Multidisciplinary Approaches Examining the Genetic Basis of Drug-Induced Locomotor Stimulation

by

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CERTIFICATE OF APPROVAL

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LIST OF ABBREVIATIONS

5-HT – 5-Hydroxytryptamine

AMPA – Amino-3-Hydroxy-5-Methylisoxazole-4-Propionic Acid

ANOVA – Analysis of Variance

B6 – C57BL/6 Mice

BEC – Blood Ethanol Concentration

BXD RI – Recombinant Inbred Strains Derived from the C57BL/6J and DBA/2J Inbred Strains

C_t – Crossing Threshold

D2 – DBA/2 Mice

DEPC – Diethylpyrocarbonate

dfw – Degrees of Freedom Within

DSM-IV – Diagnostic and Statistical Manual of Mental Disorders – Fourth Edition

EC_{50} - the concentration of agonist at which 50% of the maximum response is achieved

GABA – γ-Amino Butyric Acid

i.p. – Intraperitoneal

ISCS – Interval-Specific Congenic Strains

i.v. – Intravenous

LOD – Logarithm of the Odds

NMDA – N-Methyl-D-Aspartate

RI – Recombinant Inbred

RNAi – RNA interference

qRT-PCR – Quantitative Real Time-PCR

QTG – Quantitative Trait Gene

QTL – Quantitative Trait Locus (Loci)

siRNA – small interfering RNA

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ABSTRACT

Drug abuse is a complex trait that is influenced by both genetic and environmental factors. Endophenotypes are simple traits that are genetically related to complex traits. Understanding the biological basis of an endophenotype is thought to be a way to understand a partial component of a complex trait. The acute locomotor responses to ethanol and psychostimulants are examined here as putative endophenotypes for drug and alcohol abuse.

In this dissertation I set out to determine if nicotinic acetylcholine receptors are involved in ethanol, cocaine, and methamphetamine stimulation, and to confirm the presence of a quantitative trait locus (QTL) for the acute locomotor responses to these drugs on mouse chromosome 9 using congenic strains of mice. In this region of chromosome 9, there is a cluster of genes encoding acetylcholine receptor subunits. Therefore, the α3, α5, and β4 subunits of the nicotinic receptor (*Chrna3, Chrna5,* and *Chrnb4,* respectively) were examined as candidate genes for the acute locomotor response to ethanol. To achieve this goal, gene and protein expression assays were used.

Behavioral pharmacological studies provided evidence that neuronal nicotinic acetylcholine receptors are involved in the acute locomotor response to ethanol, but not cocaine or methamphetamine. Mecamylamine, a nonspecific acetylcholine receptor antagonist, decreased ethanol-induced locomotor activation in two genetic models of enhanced sensitivity (DBA/2J [D2] and FAST mice). Hexamethonium, a nicotinic acetylcholine receptor antagonist which does not cross the blood brain barrier, did not attenuate this response. Antagonists specific for α4β2 and α7 receptors did not affect stimulation, therefore providing evidence against the role of these receptors. A QTL on

mouse chromosome 9 was confirmed for ethanol, cocaine, and methamphetamine stimulation. The location of the QTL for ethanol and methamphetamine stimulation was further narrowed using a congenic F_2 population and interval-specific congenic strains (ISCS), respectively. To determine if nicotinic acetylcholine receptors containing an α3 subunit were involved, molecular assays were utilized since antagonists specific for these receptors are not commercially available. The D2 strain of mice, which is robustly stimulated by ethanol, had less *Chrna3* expression compared to the chromosome 9 D2.B6 congenic mice, which are less stimulated by ethanol. Mice selectively bred for sensitivity (FAST) and insensitivity (SLOW) to the stimulant effects of ethanol had similar *Chrna3* expression. The assays currently used to assess protein expression of nicotinic acetylcholine receptors subunits are inadequate; therefore the question of differences in *Chrna3* protein levels remains unresolved.

These data provide evidence that a gene on chromosome 9 accounts for part of the variation in the acute locomotor responses to ethanol and psychostimulants. There is evidence that *Chrna3* is a candidate gene for the acute response to ethanol, but this gene does not appear to be involved in psychostimulant-induced locomotor activation. Further work is needed to determine if *Chrna3* is the quantitative trait gene (QTG) on chromosome 9 for ethanol stimulation, and to identify the QTG on chromosome 9 for the locomotor responses to cocaine and methamphetamine.

Chapter 1: General Introduction

Drug and alcohol abuse are major health problems in the United States. The National Comorbidity Survey – Replication, conducted between February 2001 and April 2003 surveyed 9,282 individuals living in the United States. In this survey, diagnosis of drug use and dependence were defined by the Diagnostic and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV) criteria. The prevalences of lifetime alcohol and drug abuse (13.2 and 7.9%, respectively) were greater than those of alcohol and drug dependence (5.4 and 3%, respectively; Kessler et al., 2005b). The 12-month prevalences were similar to lifetime prevalences; abuse was greater than dependence. Furthermore, people who abused or were alcohol dependent at the time of this survey were more likely to abuse or be dependent on other drugs, as indicated by significant correlations between the traits (Kessler et al., 2005a).

The societal costs of drug abuse are substantial. In 1998, the cost of illicit drug abuse, as defined by loss of productivity, health care costs, and social services rendered, was 143.4 billion dollars (Office of National Drug Control Policy, 2001). The cost associated with alcohol abuse during the same time period was estimated to be 184 million dollars (Harwood, 2000). Approximately 33.4 – 49.8% of the people diagnosed with abuse or dependence of drugs and alcohol sought medical care for their condition, visiting a medical care worker a median of 5.3 times, in the year prior to the National Comorbidity Survey – Replication (Wang et al., 2005). Thus, research aimed at understanding the biological basis of drug and alcohol addiction is warranted.

Behavioral Stimulation: Humans

Amphetamine abusers who received a single 200 mg intravenous dose of amphetamine reported euphoria that peaked 15 min after drug administration and decreased over the following 10 hours. Plasma levels of amphetamine peaked by 30 min (blood levels were not assessed at 15 min post-amphetamine injection) and decreased over the next 10 hours of observation, suggesting that the subjective effects of amphetamine administration correlate with plasma amphetamine levels (Jonsson et al., 1971). Subcutaneous and oral administration of amphetamine causes dose-dependent increases in ratings of euphoria and liking of the drug (Jasinski et al., 1974). Subjects reported greater positive subjective effects at lower amphetamine doses with subcutaneous drug administration than with oral administration, but with higher doses of amphetamine the subjects reported similar effects with both administration routes. In addition to the subjective effects of amphetamine, physiological effects were also observed. Heart rate and blood pressure increased in the same dose responsive pattern as the subjective effects (Jasinski et al., 1974). Since these early studies on amphetamine administration, other studies have reported similar findings; humans report greater stimulant-like feelings after administration of amphetamine compared to placebo (Holdstock and de Wit, 2001; Veenstra-VanderWeele et al., 2006). In 2004, Vollm and colleagues reported that subjects reported an increased feeling of "mind racing" following intravenous methamphetamine administration compared to when they received saline (Vollm et al., 2004).

In this dissertation, studies using amphetamine and methamphetamine will be described together. These drugs are similar in structure and have similar

pharmacokinetic properties as well as elimination rates (Melega et al., 1995). Amphetamine and methamphetamine elevate dopamine levels in the nucleus accumbens to a similar extent (Melega et al., 1995), but amphetamine increases dopamine levels in the prefrontal cortex significantly more than methamphetamine (Shoblock et al., 2003). Additionally, amphetamine is more potent than methamphetamine, producing greater behavioral stimulation with the same milligram dosage (Shoblock et al., 2003).

