared to WT controls. In the current experiments, we found that KO mice of both genotypes on the predominantly B6 background only showed sedation to ethanol, as did their WT counterparts. However, *Chrm4* mice backcrossed for 2 generations onto the more ethanol-stimulant sensitive D2 strain showed stimulation to ethanol at a moderate dose, but there was no difference in stimulant response between *Chrm4*-D2 KO and WT mice. Mice lacking the m5 receptor gene on the DBA/2J background lacked a stimulant response to

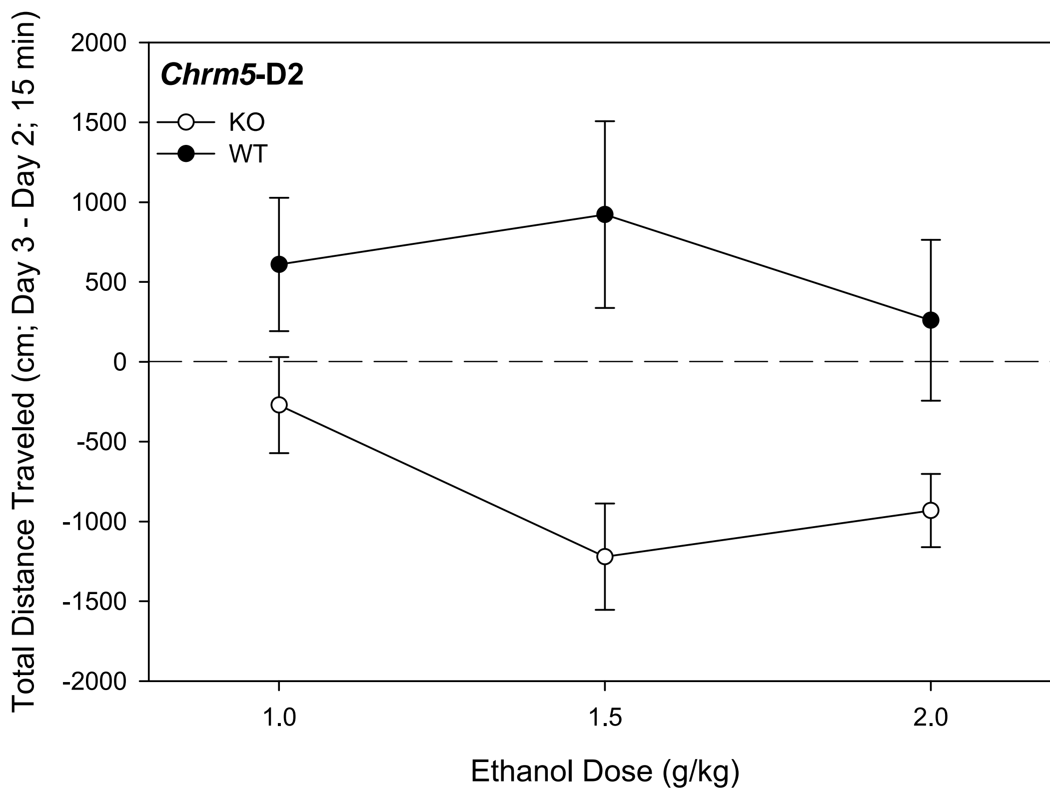

Figure 4.8. *Chrm5*-D2 KO mice, unlike WT mice, do not display stimulation to ethanol. Shown are data for total distance traveled (cm) on Day 3 corrected for Day 2 baseline activity for 15 min. Data above the dotted line indicate a stimulant response after correction for basal activity, while data below the solid line indicate net sedation. n = 24 – 27 mice/genotype/dose, N = 155. Mice were 73 ± 1 days old.

ethanol, while their WT counterparts were stimulated by ethanol. There were no differences between KO and WT mice in ethanol consumption for either mAChR gene. Therefore, it appears that the *Chrm5*, but not *Chrm4*, gene is involved in the stimulant response to ethanol, and that neither gene has a role in ethanol consumption.

In the original descriptions of the *Chrm4* (Gomeza et al., 1999) and *Chrm5* (Yamada et al., 2001) KO mice, 129SvEv (TC1) stem cells with targeted disruption of the m4 or m5 gene were implanted in B6 blastocysts. Male B6 were then mated with outbred CF-1 females, and carried out for 2 to 3 filial generations for testing. The C57BL/6Tac has been used as the WT for comparison to the KO on the mixed C57BL/6Tac/CF-1/129SvEv, which have been maintained as homozygous breeding pairs. Using C57BL/6Tac as the WT mouse is not an ideal control, as these mice lack the flanked passenger region imparted by the ES cells, and will not experience genetic drift to the same extent as the separate homozygous KO line (Wolfer et al., 2002). It is convention in our lab to maintain heterozygote breeders, as this ensures that non-linked alleles remain in about the same frequency among the genotypes (Palmer et al., 2003).

It is well-established in the literature that mice of different inbred strains differ in sensitivity to the effects of ethanol on locomotor activity (Crabbe, 1983; Crabbe, 1986; Dudek et al., 1991; Kiianmaa et al., 1983; Tabakoff and Kiianmaa, 1982). D2 mice display robust stimulation to increasing doses of ethanol, while B6 mice remain largely unactivated, or even sedated to the same doses of ethanol (Crabbe et al., 1980; Dudek et al., 1991; Lister, 1987). B6 mice do not show activation to ethanol (Dudek and Tritto, 1994). As we saw, the mice on the B6 background only showed sedation to ethanol. Therefore, we backcrossed the *Chrm4* and *Chrm5* KO lines for 2 generations onto the more ethanol stimulation permissive D2 background in order to more accurately determine if these genes influenced the stimulant effects of ethanol. This approach has been previously

used to study the effect of the DA D2 receptor gene on ethanol-induced conditioned place preference (Cunningham et al., 2000). CF-1 mice showed robust locomotor activation to both amphetamine and scopolamine (Wenger, 1989). Backcrossing these mice enabled us to reveal that the *Chrm5* gene is involved in acute locomotor stimulation to ethanol, as mice lacking this gene were insensitive to the stimulant effects of ethanol, whereas WT mice exhibited some stimulation. However, there is a lack of published ethanol locomotor data on 129S6/SvEvTac mice. As in the original paper, we also found that *Chrm4*-B6 KO mice displayed enhanced basal locomotor activity (Gomeza et al., 1999). There were no differences in basal locomotor activity in *Chrm5* mice in the original description, nor in our study (Yamada et al., 2001).