Similar to subjective reports after amphetamine administration, subjects administered cocaine intravenously or intranasally in a laboratory setting exhibited subjective and physiological effects of the drug. In response to cocaine, heart rate and blood pressure increased in a dose-dependent manner and subjects reported feeling high and amphetamine-like effects (Resnick et al., 1977). Unfortunately, in this study plasma concentrations of cocaine were not taken to determine if the subjective and physiological effects were consistent with the temporal pattern of plasma cocaine levels. However, other studies evaluating plasma concentrations of cocaine and the subjective and physiological effects of cocaine in the same individuals have shown that the temporal pattern of the effects of cocaine mirrors cocaine plasma levels (Javaid et al., 1978; Van Dyke et al., 1978). More recent studies have examined the activating effects of cocaine administration in the laboratory; feelings of high, stimulated, talkative, liking, and good drug effect all dose-dependently increased with cocaine administration (Foltin and Fischman, 1991; Foltin and Haney, 2004; Lynch et al., 2006).

Amphetamines and cocaine are purely psychostimulants (they increase motor activity such as locomotion and stereotypic behaviors), however ethanol has both stimulant and depressant effects. In an early study of the effects of alcohol in human

subjects, alcohol increased talkativeness, a behavior that may reflect the euphoric effects of this drug (Ahlenius et al., 1973), but blood alcohol concentrations were not analyzed. Further studies have demonstrated that the effects of ethanol on mood appear to be related to blood ethanol concentration. When subjects reported mood at several time points throughout the blood alcohol curve, positive moods were associated with ascending blood alcohol concentrations, while negative mood states were associated with the descending portion of the curve (Babor et al., 1983). In 1988, Lukas and Mendelson showed that male volunteers given 0.695 g/kg alcohol reported euphoria following ethanol consumption. This study also linked the increased reports of euphoria to the rising phase of the blood alcohol curve (Lukas and Mendelson, 1988). Sons of alcoholics had greater gross motor activity following alcohol (0.5 g/kg) consumption than sons of nonalcoholics (Newlin and Thomson, 1991, 1999). Recently, the locomotor stimulant effects of alcohol were observed in humans during the ascending limb of the blood alcohol curve. Activity in human subjects was assessed via accelerometers worn on the subject's wrists, and was monitored throughout the session (before, during, and after alcohol administration). Subjects that consumed wine were more activated by alcohol during the ascending limb of the blood alcohol curve (defined as the first 15 min post-ethanol consumption) than subjects that received alcohol-free wine or soda (Davidson et al., 2002). Activity was not monitored during the descending portion of the blood alcohol curve. Together these data show that in human subjects amphetamines, cocaine, and ethanol all increase self-reports of euphoria.

Behavioral Stimulation: Rodents

Amphetamines, cocaine, and ethanol also stimulate rodent behavior. The locomotor stimulant response is the primary behavioral measure of activation in rodents. The behavioral response to amphetamines in animals is dependent upon the dose administered. Low doses increase locomotor activity (Brien et al., 1978; Meng et al., 1999; Atkins et al., 2001), but higher doses increase stereotypic behaviors (motor behaviors that are repetitively performed for no obvious purpose; Brien et al., 1978). In Lewis rats, amphetamine caused a dose-dependent increase in locomotor activity up to doses of 10 mg/kg, but the increase in response to 17.8 mg/kg amphetamine was diminished, although this could have been due to increased stereotypy in these rats (George et al., 1991). Sensitivity to the stimulant effects of amphetamines are strain-dependent in both rats and mice (Moisset and Welch, 1973; Anisman et al., 1975; Anisman, 1976; Remington and Anisman, 1976; Moisset, 1977; Kitahama and Valatx, 1979; Hamburger-Bar et al., 1986; George et al., 1991; Camp et al., 1994), but patterns of strain sensitivity are dependent upon dose and testing parameters.

Similar to the work with amphetamines, cocaine also increases locomotor activity in rodents (Elliott et al., 1987), an effect which appears to be dose-dependent (Kalivas et al., 1988; Downing et al., 2003a). The time course of cocaine stimulation and brain cocaine levels follow a similar temporal pattern, peaking approximately 5-min after an acute injection. Furthermore, brain cocaine levels are significantly correlated with the locomotor response to cocaine when administered into the peritoneal cavity (i.p.; Benuck et al., 1987). The strain of rat (George et al., 1991; Camp et al., 1994) or mouse (Ruth et al., 1988; Jones et al., 1993; Tolliver and Carney, 1994; Womer et al., 1994; Henricks et

al., 1997; Rocha et al., 1998; Downing et al., 2003a) is also an important factor in determining the response to cocaine.

Many studies have assessed the acute locomotor response to ethanol in rodents. Similar to work done in humans, the behavioral activating effect of ethanol in rodents appears to be related to blood ethanol concentration. Low doses of ethanol have been shown to increase locomotor activity in both rats and mice (Ahlenius et al., 1974; Pohorecky, 1977; Frye and Breese, 1981; Erickson and Kochhar, 1985; Imperato and Di Chiara, 1986; Crabbe et al., 1994), but higher doses of alcohol cause locomotor depression (Pohorecky, 1977; Frye and Breese, 1981). In addition to ethanol having biphasic effects depending on dose administered, ethanol also produces biphasic effects that are dependent upon the time after administration. In Swiss mice, ethanol stimulated locomotor activity during the first 15 min post-ethanol injection then decreased activity thereafter (Smoothy and Berry, 1985). Similar results have been observed in the inbred DBA/2N (D2; this abbreviation will be used for any DBA/2 subline) strain (Crabbe et al., 1982).

The acute response to ethanol in rodents is also strain-dependent (Randall et al., 1975; Tabakoff and Kiianmaa, 1982; Erickson and Kochhar, 1985; Crabbe, 1986; Lister, 1987; Dudek and Phillips, 1990; Crabbe et al., 1994; Dudek et al., 1994; Tritto and Dudek, 1994; Glick et al., 2002). As with psychostimulants, the pattern of strain differences is likely dependent on dose and testing procedures. Similar to the effects observed in humans, amphetamine, cocaine, and ethanol all stimulate locomotor activity in rodents.

Behavioral Stimulation as an Endophenotype

An endophenotype is a measurable trait that is genetically related to a complex phenotype. The use of endophenotypes to identify the biological basis of complex phenotypes was first described for psychiatric traits over 30 years ago (Gottesman and Shields, 1973). The theory behind the use of endophenotypes is that a complex trait is influence by many genes, as well as environmental influences, and an endophenotype is a relatively simple trait that is influenced by fewer genetic and environmental factors (Gottesman and Gould, 2003). Identifying genes that influence an endophenotype will lead to an understanding of a partial component of the complex trait.

The acute locomotor response to ethanol has been proposed to be an endophenotype for alcohol abuse (Gabbay, 2005). Several studies have shown a positive relationship between the acute stimulant response to alcohol and one's likelihood of abuse. For example, in 1987 de Wit and colleagues showed that subjects who reported more positive mood and arousal following alcohol administration were more likely to choose an alcohol containing drink over placebo compared to individuals who report dysphoria following consumption of a beverage containing alcohol (de Wit et al., 1987). Moreover, heavy drinkers were more stimulated by alcohol than light drinkers, and less sedated (Holdstock et al., 2000; King et al., 2002), even when blood alcohol concentrations were not different. Recently, Thomas and colleagues (2004) showed that non-treatment seeking alcoholics were more stimulated by alcohol administered in a laboratory setting than social drinkers.

Although many studies have shown a positive relationship between alcohol stimulation and heavy use of this drug, in one case-control study high stimulation was

associated with low alcohol dependence (Poikolainen, 2000). While this study reported the opposite relationship, the methods used to assess ethanol stimulation and the populations studied were quite different. In this study, alcohol dependent subjects, in a hospital treatment program for their dependence, were compared to a representative household sample. Furthermore, in this study the subjects rating of stimulation was based on recall of the last time they were intoxicated (Poikolainen, 2000). This is different from the other studies where stimulation was assessed after subjects were administered a low dose of ethanol (0.34 – 0.8 g/kg) in a laboratory setting.

The positive relationship between the acute stimulant response and further drug intake has also been observed with psychostimulants. Drug preference studies have provided evidence that the initial stimulant response to amphetamine predicts the likelihood of further drug use (de Wit et al., 1986; Gabbay, 2003). Similarly, Davidson and colleagues showed that there was a significant positive relationship between subjects ratings of positive cocaine effects, including euphoria, and lifetime cocaine use (Davidson et al., 1993). These data provide evidence that, similar to the acute locomotor response to ethanol being an endophenotype for alcohol abuse, the stimulant response to amphetamines and cocaine may be an endophenotype for psychostimulant abuse. Thus, understanding the biological basis of the acute response to these drugs may lead to a partial understanding of drug and alcohol abuse.

Behavioral Stimulation is a Complex Trait

There is little work in humans regarding the amount of variation in the behavioral response to ethanol and psychostimulants that is attributed to genes or environmental factors. As mentioned in the previous section, a number of studies have suggested a

positive relationship between the acute stimulant response to these drugs and further drug intake, but these results do not provide information regarding the basis of these findings. This relationship could be influenced by genes, the environment, or interactions among these factors (e.g., gene X gene, gene X environment, and environment X environment).

In contrast to human studies, there is ample evidence in mice that the acute locomotor responses to amphetamines, cocaine, and ethanol are influenced by multiple genes. The first evidence that the locomotor response to amphetamine was influenced by polygenic factors came from a study using recombinant inbred (RI) strains of mice. Seven RI strains derived from the C57BL/6By (B6; this abbreviation will be used for any C57BL/6 subline) and BALB/cBy inbred strains were tested for their acute response to amphetamine. The strain distribution pattern of the behavioral response in these mice provided evidence that amphetamine-induced stimulation was influenced by multiple genetic factors (Oliverio et al., 1973). Similar results for the acute locomotor response to cocaine were found a few years later (Shuster et al., 1977).

Although an original study in RI strains derived from the B6 and BALB/cBy progenitor strains suggested that the acute response to ethanol was mediated by a single gene on chromosome 4 (Oliverio and Eleftheriou, 1976), there has been substantial data since this original study that this trait, like the acute response to psychostimulants, is influenced by multiple genetic factors. For example, in 1983 Crabbe and colleagues examined the acute response to ethanol in 20 RI strains derived from the B6 and D2 inbred strains (BXD RI). The strain distribution pattern provided evidence that several loci modulated the locomotor response to a 1.33 g/kg ethanol injection (Crabbe et al., 1983). Other studies have since shown that the stimulant response to ethanol is

influenced by many genes (Phillips et al., 1995; Phillips and Shen, 1996; Erwin et al., 1997; Demarest et al., 1999; Demarest et al., 2001; Downing et al., 2003b).

A few studies have described the genetic architecture of the acute locomotor response to ethanol. These have primarily used a diallel cross methodology. In a diallel cross study, three or more inbred strains (or sometimes selected lines) are crossed to produce all possible reciprocal F_1s. Evaluation of the relationships between the parental strains and F_1 offspring provides information regarding the sources of genetic influence on the trait. Phillips and Dudek (1991) assessed the acute locomotor response to 2 g/kg ethanol in a diallel cross of the LS (Long Sleep), SS (Short Sleep), B6, and MOLD/RkAbg lines and strains. These data suggested that the behavioral response to ethanol was under mostly additive genetic control, but that there was also a small dominant component. There was no evidence of epistasis, sex linkage, or maternal effects (Phillips and Dudek, 1991). Results from a diallel cross of the B6, D2, AU/SsAbg, and MOLD/RkAbg strains revealed the same genetic architecture (Dudek et al., 1991).

Another way to determine the make up of the genetic influences on a trait is to use a Mendelian cross design. In this type of experiment, two inbred strains are crossed to produce F_1, F_2, and reciprocal backcross animals. The mean phenotypic values from these populations of animals provide evidence regarding the mode of genetic inheritance of the trait within populations derived from the two progenitor strains. A Mendelian cross experiment with the B6 and D2 inbred strains provided evidence that only additive genetic factors influenced the acute locomotor response to ethanol in these strains (Dudek and Tritto, 1994). There has been a single diallel cross study examining psychostimulant

activation. In a diallel cross of the A/J, B6, and D2 strains, most of the genetic influence on amphetamine stimulation appeared to be additive, but there also appeared to be some maternal effects and overdominance (Anisman, 1976).

Further evidence that the acute locomotor responses to amphetamines, cocaine, and ethanol are influenced by multiple genetic factors was provided by selective breeding experiments. Lines of mice have been successfully bred for sensitivity and insensitivity to all of these drugs. The first selection experiment for the locomotor stimulant response to one of these drugs was for the response to ethanol. In 1987, Crabbe and co-workers reported on the first 4 generations of selective breeding of the FAST and SLOW lines of mice (Crabbe et al., 1999a). The FAST line was bred for sensitivity to the stimulant effects of ethanol, while the SLOW line was bred for insensitivity to this effect of ethanol. These lines were bred in replicate from a genetically heterogeneous (HS/Ibg) population of mice (McClearn et al., 1970). In the first generation of selective breeding, the FAST and SLOW lines (both replicates) differed in the locomotor response to an acute injection of ethanol. These lines continued to diverge throughout many of the 37 generations of selective breeding (Phillips et al., 1991; Shen et al., 1995; Crabbe et al., 1999a; Phillips et al., 2002b).

Similar to the lines of mice bred for ethanol stimulation, replicate CAHI and CALO lines were bred for differential sensitivity to an acute injection of cocaine (10 mg/kg; Marley et al., 1998). The CAHI lines were bred for heightened sensitivity to cocaine-induced locomotor activation, while the CALO lines were bred for reduced sensitivity. HS/Ibg mice served as the founding population. The CAHI and CALO lines

differed in the first generation of selection, and progressively diverged further throughout 12 generations of selective breeding (Marley et al., 1998).

Finally, lines of mice selectively bred for the locomotor response to methamphetamine provided evidence that the locomotor response to amphetamines is polygenic. The HMACT and LMACT lines were selectively bred for differential acute locomotor response to 2 mg/kg methamphetamine from a B6XD2 F_2 population. The HMACT line was bred for heightened sensitivity to 2 mg/kg methamphetamine, while the LMACT line was bred for decreased sensitivity. The HMACT and LMACT lines differed in response to an acute injection of methamphetamine in the first selection generation, and continued to diverge over the course of selection to the point where in the fourth selection generation they differed 5-fold in this response (Kamens et al., 2005).

While there is strong evidence that genes are important in the behavioral response to ethanol and psychostimulants, environmental factors can also modulate the locomotor response to these drugs. For example, housing can affect the locomotor response to cocaine. Long-Evans rats that were group housed were more stimulated by 10 or 20 mg/kg cocaine, even when taking into account differences in baseline locomotor activity, than rats that were housed in isolation (Boyle et al., 1991). Furthermore, the lighting in the test environment can also influence this behavioral response. For example, Crabbe and colleagues (1988) showed that in a brightly lit arena, ethanol-induced locomotor stimulation was greater than when assessed in an arena that was dimly lit. These data provide evidence that the acute locomotor response to ethanol, cocaine, and methamphetamine is influenced by both genetic and environmental factors.