We, along with others, have previously found a QTL for ethanol preference on Chromosome 2 in the same region as the acute locomotor stimulation QTL, suggesting that the *Chrm4* and/or *Chrm5* gene may be involved in ethanol consumption (Fehr et al., 2005; Phillips et al., 1998). C57BL/6J mice are among the inbred strains that strongly prefer ethanol over water, consuming in excess of 10 g/kg/day, with preferences close to 80%, when offered a 10% ethanol solution (Yoneyama et al., 2008). In contrast, 129S1/SvlmJ mice (the inbred strain from which the ES cells used to create the KO mice came) display preference ratios around 13% for a 10% ethanol solution (Yoneyama et al., 2008). There were no significant differences between the *Chrm4* KO and WT mice in our study, and their preferences ranged from 16 (20% ethanol) - 33% (6% ethanol), with mean g/kg/day values ranging from 1.6 (3% ethanol) – 6.9

g/kg/day (20% ethanol). Likewise, there were no significant differences between the *Chrm5* KO and WT mice, and their preference ratios ranged from 19 (20% ethanol) to 33% (6 and 10% ethanol), and they consumed 1.7 (3% ethanol) and 7.3 g/kg/day (20% ethanol). It is possible that the C57BL/6NTac background consumes less ethanol than the C57BL/6J substrain, which may have contributed to the lower than expected levels of ethanol consumption and preference that we saw in the WT mice of the current study. Mulligan et al. (2008) found significant differences in ethanol consumption and preference between C57BL/6J and C57BL/6NCrl, with the B6J mice exhibiting greater consumption and preference than the B6NCrl. The C57BL/6N substrain is the original substrain from which the C57BL/6NTac mice diverged, which may partially explain the lower ethanol drinking values. Likewise, Bryant et al. (2008) found that C57/BL6Tac are more uncoordinated than C57BL/6J, though Bothe et al. (2004) did not find differences between these substrains. A lack of published data on ethanol drinking in CF-1 mice makes it impossible to explore the potential contribution of that genotype in our current findings. A similar outcome was obtained for consumption and preference for novel tasting solution (tastants) as was found for ethanol. Although there was a statistical trend towards a sex by genotype interaction in *Chrm5*-B6 KO and WT mice, this did not interaction with concentration. Therefore, there was a tendency for overall greater ethanol intake in female WT than female KO, an effect not seen in males and opposite to our prediction.

The distal and proximal limits of the original ethanol stimulation QTL on Chromosome 2 are unknown; this QTL has not been fine-mapped and the relevant gene(s) is best estimated to reside in the 50 – 148 Mb segment, which contains over a thousand genes. It is possible that there are other genes in the QTL region that influence the response to ethanol besides *Chrm5*. One potential candidate gene near the QTL peak at 54 Mb that may influence the valence of ethanol's locomotor effects is the gene encoding for the subunit 3 of the G-protein gated inwardly-rectifying K+ channel (GIRK) (*Kcnj3*; Chromosome 2, 55 Mb) (Federici et al., 2009). GIRK mutant mice lacking the subunit 2 have been investigated for their role in ethanol's effects (Blednov et al., 2001). Acute locomotor stimulation to ethanol is a complex trait with multiple genetic influences. The data presented here indicate that the m5 mAChR subtype is involved in the locomotor stimulant effects of ethanol, as has been found for other stimulant drugs.

CHAPTER 5: General Discussion

The goals of this dissertation were to investigate the muscarinic acetylcholinergic-mediated effects of acute locomotor stimulation to ethanol, and to specifically investigate the role of the previously implicated m4 and m5 mAChR subtype genes in this response. To accomplish these aims, we utilized the FAST and SLOW selectively bred lines of mice, bred for extreme sensitivity (FAST) and insensitivity (SLOW) to the stimulant effects of ethanol. Data were collected that addressed innate genetic differences between the lines that may predispose them to differential sensitivity to ethanol. In addition, pharmacological investigations were performed in ethanol-treated animals to directly address ethanol's mechanism of action.

In the results described here, we found that SLOW-1 and -2 mice displayed different responses to the combination of scopolamine and ethanol. While both lines were sensitive to the locomotor stimulant effects of scopolamine, only SLOW-1 displayed locomotor depression to ethanol. When combined, scopolamine had no effect on ethanol-induced sedation in SLOW-1 mice. In SLOW-2 mice, stimulation to scopolamine was seen following all but the highest dose of ethanol (Chapter 2). The follow-up rotarod study in SLOW-1 mice revealed that scopolamine enhanced the motor incoordinating effects of ethanol. In FAST mice, it was hypothesized that the synergistic enhancement of locomotor activity following the combination of scopolamine and ethanol seen previously was due to antagonism of the m4 receptor subtype in the NAc (Scibelli

and Phillips, 2009). While microinjection of scopolamine into the NAc enhanced locomotor activity both alone and in combination with ethanol in FAST-2 mice, these effects were additive, rather than synergistic (Chapter 2). Neither scopolamine nor ethanol significantly enhanced locomotor activity in FAST-1 mice.

Potential sequence and gene expression differences between FAST and SLOW mice were examined to determine whether they partially influenced the selection phenotype. There were no consistent polymorphisms found for both sets of replicate FAST and SLOW lines for the *Chrm4* or *Chrm5* genes. There were, however, 2 sequence polymorphism differences between FAST-2 and SLOW-2 for *Chrm5* (Chapter 3). There were no differences in *Chrm4* expression in any region between FAST and SLOW. However, we found greater levels of expression of the *Chrm5* gene in VM tissue from FAST mice as compared to SLOW mice, and SLOW-1 mice had greater expression levels of *Chrm5* in the HIP as compared to FAST-1 mice (Chapter 3). Finally, KO of the m5 receptor subtype in mice on a DBA/2J background attenuated the stimulant response to ethanol as compared to WT mice, while m4 receptor KO did not impact the stimulant response to ethanol. These receptor subtypes did not appear to be significantly involved in ethanol consumption (Chapter 4). Overall, these data suggest that (1) the muscarinic cholinergic system may accentuate some effects of ethanol in FAST and SLOW mice (2) sequence polymorphisms and expression of the m5 receptor gene may underlie some of the phenotypic divergence between FAST and SLOW mice, and (3) the m5 receptor subtype is

necessary for the stimulant response to ethanol. However, these data do not support a critical role for the m4 receptor in acute locomotor stimulation to ethanol, either in the NAc or globally.

Role of mAChR in Effects of Ethanol on Locomotor Activity

Selection for differential sensitivity to the stimulant effects of ethanol in FAST and SLOW mice has also altered alleles involved in sedation. SLOW mice were more sensitive to hypothermia and displayed a longer loss of righting reflex (LORR) than did FAST mice following ethanol injection, effects that diverged along with the selection phenotype (Phillips et al., 2002b; Shen et al., 1996). SLOW mice also showed sensitivity to the depressant effects of a variety of alcohols at varying doses (Phillips et al., 1992; Palmer et al., 2002). We hypothesized that scopolamine, which has stimulant effects on its own, would attenuate ethanol-induced locomotor depression in SLOW mice. We also investigated the combination of scopolamine and ethanol, when scopolamine was microinjected into the NAc of FAST mice. We hypothesized that the robust synergistic locomotor response to this peripherally administered drug combination was a result of activity at m4 receptors in the NAc (Scibelli and Phillips, 2009). Although scopolamine centrally administered did potentiate ethanol's stimulant effects in FAST-2 mice, the response to the drug combination was additive, when responses to the two drugs given alone were considered.

In SLOW mice, the locomotor response to scopolamine, when combined with ethanol, looked identical to that of ethanol alone, despite that scopolamine

has stimulant effects. Further examination of this effect in a rotarod experiment revealed that this was in part due to increased ataxia following the combination of scopolamine and ethanol. While scopolamine alone had no effect on latency to fall off the rod, the combination of scopolamine and 1.5 g/kg ethanol significantly shortened the latency to fall as compared to mice receiving only 1.5 g/kg ethanol (Chapter 2). Although the combination of scopolamine and ethanol reduced the latency to fall on the rotarod task as compared to mice receiving ethanol alone, the combination of scopolamine and ethanol in the locomotor study in SLOW-1 mice did not result in enhanced locomotor depression as compared to the ethanol alone group, as one would expect. It is possible that the rotarod test is more sensitive than the locomotor testing procedure at detecting the combined effect of the drugs on ataxia.

Unlike the SLOW-1 mice, which displayed depression to increasing doses of ethanol, the SLOW-2 mice did not show significant locomotor depression to any dose of ethanol. We have previously seen differences in ethanol depressant sensitivity between SLOW-1 and -2 mice (Holstein et al., 2009; Palmer et al., 2002). This raises the point that the replicate lines are not identical, which will be discussed below. However, SLOW-1 and -2 mice were similar in their sensitivity to scopolamine's stimulant effects, similar to what we have previously seen (Bergstrom et al., 2003). Therefore, the locomotor differences between SLOW-1 and SLOW-2 mice following the combination of scopolamine and ethanol appear to be driven by the replicate lines' differential sensitivity to ethanol rather than scopolamine.

Only recently have investigators begun to examine the role of mAChRs on ethanol effects *in vitro*. Recent data indicate that mAChRs are both inhibited and excited by ethanol. A moderate dose of ethanol depressed the magnitude of neuronal firing in rat striatum, but when ethanol was washed out from the neurons, a prolonged enhancement of firing was seen (Adermark et al., 2011). However, the prolonged enhancement was blocked by scopolamine, suggesting that ethanol excites cholinergic neurons, increasing overall input to GABAergic neurons and decreasing striatal output. Striatal neurons also express nAChR, which may also modulate GABAergic neurons. This modulation of GABA release has been proposed to be influenced by the m4 receptor subtype (Grilli et al., 2009). Another group has shown that ethanol excites cholinergic interneurons (Blomeley et al., 2011). Activity at mAChRs can result in a multitude of effects, especially considering all the neurotransmitter systems upon which ethanol acts (Lovinger, 1997; Morikawa and Morrisett, 2010; Spanagel, 2009).

Within the microcircuitry of the striatum and NAc, there are a variety of neuronal types on which mAChRs (notably the m2 and m4 subtypes) may be expressed. A DA nerve terminal makes contact with other GABAergic medium spiny neurons, GABA projections to the VP, GABA input from the VTA, glutamatergic afferents, as well as both GABA and cholinergic interneurons. There is no anatomical evidence that proves mAChRs are located on dopaminergic axons (Threlfell and Cragg, 2010). However, m2 and m4 mAChR may be expressed on any of the aforementioned GABAergic neurons. A strong possibility, although not the only possibility, is that m2 and m4 receptors promote

DA release as autoreceptors on cholinergic interneurons. Data indicate that while both the m2 and m4 receptor subtypes influence DA release in the striatum, the m4 receptor is the sole inhibitory subtype which modulates DA release in the NAc core (Bonsi et al., 2010; Threlfell and Cragg, 2010). While these data were collected via pharmacological and genetic tools, no anatomical data exist that prove differential projections of cholinergic interneurons expressing m2 and/or m4 receptors to the striatum or NAc. However, the m4 receptor subtype is the most dominantly expressed subtype in the striatum, though most likely majorly on medium spiny neurons (Chapman et al., 2011; Vilaro et al., 1991).

A lack of expression differences for the *Chrm4* gene in FAST and SLOW in striatum makes it hard to interpret how ethanol's effects in the striatum could explain the differential stimulant sensitivity (Chapter 3). Furthermore, the locomotor data in FAST or SLOW mice do not indicate that ethanol excites cholinergic neurons, as scopolamine did not block the effects of ethanol (Chapter 2). Although SLOW mice display DA release in the NAc following ethanol administration, it is significantly less than that of FAST mice (Meyer et al., 2009). It is possible that while there may be no differences in mAChR expression in FAST and SLOW mice in the striatum, mAChR effects on differentially expressed nAChR or GABAergic receptors expressed on dopaminergic neurons result in differential locomotor activation profiles. For example, there are differences between FAST and SLOW mice in whole brain expression of both the α6 and β4

nAChR, although regional analysis has not yet been done (Kamens and Phillips, 2008).

Neurocircuitry

A key component of this dissertation work was to test NAc-specific administration of scopolamine on acute ethanol stimulation in FAST mice. Previous work in our laboratory showed that the combination of scopolamine and ethanol, when peripherally administered, in FAST mice, resulted in a robust locomotor enhancement greater than one would expect from the additive means of scopolamine and ethanol activation alone (Scibelli and Phillips, 2009). It was hypothesized that microinjection of scopolamine into the NAc would enhance ethanol-induced locomotor stimulation in a super-additive fashion, as we suspected the robust locomotor enhancement from peripheral administration of scopolamine and ethanol was influenced by inhibitory muscarinic receptors in the NAc. The NAc is rich in muscarinic autoreceptors, comprised of both the m2 and m4 subtypes. Although we found that scopolamine, when infused into the NAc, did promote locomotor activation, when combined with ethanol the activity was additive rather than synergistic (Chapter 2). There are a variety of possibilities for this outcome: 1.) that the NAc may not be the brain region responsible for the synergism seen following peripheral administration, 2.) that the NAc is the correct region, but the microinjections were not placed in the correct subregion, and 3.) that the effects were modulated via the peripheral nervous system, and not centrally.