Genetic Dissection of Complex Traits

The previous section described evidence that the acute locomotor response to ethanol and psychostimulants is a complex trait (i.e., influenced by multiple genetic and environmental factors). To identify which genes influence the locomotor responses to these drugs quantitative trait loci (QTL) have been mapped. The abbreviation QTL will be used for both quantitative trait locus (singular) and quantitative trait loci (plural) in this dissertation. A QTL refers to a chromosomal region containing a gene that influences a complex trait. An extensive review of the techniques used to map QTL is beyond the scope of this dissertation, but there are a number of reviews and book chapters that provide details on these approaches (Crabbe et al., 1999b; Wehner et al., 2001; Palmer and Phillips, 2002; Phillips et al., 2002a; Flint, 2003; Flint et al., 2005). Instead, I will talk briefly about QTL mapping approaches, with specific details given for the methods used in this dissertation.

A number of different genetic crosses can be used to map QTL. In most cases, QTL have been mapped in crosses derived from two inbred strains. For the behavioral responses to drugs of abuse, these have usually been the B6 and D2 inbred strains. Populations derived from crossing two inbred strains that can be used to map QTL include: RI strains, recombinant congenic strains, chromosome substitution strains, intercross populations (including F_2 and backcross populations), and short-term selected lines. More recently heterogenous mice, derived from crosses of four or more inbred strains, have also been used to map QTL. The strengths and weaknesses associated with using these different mapping populations can be found elsewhere (Palmer and Phillips, 2002; Flint, 2003), but the QTL mapping in all of these populations follows the same

general methods. To map QTL, one of these populations is phenotyped for the trait of interest, and the behavioral response is correlated with the variant alleles at a specific genetic marker. If there is a significant association between the genotype and phenotype of the animals, there is evidence that a gene influencing the trait is close to the genetic marker.

Congenic strains of mice provide a valuable tool for confirmation of QTL (Bailey, 1981; Bennett, 2000). Congenic strains are isogenic animals that are derived from two inbred strains (i.e., progenitor strains). One strain serves as a donor strain while the other is the recipient (Figure 1). A small region of the donor's genome is introgressed (moved onto another genomic background) via homologous recombination onto the recipient strain. In some cases reciprocal congenic strains exist, such that in one case strain X is the donor strain while strain Y is the background strain and in the opposite strain the Y strain is the donor strain and the X strain is the background strain. If the congenic strain differs from the pure background strain, a gene in the introgressed region affects the trait of interest.

Once QTL are provisionally mapped with one or more of the populations described above, finer mapping can then ensue. The goal of fine mapping is to narrow the QTL region with the ultimate goal of identifying the quantitative trait gene (QTG). A number of approaches can be used for finer mapping (Darvasi, 1998). These include using advanced intercross lines and interval-specific congenic strains (ISCS).

ISCS have been useful in narrowing the location of QTL that influence behavioral traits (e.g., Fehr et al., 2002; Ferraro et al., 2004; Shirley et al., 2004). This strategy employs multiple congenic strains containing unique overlapping introgressed segments

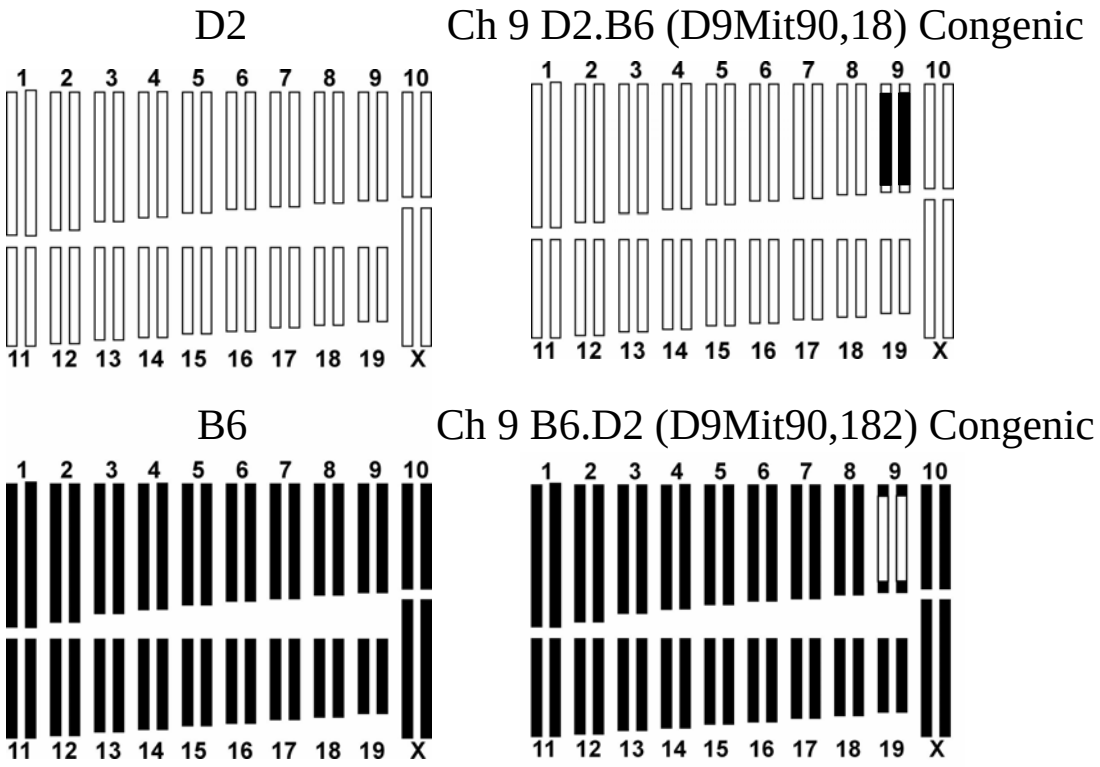


Figure 1. Reciprocal chromosome 9 congenic mice. The background strain is always listed first, and then the donor strain in the congenic designation (e.g., D2.B6). Ch = Chromosome. D9Mit90,18 = represents the makers used to define the congenic region D9Mit90 is the proximal marker and D9Mit18 is the distal genomic marker. D9Mit90,182 = D9Mit90 is the marker that defines the proximal part of the congenic region and D9Mit182 defines the distal portion of the region. This figure was provided by Nicki Walter.

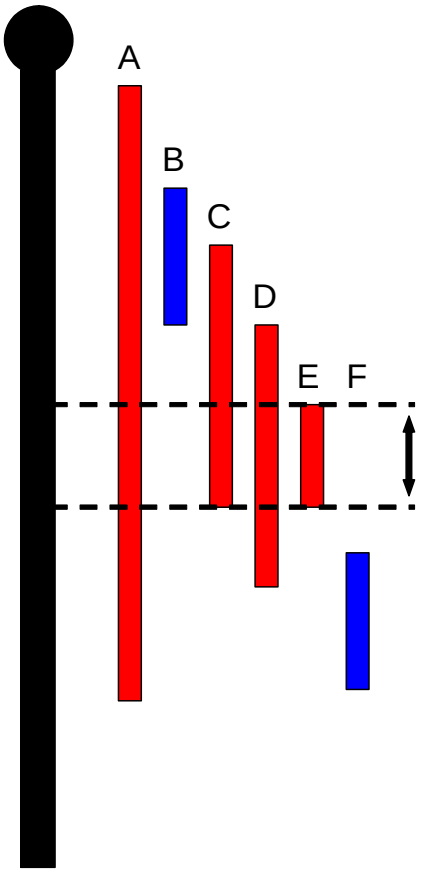
throughout the region thought to harbor the QTL (Darvasi, 1997). Each of the ISCS is tested for the behavior of interest and strains that are significantly different from the background strain (i.e., that capture the QTL) identify the location of the QTL (see Figure 2).