The first potential reason why we did not see a synergistic enhancement of ethanol-induced stimulation following scopolamine infusion into the NAc is that this may not be the brain region responsible for the synergism we saw in the peripheral administration. This begs the question: what is the correct brain area? While the NAc is a key nucleus in the circuit of drug reward and locomotor behavior, it is by no means solely responsible for drug-related phenotypes. For example, electrolytic lesioning of the NAc did not attenuate the stimulant response to ethanol in DBA/2J mice, one of the founding strains of the HS/Ibg stock used to create the FAST and SLOW lines, and one that is phenotypically similar to FAST (Gremel and Cunningham, 2008). However, FAST mice displayed greater DA efflux from the NAc than did SLOW following 2 g/kg ethanol (Meyer et al., 2009), indicating a role for DA in the selection response. In FAST and SLOW mice, ethanol-induced DA release in the NAc is genetically correlated with ethanol stimulant sensitivity, although this is only one piece of the circuitry puzzle.

One potential alternative candidate region that may have played a role in our previous work is the VM, which contains the VTA, SN, and interpeduncular nuclei. The VTA is the origin of the mesolimbic DA pathway, associated with reward and motivation, while the SN is the origin of the striatonigral pathway, associated with motor activity. Both regions contain mAChR m5 receptors. The VTA receives input from cholinergic projections stemming from the LDT/PPT, where the LDT preferentially sends projections to mesoaccumbal VTA neurons that excite DA release (Omelchenko and Sesack, 2005). Projections from the

PPT promote DA burst firing in the VTA, which signals salience (Floresco et al., 2003), though the PPT also innervates the SN (Oakman et al., 1995). There are also nicotinic, ionotropic glutamatergic, and GABA receptors in the VTA. It has been suggested that the nicotinic and glutamatergic receptors provoke a fast, transient release of DA in the NAc, while m5 mAChRs maintain a prolonged release (Lester et al., 2008; Yeomans et al., 2001). There are also μ-opioid receptors in the VTA, which disinhibit DA neurons via GABA interneurons (Johnson and North, 1992a; 1992b). Overall, activation of the various receptors expressed in the VTA that influence DA release in the NAc may enhance activity at mAChR as well. Further complicating matters, the VTA is divided into anterior and posterior regions. For example, when baclofen was injected into the anterior VTA, it attenuated the stimulant response to ethanol in FAST mice, but baclofen injected into the posterior VTA potentiated the stimulant response to ethanol in FAST mice (Boehm et al., 2002). These different outcomes are likely due to different afferent and efferent connections within these two regions.

We saw higher expression levels of the m5 gene in the VM (Chapter 3), as well as a lack of ethanol-induced stimulation in *Chrm5* KO mice as compared to their counterpart WT mice (Chapter 4). FAST mice displayed greater basal DA pacemaker firing in the VM as compared to SLOW, as well as greater spontaneous DA firing following ethanol application than SLOW mice (Beckstead and Phillips, 2009). However, the m5 receptor subtype, expressed in the VTA, is excitatory in nature. Therefore, I would hypothesize that microinjection of scopolamine into the VTA would attenuate ethanol stimulation rather than

accentuate it. Intra-VTA infusion of scopolamine reduced DA efflux in the NAc, while intra-SN infusion of scopolamine reduced DA efflux in the striatum (Miller and Blaha, 2005). Electrical stimulation of the LDT along with intra-VTA infusion of scopolamine resulted in attenuated DA efflux in the NAc (Lester et al., 2008), and scopolamine infusion in this manner also blocked cocaine (Lester et al., 2010) and morphine-induced (Miller et al., 2005) DA release from the NAc. These data indicate that scopolamine infusion into the VTA would attenuate ethanol-induced DA release, which may impact the stimulant response to ethanol. However, scopolamine, when administered systemically, resulted in an increase in DA neuron activity in the SN, an effect that was mimicked by local infusion of scopolamine into the PPT (Di Giovanni and Shi, 2009).

It is possible that the VTA projections to other brain regions, such as the amygdala, are driving stimulant sensitivity, and that these projections then impinge back on the NAc. For example, lesions of the amygdala attenuated D2 stimulation to ethanol (Gremel and Cunningham, 2008). mAChR in the basolateral amygdala are necessary for the formation of stimulus-reward drug pairings, but it is unknown what effect these receptors have on acute drug effects (See et al., 2003). Conceptualizing how an antagonist would promote, rather than attenuate, locomotor activity, points towards antagonist action at autoreceptors, of which there are 2 main subtypes in the mAChR family: m2 and m4. However, these two receptor subtypes have inhibitory properties on other neuronal types besides ACh, in which case they act as heteroreceptors rather than autoreceptors. To the best of my knowledge, there are no muscarinic

autoreceptors in the VTA, although there are m2, and maybe m4, autoreceptors in the PPT (Di Giovanni and Shi, 2009; Lester et al., 2008; Levey et al., 1991).

The m2 and m4 mAChR are of the same inhibitory class. They are the only two inhibitory-type mAChR subtypes, coupled to $G_{i/o}$. Some have suggested there are m1 autoreceptors, but data on these are scarce (Bernard et al., 1992; Smythies, 2005). The m2 receptors are widely expressed in the forebrain, striatum, NAc, tegmental areas, thalamus, hypothalamus, pons, and medulla (Lester et al., 2008; Levey et al., 1991; Smythies, 2005). The m4 receptors are expressed in the HIP, cortex, striatum, NAc, olfactory tubercle, and island of Calleja (Levey et al, 1991; Smythies, 2005). It is very possible that the systemic injection of scopolamine could be influenced by any one of these areas, although the data linking the brain reward areas, QTL studies, and existing drug and microinfusion data highlight the mesolimbic DA pathway as the most likely locale for mediating scopolamine’s effects.

Also, with regard to other brain regions influencing the role of ACh in ethanol stimulation, there are cholinergic circuits in other regions than those that would be affected by placement of scopolamine into the NAc. These circuits revolve around the nucleus basalis of Meynert, and the medial septum/diagonal band. There are glutamatergic projections from the LDT/PPT to the nucleus basalis of Meynert, which projects to the cortex. The nucleus basalis also projects to the amygdala and the thalamus (Smythies, 2005). The medial septum/diagonal band projects to the HIP, mPFC, and olfactory tubercle

(Smythies, 2005). Besides the cholinergic interneurons of the striatum and NAc, they are also in the neocortex, amygdala, and olfactory tubercle (Smythies, 2005). While any of these areas may influence reward response and maybe locomotor activity, it seems unlikely that they are driving either the previous or current results, given the data that suggests the mesolimbic DA pathway as the more attractive candidate.

The NAc, although frequently described as a stand-alone nucleus, is the ventral part of the striatum. It is possible that the dorsal striatum is the area truly mediating the synergism we previously saw following peripheral injection of scopolamine (Scibelli and Phillips, 2009). The striatum is very similar in composition to the NAc in relation to expression of m1, m2, and m4 receptors (Bernard et al., 1992; Hersch et al., 1994). Recent data indicate 30% of the striatum contains m1 receptors, while the remaining types are m4 with a slight m2 contribution (Chapman et al., 2011). The striatum is relatively large, with functionally distinct regions that have differential circuits. For example, the dorsal striatum is involved in habit learning and sensory motor function (Everitt and Robbins, 2005; Sesack and Grace, 2010; Threlfell and Cragg, 2011). However, looking at striatal “misses” (attempted NAc hits that were too dorsal) did not reveal any synergistic effects (data not shown). Furthermore, there were no differences in *Chrm4* gene expression levels between FAST and SLOW mice in the striatum (Chapter 3). It is also possible that the synergistic effect could be due to a combination of mAChR in the striatum and the NAc, although again, the “misses” did not indicate this. Overall, it is hard to imagine a more attractive

candidate for target than the mesolimbic DA pathway in mediating the synergistic effects seen previously, although these alternatives are hard to disprove without experimental data.

With regard to the second possible alternative explanation for the microinfusion results, that the NAc is the region responsible for the synergistic effect of the scopolamine and ethanol combination, as the striatum is functionally distinct, so is the NAc. The NAc is comprised of at least two regions: the core and the shell (the rostral pole is also designated) (Zahm and Brog, 1992). The NAc core projects to the dorsolateral VP and the SN-pr, while the NAc shell projects to the ventromedial VP, SN-pc, VTA, and PPT (Sesack and Grace, 2010). Also, the NAc shell sends projections to the VTA, which in turn influence DAergic neurons that impact the NAc core (Sesack and Grace, 2010). Based on circuitry differences and experimental data, some have suggested that the shell is involved in primary reinforcement, while the core is involved in conditioned reinforcement (Crespo et al., 2006; Everitt and Robbins, 2005). Both amphetamine and DA infusion into either the core or shell elicited locomotor activation, although only cocaine infusion into the shell resulted in activation (Ikemoto, 2002). However, Ikemoto (2002) concluded that both the core and the shell are important for locomotor activity, relative to drug profile. Animals will self-administer drugs into both the shell and core, indicating that both areas influence reward (Ikemoto and Sharpe, 2001; Mark et al., 2006). Also, infusion of scopolamine into either the shell or the core attenuated cocaine-primed reinstatement, although scopolamine infusion into the core also attenuated

sucrose reinstatement (Yee et al., 2011). Therefore, the shell may be more specific to drug reward, while the core may be a more general role in motor and motivation. We were aiming for the “shore,” as it is debatable whether one of those specific regions could be targeted in the mouse without drug diffusing to the other area (GP Mark, personal communication). We wished to obtain gene expression results for the NAc in FAST and SLOW mice, but technical issues with RNA quality prevented us from attaining that aim.

Finally, alternative explanation number 3 is that the synergistic effects could be solely peripherally mediated. Scopolamine is a non-selective antagonist, and there are mAChR in virtually all brain and tissue types. The m1, m4, and m5 receptors are predominantly expressed in the central nervous system, while the m2 and m3 subtypes are represented in both the central nervous system and the periphery (Wess et al., 2007). Generally, mAChRs in the brain underlie cognition as well as drug reward, while in the periphery, they modulate heart rate, vasodilation, and smooth muscle control (Wess et al. 2007). However, muscarinic antagonism, as with scopolamine, would reduce smooth muscle activity. ACh causes a slowing of the heart rate and stimulates smooth muscle contraction (Eglen, 2005). Muscarinic agonists have analgesic effects (Gomeza et al., 1999; Wess et al., 2007). In mice, scopolamine pretreatment elevated respiration (Collins et al., 1990). So, while scopolamine could potentially raise heart rate and respiration, it would reduce smooth muscle activation. As peripheral mAChR are expressed widely throughout the body, it is hard to say how these peripheral effects of scopolamine would impact locomotor activity.

As scopolamine has equal affinity for all five mAChR subtypes (Hammer et al., 1980), and selective pharmacological agents do not exist with certainty, investigators have recently focused on the development of allosteric modulators. These agonist drugs bind to allosteric sites that are distinct from classic orthosteric binding sites, but that are able to influence the binding properties of the orthosteric site (Stahl and Ellis, 2010). These have been described for the m1 (Davis et al., 2009; Ma et al., 2009), m4 (Brady et al., 2008; Leach et al., 2010), and m5 (Bridges et al., 2009; Stahl and Ellis, 2010) mAChR subtypes. These drugs are promising, although they only function to enhance mAChR binding, and have not been widely characterized in behavioral models. There are some muscarinic antagonists with relatively selective action at m1 and m4 receptors, but they are derived from snake venom. These drugs are cost-prohibitive, challenging to work with, and are not well characterized *in vivo*. The lack of selective pharmacological tools has necessitated a genetic approach towards elucidating the roles of mAChR in behavior.

Genetic Differences of mAChR in FAST and SLOW Mice

A previously mapped QTL on Chromosome 2 for acute locomotor stimulation to ethanol contains within the implicated region the genes for the m4 and m5 mAChR subtypes (Demarest et al., 1999b; Matsui et al., 1999; Palmer et al., 2006). While there are over a thousand genes located in this QTL region on Chromosome 2, the m4 and m5 receptor subtype genes are near the peak of the QTL (54 Mb), increasing the likelihood to some degree (albeit cautiously) that

they underlie the locus for this quantitative trait. Also, as has been discussed, the m4 and m5 receptor subtypes are expressed in regions known to play a role in reward and reinforcement.

The *Chrm4* and *Chrm5* genes were sequenced in FAST-1, SLOW-1, FAST-2, and SLOW-2 mice in an effort to determine whether sequence differences in these genes could lead to altered forms of m4 and/or m5 receptor protein, and thus the potential for altered function in sensitivity to ethanol's stimulant effects. Also, because these lines were derived from an 8-way inbred strain cross, it would be beneficial for future molecular biology experiments to know if the *Chrm4* and *Chrm5* sequences were different compared to the commonly used B6 reference strain (which was one of the strains in the 8-way cross).

There were no consistent sequence differences between FAST and SLOW mice for *Chrm4*. There was one synonymous SNP between B6 and FAST/SLOW in the coding region. Three nucleotides varied from the B6 reference in the 3' untranslated region. For two of these, the variation was the same in all FAST and SLOW samples. For the third, one FAST-1 sample was heterozygous, while the other 14 matched the B6 allele. As the SNP was synonymous, this sequence difference did not result in different protein products between B6 and FAST/SLOW. Furthermore, FAST and SLOW did not differ from one another for any SNP measured in the *Chrm4* gene. These negative SNP data, combined with the lack of differentially expressed *Chrm4* gene expression

levels between FAST and SLOW mice in any region tested, indicates no variation between FAST and SLOW for the *Chrm4* gene.