This strategy was successful in fine mapping a QTL for ethanol withdrawal severity as measured by handling-induced convulsions. ISCS narrowed the location of a QTL for ethanol withdrawal severity to an interval of approximately 1.8 Mb, which contained only 15 genes (Fehr et al., 2002; Shirley et al., 2004). This group nominated the *Mpdz* (multiple PDZ domain protein) gene as the quantitative trait gene (QTG), which they were then able to confirm through gene and protein expression assays.

The QTG can influence the behavior in at least two ways. First, there could be a polymorphism (a difference in DNA sequence) in the gene that changes the function of the protein. Second, there could be differences in gene expression leading to differences in the phenotype of interest (Flint, 2003). Recently, gene-expression profiles in strains of mice that differ in the trait of interest have been widely used (e.g., Hitzemann et al., 2004; MacLaren et al., 2006). Genes that are differentially expressed in these strains, and map in the QTL region, are considered candidate genes (Flint et al., 2005). Multiple steps go into mapping QTL with the ultimate goal of identifying the genes that influence a quantitative trait.

QTL for the Acute Locomotor Response to Amphetamines, Cocaine, and Ethanol

There is evidence that a number of genes influence the acute locomotor response to ethanol and psychostimulants. One or more QTL have been mapped on all mouse



Location of the quantitative trait locus (QTL) if strains A, C, D, and E are different from the background strain.

Figure 2. An illustration of mapping QTL with interval-specific congenic strains.

The segment of introgressed donor alleles for congenic A – F is represented as red or blue boxes relative to the chromosome on the left.

chromosomes for the acute locomotor responses to amphetamines, cocaine, or ethanol (Table 1). The strength of the evidence associated with these QTL varies. For example, experiments that used the BXD RI strains reported evidence for a number of QTL, including those in which a single genetic marker was associated with the phenotype of interest. In contrast, some of the QTL exceed the accepted Lander and Kruglyak's threshold for significant QTL (1995).

Some QTL have been mapped in a single genetic population while others have been mapped on multiple genetic backgrounds or in multiple crosses derived from the same genetic population. There are some cases where a single mapping population provides evidence of a significant QTL, but in other cases evidence from a single mapping population provides only suggestive evidence of QTL. In cases where there is suggestive evidence of a QTL, when a QTL is mapped in multiple crosses from the same genetic population (e.g., mapped in a BXD RI and a B6D2 F_2 population) these data may provide additional evidence for the QTL. An example of this can be found in a recent paper by Palmer and colleagues (2006). In this paper a QTL for ethanol stimulation on mouse chromosome 2 was mapped in four different populations of animals derived from the B6 and D2 inbred strains. Alone, data from the BXD RI cross provided only suggestive evidence for this QTL, but when these data were combined with data from three other mapping populations there was significant evidence of a QTL on chromosome 2 for this trait (Palmer et al., 2006). Furthermore, when a QTL is mapped in multiple genetic crosses, this may also provide additional evidence for the QTL. For example, a QTL for ethanol-induced stimulation has been mapped on chromosome 2 in six different

Table 1. QTL mapped for the acute locomotor response to amphetamines, cocaine, and ethanol on mouse chromosomes. Listed are the QTL defined by the authors of the respective studies. The strength of statistical evidence in support of the QTL varies. Those that exceeded Lander and Kruglyak's (1995) threshold for significant QTL (1.0 X 10-4 for a cross with 1 degree of freedom or 5.2 X 10-5 for a cross with 2 degrees of freedom) are denoted by a *.

Chr	Amphetamines	Cocaine	Ethanol
1	4 mg/kg MA in BXD RI[12] 5 mg/kg AMP AcB/BcA RCS[25] 16 mg/kg MA in BXD RI[12]	5 mg/kg BXD RI[21] 10 mg/kg BXD RI[21] *20 mg/kg AcB/BcA RCS[9] 40 mg/kg BXD RI[21] 32 mg/kg BXD RI[24]	1.5 g/kg BXD RI[4, 5] *1.5 g/kg B6XD2 F2[4, 14, 15, 17] *1.5 g/kg B6XC3H F2[7] *1.5 g/kg B6XLP F21[7] *1.5 g/kg B6XBALB F2[15, 17] *1.5 g/kg HS4[17] *1.5 g/kg HS/Npt[4] *2 g/kg B6XD2 F2[19] 2 g/kg LSXSS RI[8]
2	5 mg/kg AMP AcB/BcA RCS[25]	5 mg/kg BXD RI[21] 10 mg/kg BXD RI[21] 20 mg/kg AXB/BXA RI[2] 40 mg/kg BXD RI[21]	1.33 g/kg BXD RI[11] 1.5 g/kg BXD RI[4, 5] *1.5 g/kg B6XD2 F2[4, 13, 14] 1.5 g/kg B6XC3H F2[7] *1.5 g/kg HS4[17] *1.5 g/kg HS/Npt[4] 1.8 g/kg LXS RI[6] 2 g/kg BXD RI[3] *2 g/kg B6XD2 F2[19] *2 g/kg B6.D2 Congenic[19] *2 g/kg D2.B6 Congenic[19] 2 g/kg LSXSS RI[8] 2 g/kg AXB/BXA RI[10]
3	4 mg/kg MA in BXD RI[12] 5 mg/kg AMP AcB/BcA RCS[25] 8 mg/kg MA in BXD RI[12]	5 mg/kg BXD RI[21] 10 mg/kg BXD RI[18, 21] 40 mg/kg BXD RI[21]	1.5 g/kg B6XC3H F2[7] *1.5 g/kg D2XBALB F2[17] 1.5 g/kg LPXBALB F2[17] 1.8 g/kg LXS RI[6] 2 g/kg BXD RI[3, 22, 23] 2 g/kg B6XD2 F2[19] 2 g/kg AXB/BXA RI[10]
4	5 mg/kg AMP in BXD RI[1]	5 mg/kg BXD RI[21] 10 mg/kg BXD RI[18, 21] 40 mg/kg BXD RI[21]	1.33 g/kg BXD RI[11] 1.5 g/kg BXD RI[5] 1.8 g/kg LXS RI[6] 2 g/kg BXD RI[23]
5	2 mg/kg MA STSL[20] 5 mg/kg AMP in BXD RI[1] 5 mg/kg AMP AcB/BcA RCS[25] 8 mg/kg MA in BXD RI[12]	10 mg/kg BXD RI[18] 15 mg/kg BXD RI[16] 20 mg/kg AXB/BXA RI[2] *20 mg/kg AcB/BcA RCS[9] 40 mg/kg BXD RI[21] 45 mg/kg BXD RI[16]	1.5 g/kg B6XD2 F2[4] 1.5 g/kg B6XC3H F2[7] 2 g/kg BXD RI[3] 2 g/kg B6XD2 F2[19]