For *Chrm5*, there were no differences between the B6 sequence and that of FAST/SLOW mice in the 3' or 5' UTR. However, there were three SNPs between B6 and FAST/SLOW in the coding region. For the first non-synonymous SNP, all 16 FAST/SLOW mice differed from B6, although FAST and SLOW did not differ from each other at all. For the remaining two SNPs, the majority of SLOW-1, FAST-1 and SLOW-2 matched the B6 sequence, although 2 SLOW-1 mice were heterozygous. However, all 4 FAST-2 were different from B6 as well as SLOW-2 mice for the 2 remaining non-synonymous SNPs. These two non-synonymous SNPs result in different amino acids within the *Chrm5* sequence, and could possibly underlie some of the differential sensitivity to ethanol between FAST-2 and SLOW-2 mice. It is unknown whether these SNPs have functional implications or not. However, the alteration of amino acids within a protein may render the protein or receptor non-functional. The divergent response to ethanol in FAST-2 and SLOW-2 mice suggests that the SNPs may have functional consequences. These SNPs are not required for the divergent response to ethanol as they are not present in the replicate-1 line. However, the replicate lines have almost certainly arrived at their differential sensitivity to ethanol via different genetic mechanisms. It is intriguing that the sequence differences in the *Chrm5* gene were found in replicate-2, the same replicate that showed differential sensitivity to the locomotor stimulant effects of scopolamine (Bergstrom et al., 2003). The promoter regions of the *Chrm4* and *Chrm5* genes

have not yet been sequenced, but that work is in progress. As others have shown that sequence polymorphisms in the promoter region may have functional implications, we are eager to obtain those results (Barr et al., 2009; Hansson et al., 2006; Mexal et al., 2007).

The gene expression data in Chapter 3 indicate that there are basal expression differences between FAST and SLOW for the *Chrm5* gene in the VM. The VM region consists of the VTA, SN, and interpeduncular nuclei. These data are intriguing for several reasons. First, the VTA is the origin of the mesolimbic DA pathway, and the SN, the origin of the nigrostriatal motor pathway (Omelchenko and Sesack, 2006). The m5 receptor subtype is the least expressed of all the mAChRs in the brain, but is known to reside in these areas in greater numbers than any of the other subtypes (Weiner et al., 1990). Secondly, blockade of muscarinic receptors in the VTA (presumably m5) blocked the enhancement of extracellular DA levels in the NAc following morphine (Miller et al., 2005). Muscarinic receptors in the VTA modulate DA release in the NAc, and those in the SN modulate DA release in the striatum (Miller and Blaha, 2005). Third, we have previously shown that FAST mice had greater pacemaker firing of DA cells in the SN than did SLOW mice (Beckstead and Phillips, 2009). These data point to the *Chrm5* in the VM as a seat of drug reward and a potential candidate underlying differences between FAST and SLOW. Additionally, in Chapter 4 we found that KO of the m5 receptor subtype gene abolished the stimulant response to ethanol seen in WT mice.

We also found that SLOW-1 mice had greater expression levels of *Chrm5* in the HIP than FAST-1 mice, although this difference was not present in replicate-2 mice. This result is in the opposite direction to what was hypothesized. We lack published data on m5 expression in the hippocampus, but *in situ* hybridization figures for *Chrm5* in the Allen Brain Atlas clearly show staining for *Chrm5* along the CA1 region (www.mouse.brain-map.org). Recently published data indicated that mAChR (M1-type) activation reduced small conductance calcium-activated potassium channel sensitivity, increasing synaptic potentials in the HIP, and promoting long-term potentiation (Buchanan et al., 2010; Giessel and Sabatini, 2010). While we are interested in acute effects of ethanol, the literature has indicated that muscarinic antagonism in the HIP prevents acquisition of learning about stimulus-reward pairings (Crespo et al., 2006; Klinkenberg and Blokland, 2010; See et al., 2003; Sharf et al., 2006). SLOW mice developed conditioned taste aversion to saccharin following pairing with ethanol more readily than FAST mice, indicating no differences in learning ability along with *Chrm5* expression differences. However, the line difference for conditioned taste aversion appeared to be driven by SLOW-2 animals (Risinger et al., 1994), whereas the greater expression of *Chrm5* in the HIP was seen only in SLOW-1 mice, vs. FAST-1.

Gene expression levels do not necessarily indicate differences in receptor protein amounts. Unfortunately, we were unable to pursue follow-up Western blot analyses because of unreliable antibodies for mAChRs (Jositsch et al., 2008). The close homology of the mAChR subtypes has hampered the

development of subtype-selective pharmacological agents as well as precise subtype-selective antibodies. We also took the NAc, olfactory tubercle, and the cerebellum for gene expression analysis, but a lack of quality RNA made these measurements impossible. The original goal was to use the Leica laser-capture dissecting microscope to obtain these regions, but that equipment was unavailable at the time these experiments were performed.

FAST and SLOW Mice as a Genetic Model of Acute Locomotor Stimulation to Ethanol

We have previously shown that both FAST and SLOW mice were sensitive to the stimulant effects of peripherally administered scopolamine (Bergstrom et al., 2003). In that study, FAST-2 and SLOW-2 mice significantly differed in magnitude of stimulation to scopolamine (with FAST-2 mice displaying greater activity than SLOW-2); FAST-1 and SLOW-1 displayed equivalent stimulation to the drug. One may hypothesize that if the muscarinic cholinergic system somehow underlies the divergence in stimulation to ethanol in FAST and SLOW, that only FAST would show a stimulant response to scopolamine, or that this mechanism may only be relevant in replicate-2. However, it may be true that receptor sensitivity under normal conditions is mechanistically distinct from how those receptors respond following ethanol administration. That appears to be true when examining the data collected in Chapter 2. Moreover, we found evidence that FAST and SLOW mice differed in basal expression of the *Chrm5* gene, at least in an area (the VM) known to impact drug stimulant sensitivity

(Oakman et al., 1995; Lester et al., 2008; Zhou et al., 2003). While there was no main effect or interaction of replicate line, and FAST-2 mice had greater expression of *Chrm5* than SLOW-2, the difference between FAST-1 and SLOW-1 appeared to driving the differential expression in the VM.

There are examples in this dissertation where the replicate lines are not identical. SLOW-1 and -2 mice were differentially sensitive to the sedative effects of ethanol, and FAST-1 and -2 mice were differentially sensitive to the stimulant effects of ethanol (Chapter 2), only replicate-2 had non-synonymous SNP sequences for the *Chrm5* gene (Chapter 3), and only one set of replicate lines displayed different *Chrm5* levels in the HIP (replicate-1) (Chapter 3). When only one pair of replicate lines displays a response difference, it is considered weak to moderate evidence for a genetic correlation between the phenotype and the original selection phenotype (Crabbe et al., 1990). However, there are a variety of reasons why one may see these effects.

Selected lines are not identical; the ability to selectively breed depends on starting with a genetically diverse starting population. These individual genetic differences are a source of allele frequency differences in pairs of selected lines. In addition, theoretically, alleles relevant to the selection phenotype become fixed, while trait-irrelevant alleles remain segregating at the original frequency. However, it is certain that some trait-irrelevant alleles become fixed, which may impact the response of the line. Those that become fixed due to inbreeding of this nature may be different in the different lines. The rigorous approach of

creating replicate lines is that one is able to determine whether a phenotype or response is due to chance fixation, or truly an effect of selection (Crabbe et al., 1990; Henderson, 1989; 1997). Furthermore, the same trait may be affected by different genes, different biochemical mechanisms, or a combination of the two (Crabbe et al., 1990). This seems likely in the current work, as we saw both replicate-dependent gene expression differences as well as sequence differences, though these were not consistently within only one replicate set of lines (i.e. sequence differences for *Chrm5* were seen between FAST-2 and SLOW-2, while gene expression differences for *Chrm5* in the HIP were seen between FAST-1 and SLOW-1, and *Chrm5* differed between both replicate lines). Also, the time course of selection response may be different in the replicate lines. However, as selection was relaxed in G_{37}, this is most likely not a possibility in the current work. Some relevant genes may have been lost in one set of lines, given the relatively small closed breeding populations used. In a way, it seems appropriate that the FAST and SLOW replicate lines are not identical, as this more adequately models the human condition.

mAChR Gene Deletion and Ethanol Phenotypes

While the m4 and m5 KO and WT mice on the predominantly B6 background displayed only sedation to ethanol, backcrossing these mice for 2 generations to the more ethanol stimulant-sensitive D2 line revealed the importance of the m5 subtype in ethanol-induced stimulation. Mice lacking the m5 receptor on the D2 background did not show stimulation to ethanol, while the

m5 WT did. There were no differences between the m4 KO and WT mice; both m4 KO and WT displayed stimulation to the moderate dose of ethanol (1.5 g/kg).

The mixed background of these mice [C57BL/6Tac x CF-1 x 129SvEv or C57BL/6Tac x CF-1 x 129SvEv x DBA/2J] makes it challenging to truly determine if the results seen in the *Chrm5*-D2 KO mice are due to a lack of that receptor, or a background effect of one of the other genetic strain contributions. Furthermore, in their home colony (not in Portland), these mice are maintained as homozygous breeding pairs, with the “WT” mice as separately housed C57BL/6Tac mice. Hence, these mice are not maintained as littermates, nor do the WT contain the flanked region from the ES cells (Wolfer et al., 2002). In our hands, they were set up as heterozygous breeding pairs to reduce the potential impact of use of non-littermates, systematically different maternal environments (KO vs. WT dam), and poorly matched background genotype. As littermates were used, the genetic background for all genes except the mutation and genes linked to the mutation should be well-matched.

Our lab and others have previously found a QTL for ethanol consumption on Chromosome 2 from 50 – 106 Mb that partially overlaps with the ethanol-induced stimulation QTL (Fehr et al., 2005; Phillips et al., 1998; Tarantino et al., 1998). The *Collaborative Study on the Genetics of Alcoholism* indicated that polymorphisms in the *CHRM2* (human m2 mAChR subtype gene) were associated with alcohol dependence (Dick et al., 2006). While we did not directly investigate this receptor subtype in our targeted approach in mice, this

receptor subtype functions as an inhibitory autoreceptor in the striatum, much like m4. However, the m2 receptor subtype gene is not nearly as widely expressed in the brain as is the m4 receptor subtype. As previously mentioned, the m2 receptor subtype has been shown *in vitro* to modulate probability of DA release in the striatum (Threlfell et al., 2010). Follow-up studies in humans have been conflicting as to the precise role of this muscarinic subtype in alcohol dependence. It has been associated with a "general externalizing phenotype" – a measure including alcohol dependence as well as conduct disorder and antisocial personality disorder (Dick et al., 2008). Another study found no association with *CHRM2* SNPs and alcohol dependence after performing statistical correction for multiple comparisons (Jung et al., 2011). Mice lacking the m2 receptor did not differ in ethanol consumption as compared to m2 WT mice (J. Wess, personal communication). We investigated m4 and m5 receptor KO and WT mice for differences in ethanol consumption, but found none.

Summary and Conclusions

We took a partial candidate gene approach to assess whether the muscarinic acetylcholinergic system differed between FAST and SLOW mice, potentially underlying their differential stimulant sensitivity to ethanol. Preliminary data for this research question came from consideration of the literature for the effects of mAChR on relevant neurochemical systems and from a QTL study implicating a large region on Chromosome 2 in acute locomotor stimulation to ethanol (Demarest et al., 1999; Palmer et al., 2006). The *Chrm4* and *Chrm5*

genes reside within this region (Matsui et al., 1999). However, there are over 1000 genes within this region. What made these two receptor genes attractive for study was their areas of expression in the brain, as well as the fact that they have remained understudied because of the lack of selective agonists and antagonists. We had some preliminary behavioral data that indicated these genes were worthwhile to pursue (Bergstrom et al., 2003; Scibelli and Phillips, 2009). However, the lack of additional data made this investigation truly uncharted.

Overall, the data presented here indicate that the *Chrm4* gene is not a candidate gene for acute locomotor stimulation to ethanol. While both FAST and SLOW mice were sensitive to the stimulant effects of scopolamine, there did not appear to be any mAChR-influenced consequences to the respective ethanol locomotor phenotypes in these mice. Scopolamine did appear to enhance ethanol's ataxic properties in SLOW-1 mice, but further studies of ataxia would be needed to truly know if scopolamine was enhancing ataxia or initiating a competing behavior. Only FAST-2 mice were sensitive to the stimulant effects of scopolamine when injected intraaccumbally. FAST-1 mice were also not stimulated to the sub-threshold dose of ethanol, although this may have been due to the high basal activity group. In FAST-2 mice, the combination of scopolamine and ethanol was merely additive, and not synergistic as we had hypothesized. The NAc contains m1, m2, and m4 receptors, although we hypothesized that the synergistic locomotor response was due to m4 inhibitory-type receptors. They had no effect in this study. This is not to say that mAChR

do not influence ethanol activation, as they clearly did in the peripheral study. However, they do not appear to in the NAc.

In addition, there were no SNPs between B6 and FAST/SLOW or between FAST and SLOW themselves. There were also no gene expression differences in *Chrm4* levels in any region tested. m4 gene deletion on two different genetic backgrounds did not alter ethanol-induced locomotor activity or consumption. There were no results in this dissertation indicating a role for *Chrm4* in acute locomotor stimulation to ethanol.