Chr	Amphetamines	Cocaine	Ethanol
6	4 mg/kg MA in BXD RI[12] 5 mg/kg AMP in BXD RI[1] 5 mg/kg AMP AcB/BcA RCS[25] 8 mg/kg MA in BXD RI[12]	10 mg/kg BXD RI[18] 20 mg/kg AXB/BXA RI[2] *20 mg/kg AcB/BcA RCS[9]	1.5 g/kg BXD RI[4, 5] *1.5 g/kg B6XC3H F2[7] *1.5 g/kg BALBXLP F2[14] 2 g/kg BXD RI[3, 23] 2 g/kg B6XD2 F2[19] 2 g/kg LSXSS RI[8]
7	4 mg/kg MA in BXD RI[12] 8 mg/kg MA in BXD RI[12]	10 mg/kg BXD RI[18] *20 mg/kg AcB/BcA RCS[9] 40 mg/kg BXD RI[21]	1.8 g/kg LXS RI[6] 2 g/kg BXD RI[3, 23] 2 g/kg B6XD2 F2[19]
8	4 mg/kg MA in BXD RI[12] 5 mg/kg AMP in BXD RI[1] 5 mg/kg AMP AcB/BcA RCS[25]	5 mg/kg BXD RI[21] 10 mg/kg BXD RI[18, 21]	1.5 g/kg LPXB6 F2[17] 1.8 g/kg LXS RI[6] 2 g/kg AXB/BXA RI[10]
9	2 mg/kg MA STSL[20] 5 mg/kg AMP AcB/BcA RCS[25] 8 mg/kg MA in BXD RI[12] 16 mg/kg MA in BXD RI[12]	10 mg/kg BXD RI[18, 21] *20 mg/kg AcB/BcA RCS[9] 32 mg/kg BXD RI[24] 40 mg/kg BXD RI[21]	1.33 g/kg BXD RI[11] 1.5 g/kg BXD RI[5] 1.5 g/kg B6XC3H F2[7] *1.5 g/kg LPXB6 F2[17] *1.5 g/kg LPXBALB F2[17] 2 g/kg BXD RI[3] 2 g/kg B6XD2 F2[19] 2 g/kg LSXSS RI[8]
10	5 mg/kg AMP AcB/BcA RCS[25] 16 mg/kg MA in BXD RI[12]	10 mg/kg BXD RI[18, 21]	2 g/kg BXD RI[22] 2 g/kg B6XD2 F2[19] 2 g/kg LSXSS RI[8]
11	2 mg/kg MA STSL[20]	32 mg/kg BXD RI[24]	2 g/kg BXD RI[3, 22] 2 g/kg LSXSS RI[8]
12	2 mg/kg MA STSL[20] 4 mg/kg MA in BXD RI[12]	10 mg/kg BXD RI[21] 20 mg/kg AXB/BXA RI[2]	1.8 g/kg LXS RI[6] 2 g/kg BXD RI[3, 22, 23] 2 g/kg LSXSS RI[8]
13		5 mg/kg BXD RI[21] *20 mg/kg AcB/BcA RCS[9]	1.33 g/kg BXD RI[11] *1.5 g/kg LPXB6 F2[17] 1.8 g/kg LXS RI[6] 2 g/kg BXD RI[23] 2 g/kg B6XD2 F2[19] 2 g/kg LSXSS RI[8] 2 g/kg AXB/BXA RI[10]
14	5 mg/kg AMP in BXD RI[1] 8 mg/kg MA in BXD RI[12] 16 mg/kg MA in BXD RI[12]	10 mg/kg BXD RI[21] 40 mg/kg BXD RI[21]	1.5 g/kg LPXBALB F2[17] 2 g/kg LSXSS RI[8]
15	2 mg/kg MA STSL[20] 8 mg/kg MA in BXD RI[12] 16 mg/kg MA in BXD RI[12]	5 mg/kg BXD RI[16] 10 mg/kg BXD RI[21] 20 mg/kg AXB/BXA RI[2]	*1.5 g/kg B6XC3H F2[7, 17] 2 g/kg BXD RI[23]
16	8 mg/kg MA in BXD RI[12]	5 mg/kg BXD RI[21] 10 mg/kg BXD RI[18, 21] *20 mg/kg AcB/BcA RCS[9] 32 mg/kg BXD RI[24] 40 mg/kg BXD RI[21]	1.5 g/kg LPXBALB F2[17] 2 g/kg B6XD2 F2[19] 2 g/kg AXB/BXA RI[10]
17	5 mg/kg AMP AcB/BcA RCS[25] 16 mg/kg MA in BXD RI[12]	10 mg/kg BXD RI[18] 20 mg/kg AXB/BXA RI[2] *20 mg/kg AcB/BcA RCS[9] 32 mg/kg BXD RI[24]	2 g/kg BXD RI[3, 22, 23] 2 g/kg LSXSS RI[8]

Chr	Amphetamines	Cocaine	Ethanol
18	5 mg/kg AMP in BXD RI[1]	20 mg/kg AXB/BXA RI[2] *20 mg/kg AcB/BcA RCS[9] 10 mg/kg BXD RI[18] 10 mg/kg BXD RI[21]	2 g/kg BXD RI[3, 22, 23] 2 g/kg B6XD2 F2[19] 2 g/kg AXB/BXA RI[10]
19		5 mg/kg BXD RI[21] 10 mg/kg BXD RI[21]	2 g/kg AXB/BXA RI[10]
X	5 mg/kg AMP AcB/BcA RCS[25]		

A = A/J; AMP = amphetamine; BALB = BALB/cJ; C3H = C3H/HeJ; Ch = chromosome; LP = LP/J; LXS = inbred long sleep X inbred short sleep; LSXSS = long sleep X short sleep; MA = methamphetamine; RCS = recombinant congenic strain; STSL = short term selected lines.

[1](Alexander et al., 1996)
[2](Boyle and Gill, 2001)
[3](Cunningham, 1995)
[4](Demarest et al., 2001)
[5](Demarest et al., 1999)
[6](Downing et al., 2006)
[7](Downing et al., 2003b)
[8](Erwin et al., 1997)
[9](Gill and Boyle, 2003)
[10](Gill et al., 2000)
[11](Gora-Maslak et al., 1991)
[12](Grisel et al., 1997)
[13](Hitzemann et al., 1998)
[14](Hitzemann et al., 2000)
[15](Hitzemann et al., 2002)
[16](Jones et al., 1999)
[17](Malmanger et al., 2006)
[18](Miner and Marley, 1995)
[19](Palmer et al., 2006)
[20](Palmer et al., 2005)
[21](Phillips et al., 1998)
[22](Phillips et al., 1995)
[23](Phillips et al., 1996)
[24](Tolliver et al., 1994)
[25](Torkamanzehi et al., 2006)

genetic backgrounds (Gora-Maslak et al., 1991; Cunningham, 1995; Erwin et al., 1997; Hitzemann et al., 1998; Demarest et al., 1999; Gill et al., 2000; Hitzemann et al., 2000; Demarest et al., 2001; Downing et al., 2006; Malmanger et al., 2006; Palmer et al., 2006). These data provide strong evidence for a QTL for ethanol-induced stimulation on mouse chromosome 2. A significant QTL in a single mapping population is strong evidence of a QTL, but when a QTL has been mapped in multiple genetic crosses, like this QTL for ethanol sensitivity, these data provide a replication of the QTL finding and allow for finer resolution of the QTL location (Hitzemann et al., 2002).

In some cases there are QTL mapped on the same chromosome for more than one trait. In this situation there could be one gene that has pleiotropic effects on more than one phenotype, or different genes in the same region could affect the different traits. Case in point, there is evidence that a gene on mouse chromosome 9 accounts for part of the phenotypic variation in the response to amphetamines, cocaine, and ethanol. Genetic correlations have been observed between the acute locomotor responses to these drugs (Marley et al., 1998; Bergstrom et al., 2003; Kamens et al., 2005, 2006), providing evidence that a common gene (or genes) influences these behaviors. Therefore, it is possible that a single gene on mouse chromosome 9 may have pleiotropic effects on the acute locomotor responses to amphetamines, cocaine, and ethanol.