In contrast, the m5 receptor gene still appears to be an attractive candidate gene for ethanol-induced stimulation. The effect of the m5 receptor was not tested in a locomotor test of behavior, as the RNA interference study was meant to accomplish this (see *Future Directions*). However, other compelling data implicate the receptor in acute locomotor stimulation to ethanol. There were two non-synonymous SNPs in *Chrm5* conserved between FAST-2 and SLOW-2 that may underlie their significantly different locomotor response to scopolamine (Bergstrom et al., 2003). Both FAST and SLOW replicate lines differed in expression levels of *Chrm5* in the VM, the seat of mesolimbic DA propagation, heavily implicated in drug reward and locomotor activity. Furthermore, this difference was in the direction we hypothesized; the stimulant sensitive FAST mice had higher levels of the excitatory *Chrm5* gene. Unexpectedly, FAST-1 and SLOW-1 also differed in HIP *Chrm5* levels, although in the opposite direction to what we hypothesized (SLOW-1 had greater *Chrm5*

levels in the HIP than FAST-1). Once backcrossed to a stimulant-sensitive background, deletion of the m5 receptor gene ablated ethanol-induced locomotor stimulation as compared to WT mice.

Assessing candidate genes takes a multipronged approach, combining genotype-driven, hybrid, and phenotype-driven efforts (Phillips et al., 2002). In the current work, we used all three. Genotype-driven usage of m4 and m5 knockout mice helped to identify the global contribution of these receptor subtypes in ethanol activation. However, a more precise genotype-driven approach is still needed, proposed to be accomplished via RNA interference (see *Future Directions*). For the hybrid approach, we measured gene expression levels as well as sequence profiling. As for phenotype-driven approaches, we used mice selectively bred for extreme sensitivity (FAST) and insensitivity (SLOW) to the stimulant effects of ethanol. The combination of these techniques has allowed us to confidently interpret the results described within. In summary, the *Chrm5* receptor subtype appears to be of prime influence on ethanol-induced locomotor stimulation. The m4 receptor subtype does not appear to be uniquely involved in the stimulant response to ethanol. In general, these data contributed to the knowledge of neurotransmitter receptor subtypes involved in the differential response to ethanol in FAST and SLOW mice, and are the first data to focus on ethanol-related phenotypes for the m4 and m5 receptors.

Future Directions

There are many future directions to explore in the investigation of muscarinic acetylcholine influences on ethanol-induced stimulation. Furthermore, the complicated circuitry of mAChR/DA interactions, coupled with the lack of selective pharmacological agents for the different receptor subtypes, leaves much to be learned about basic muscarinic effects on drug reward and locomotor activity.

Because of the lack of selective pharmacological agents, we initially wished to selectively knockdown the *Chrm4* and/or *Chrm5* receptor genes using RNA interference techniques. The overall approach was to design 3 – 4 selective oligonucleotides that targeted the *Chrm5* gene (our initial candidate), ligate these oligonucleotides into a plasmid co-expressing green fluorescent protein, and clone the plasmid. We accomplished this step. The next step would have been to co-transfect these oligonucleotides into cells, and determine which of the four oligonucleotides produced the most substantial knockdown *in vitro*. Following that, the winning oligonucleotide would have been packaged into a viral vector, which would then have been injected into a specific brain region (in this case, the VTA) and animals tested for locomotor response to ethanol. In order to accurately determine knockdown of the m5 receptor in cells, one would need to find a cell line that endogenously expressed the m5 receptor in sufficient amounts, or co-transfect that receptor cDNA into cells. I attempted to amplify and clone the m5 receptor cDNA, with no success. One factor that necessitated

the attempt to clone m5 receptor cDNA was the inability to test cell lines for expression of the m5 receptor. While gene expression analysis of cell lines may have been an option, the lack of cell lines in which it is known that *Chrm5* receptors are expressed would have made interpretation of the gene expression results difficult. Without that knowledge, it would be challenging to interpret data as no positive controls exist (knowledge that a cell line absolutely expresses m5 receptors, enabling one to compare an unknown to a known). In addition, commercial resources are scarce. For example, one company sells rat cDNA for the *Chrm5* gene, but it does not cover the entire reading frame for the gene, which limits the range of potential oligonucleotide transcripts one could use for RNA interference. There is a line of cells that purportedly endogenously expresses the m4 receptor; NG108-15 cells (Leach et al., 2010; Yasuda et al., 1992), although they are a fusion of mouse neuroblastoma cells and rat glioma cells, so they may not adequately model potential effects in mouse neurons.

While I was able to amplify genomic DNA from both B6 and D2 mice using the primers targeted to the *Chrm5* open reading frame, I was unable to amplify FAST/SLOW DNA at the correct molecular weight. However, a test cut using the restriction enzyme *EcoRI* did cleave FAST and SLOW gDNA samples into segments corresponding to the correct sizes for *EcoRI* restriction sites in the *Chrm5* gene. However, I did not see cuts in the FAST/SLOW cDNA, but this may be due to an inadequate amount of cDNA loaded onto the gel.

As a pilot study, one could directly inject the double-stranded RNA transcripts into the brain, without packaging it into a viral vector, in order to determine if it would be a fruitful area to pursue. The packaging into a viral vector keeps the complex stable. When RNA is injected directly into the brain, it only lasts for 36 hours (AW Lasek, personal communication). This would not allow for recovery time following brain puncture. As in Boehm et al. (2002), the ventricles could initially be targeted, followed by vector targeting of the VTA if promising results were obtained.

Gene expression analyses would be better served using dissections from a laser-capture dissecting microscope rather than hand dissections. This would enable one to precisely target brain regions known to express the subtypes of interest. In addition to cleanly and accurately dissected brain regions, the dissecting microscope allows one the capacity to label cell types. For example, one could dissect only choline acetyltransferase-containing cells of the NAc prior to performing gene expression assays on those cells. This approach would aid in anatomical knowledge of mAChR. However, comparison of mRNA values is just one part of the story; analyses of protein levels using Western blotting would provide information about receptor proteins. One could also dissect regions used for Western blotting with the laser capture dissecting microscope. However, only the antibody for the m2 receptor subtype is reliable (Jositsch et al., 2008). This is because of the high sequence homology among the five mAChR subtypes. Another approach that may improve the gene expression studies is to pool the smaller brain regions, such as the NAc and OT. Furthermore, the kit used for

these regions (weight between 5 and 40 mg) was probably not well suited for cerebellar extracts (800 mg), despite the company's assurances.

In general, the major future direction should be to complete selective knockdown of the m5 receptor using RNA interference, as it will allow selective pinpointing of the gene and its precise location without any developmental effects. This remains the critical unanswered question of the current work. The lack of subtype-selective drugs could be circumvented by the genotype-driven approach, and it would not only provide information about ethanol-induced stimulation, but advance the field of mAChR research as well.

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