The studies in this dissertation sought to determine if a common gene on mouse chromosome 9 influenced the locomotor responses to cocaine, ethanol, and methamphetamine. The initial interval mapping data of these QTL are large with over 1,000 genes in the QTL regions (www.genome.ucsc.edu). Of the genes located in the QTL region, the α3 subunit of the nicotinic acetylcholine receptor (*Chrna3*) was

examined as a candidate gene. This gene was chosen as the candidate because it mapped to distal chromosome 9 (the location that the QTL mapping data had suggested were the most likely QTL location), and because there is evidence that nicotinic acetylcholine receptors are involved in the responses to these drugs (see below).

Neural Basis of Stimulation

Activation of the mesolimbic dopamine system is thought to be a common response to many abused drugs. Amphetamines, cocaine, and ethanol cause an increase in dopamine levels in the nucleus accumbens (a principal nucleus in this system) at doses which stimulate locomotor activity (Imperato and Di Chiara, 1986; Di Chiara and Imperato, 1988; Carboni et al., 1989; Robinson and Camp, 1990; Broderick, 1991). A simplified diagram of this circuitry can be found in Figure 3 (Amalric and Koob, 1993; Ikemoto, In Press; Pierce and Kalivas, 1997; Kalivas and Nakamura, 1999; Kalivas and Volkow, 2005; Nestler, 2005). The mesolimbic dopamine system is comprised of the ventral tegmental area, from which dopamine neurons project to the nucleus accumbens, prefrontal cortex, amygdale, and ventral pallidum. Within the ventral tegmental area, γ-amino butyric acid (GABA) interneurons provide negative input. Furthermore, GABA neurons from the nucleus accumbens and ventral pallidum provide negative feedback to the ventral tegmental area, while excitatory glutamate projections come from the prefrontal cortex. Excitatory glutamate neurons project from the amygdala to the nucleus accumens. There are reciprocal GABAergic projections between the nucleus accumbens and ventral pallidum. The ventral pallidum is an important structure because it connects the mesolimbic dopamine pathway with basal ganglia nuclei and motor cortices (via the

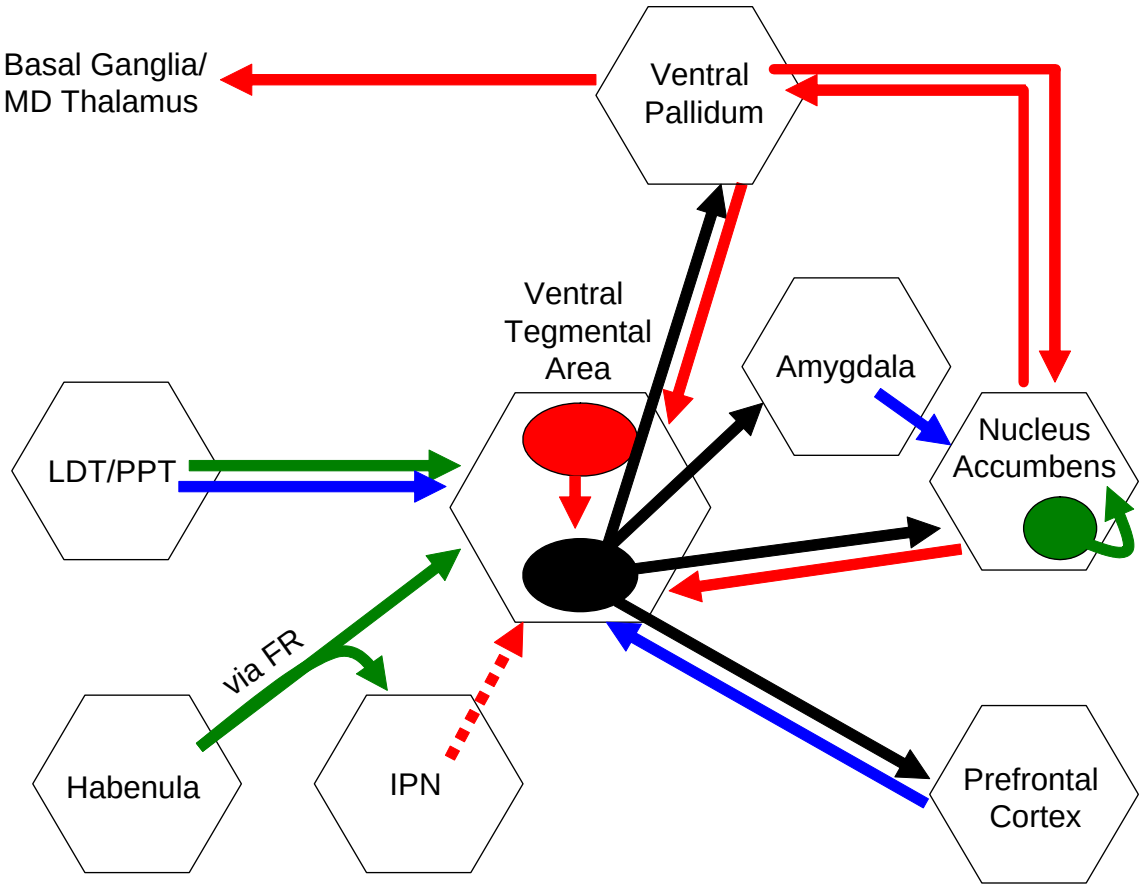


Figure 3. The mesolimbic circuitry and some associated neural connections. Red arrows indicate GABAergic neurons, blue arrows indicate glutamatergic neurons, black arrows indicate dopaminergic neurons, and green arrows indicate cholinergic neurons. Dotted red arrow indicates an assumed GABAergic projection neuron. MD = mediodorsal; LDT/PPT = laterodorsal tegmentum/pedunculopontine tegmentum; IPN = interpeduncular nucleus; FR = fasciculus retroflexus.

mediodorsal thalamus), which are important in controlling movement (for review see Albin et al., 1989; Hauber, 1998).

There are at least three of ways in which cholinergic neurons may modulate the mesolimbic dopamine circuit. Two cholinergic pathways innervate the ventral tegmental area and therefore, may modulate dopamine neurotransmission. Cholinergic interneurons in the striatum may also modulate this circuit (see Figure 3).

The ventral midbrain receives cholinergic input from the laterodorsal tegmental and pedunculopontine tegmental nuclei (Oakman et al., 1995). Cholinergic neurons from these areas innervate dopamine and GABA neurons in the ventral tegmental area and substantia nigra (Oakman et al., 1995; Blaha et al., 1996; Omelchenko and Sesack, 2005). When the cholinesterase inhibitor, neostigmine, was infused into the ventral tegmental area, dopamine levels in the nucleus accumbens increased, an effect which was blocked by lesions of the laterodorsal tegmentum, but not by lesions of the pedunculopontine tegmentum (Blaha et al., 1996). In contrast, pedunculopontine tegmentum lesions attenuated dopamine increases when neostigmine was infused into the substantia nigra (Blaha and Winn, 1993; Blaha et al., 1996). In addition to cholinergic neurons, the laterodorsal tegmental and pedunculopontine tegmental nuclei also send excitatory glutamate projections to the ventral midbrain (Oakman et al., 1995; Lanca et al., 2000; Omelchenko and Sesack, 2005). It appears that the cholinergic projection is involved in the initial stimulatory effect of the laterodorsal tegmentum on ventral tegmental area dopamine neurons, since intra-ventral tegmental area mecamylamine, a nonspecific nicotinic acetylcholine receptor antagonist, blocks the laterodorsal tegmentum stimulated dopamine increase in the nucleus accumbens (Forster and Blaha, 2000). Thus,

cholinergic neurons from the laterodorsal tegmentum and pedunculopontine tegmentum may be important for activation of mesolimbic dopamine system.

The habenulointerpeduncular pathway, also known as the dorsal diencephalic pathway, is another cholinergic pathway that feeds into the ventral midbrain. The major afferent pathway into the habenula is the stria medullaris (Sutherland, 1982; Ellison, 1994; Klemm, 2004). Some fibers of the stria medullaris make connections in the habenula while others course through the area to downstream targets, including the interpeduncular nucleus (Contestabile and Fonnum, 1983). Leaving the habenula, efferent fibers travel in the fasciculus retroflexus to the interpeduncular nucleus, ventral tegmental area, substantia nigra, and thalamic and hypothalamic nuclei (Sutherland, 1982; Ellison, 1994; Klemm, 2004). This pathway provides an alternative circuit for cholinergic modulation of dopamine neurons in the ventral tegmental area.

The habenulointerpeduncular pathway provides a negative tone on dopamine neurons in the ventral tegmental area. Lesions of the habenula have been shown to increase (Lisoprawski et al., 1980), while stimulation of the lateral habenula has been shown to inhibit dopamine cell firing (Christoph et al., 1986). Additionally, lesions of the stria medullaris and fasciculus retroflexus have been shown to increase dopamine metabolites in dopamine terminal fields such as the nucleus accumbens and prefrontal cortex (Nishikawa et al., 1986). Due to the overall negative tone that the habenulointerpeduncular pathway has on dopamine levels, it is accepted that there is a GABA projection from the interpeduncular nucleus to the ventral tegmental area, although clear neurochemical evidence of this is yet to be shown.

Finally, there are cholinergic interneurons located within the striatum (Fibiger, 1982; Woolf, 1991; Di Chiara et al., 1994). The striatum is involved in motor behavior; therefore, nicotinic acetylcholine receptors located in this brain region may also modulate drug-induced locomotor activation. Since cholinergic neurons innervate key structures of the mesolimbic dopamine system, nicotinic acetylcholine receptors may modulate the acute locomotor response to drugs of abuse.

Nicotinic Acetylcholine Receptors

A number of recent reviews have focused on nicotinic acetylcholine receptors (McGehee, 1999; Romanelli and Gualtieri, 2003; Dajas-Bailador and Wonnacott, 2004; Jensen et al., 2005; Arias et al., 2006; Romanelli et al., 2007). There are currently seventeen α, β, δ, γ, and ε nicotinic receptor subunits that form ligand-gated ion channels. The α1, β1, δ, γ, and ε subunits are found exclusively in muscle acetylcholine receptors, while the α2 – α10 and β2 – β4 subunits are found in the nervous system. These subunits form pentameric ion channels that are permeable to cations. Acetylcholine receptors come in two varieties: homomeric and heteromeric. In the central nervous system the primary homomeric receptor is the α7 receptor. Heteromeric receptors are usually comprised of two α and three β subunits with the most abundant in the brain being the α4β2 receptor. Table 2 provides details on which subtypes form heteromeric versus homomeric channels. The acetylcholine binding site on the heteropentameric receptors is at the interface of α and β subunits, thus there are two binding sites per receptor. There are five binding sties on the homopentameric receptors at the interface of the α subunits. Acetylcholine receptors are localized on both the pre- and post-synaptic terminals where

Subunit	Homopentameric	Forms Functional Receptors With	Involved in Agonist Binding
α2	No	α5, β2, β3, β4	Yes
α3	No	α5, β2, β3, β4	Yes
α4	No	α5, β2, β3, β4	Yes
α5	No	α2, α3, α4, α6, β2, β4	No
α6	No	α4, α5, β2, β3	Yes
α7	Yes	α7	Yes
α8	Yes	α8	Yes
α9	Yes	α9, α10	Yes
α10	No	α9, α10	Yes
β2	No	α2, α3, α4, α5, α6	Yes
β3	No	α2, α3, α4, α6, β2, β4	No
β4	No	α2, α3, α4, α5	Yes

Table 2. Nicotinic acetylcholine receptor subunits found in the nervous system. Listed are the twelve nicotinic acetylcholine receptors found in the nervous system. Next to each subunit there is information regarding whether that subunit forms homopentameric receptors or not, what other subunits it can form functional receptors with, and if the subunit is known to be involved in agonist binding.

they depolarize the membrane by allowing the influx of Na^+ or Ca^{2+} ions. A number of antagonists currently exist that block acetylcholine receptors, those discussed in this dissertation are listed in Table 3 (McGehee, 1999; Romanelli and Gualtieri, 2003; Dajas-Bailador and Wonnacott, 2004; Jensen et al., 2005; Arias et al., 2006; Romanelli et al., 2007).

The nicotinic acetylcholine receptor subunits can have differential effects on receptor function. For example, α3β2 receptors desensitize more rapidly than α3β4 receptors (Bohler et al., 2001). The α subunit also appears to mediate the rate of desensitization since receptors containing an α3 subunit desensitize faster than those containing either a α2 or α4 (Le Novere et al., 2002). Not only is the rate of desensitization influenced by the subunit composition, but the other channel properties are also affected. For example, the subunit composition is important for receptor function. The pattern of channel functioning as defined by the EC_{50}, or concentration of agonist at which 50% of the maximum response is achieved, appears to be influenced by the combination of α and β subunits in the receptor (Le Novere et al., 2002). In regards specifically to α3 containing nicotinic acetylcholine receptors, acetylcholine is more potent at α3β2 receptors than α3β4 receptors (Cachelin and Rust, 1994).

Nicotinic Acetylcholine Receptors are Involved in Behavioral Stimulation

The role of nicotinic acetylcholine receptors in ethanol-induced stimulation has been examined in both humans and animals. In humans, mecamylamine, a nonspecific nicotinic acetylcholine receptor antagonist, was shown to decrease the subjective stimulant effects of alcohol (Chi and de Wit, 2003) and the physiologic stimulant effects (Blomqvist et al., 2002). A decrease in ethanol-induced locomotor stimulation has also

Antagonist	Receptor Specificity	Crosses the Blood Brain Barrier
18-Methoxycoronaridine	α3β4	Yes
α-Conotoxin AuIB	α3β4	No
α-Conotoxin MII	α3β2, β3, and α6	No
α-Conotoxin OmIA	α3β2	No
α-Conotoxin PIA-analogue	α6	No
α-Conotoxin Vc1.1	α3	No
Dihydro-β-erythroidine	α4β2	Yes
Hexamethonium	Nonspecific	No
Mecamylamine	Nonspecific	Yes
Methyllycaconitine	α7	Yes

Table 3. The acetylcholine receptor antagonists referred to in this dissertation. Listed next to each antagonist is the information regarding which receptors the drug blocks and if it is capable of crossing the blood brain barrier.

been observed in animals. Systemic mecamylamine decreased ethanol-induced stimulation as well as ethanol-induced increase in extracellular dopamine in the nucleus accumbens (Blomqvist et al., 1992; Larsson et al., 2002), and this effect on dopamine was localized to acetylcholine receptors in the ventral tegmental area (Blomqvist et al., 1997). In the ventral tegmental area, a number of nicotinic acetylcholine subunits have been detected. Both dopamine and GABA neurons in this region express the α3-α7 and β2-β4 nicotinic acetylcholine receptor subunits (Klink et al., 2001; Azam et al., 2002), and receptors containing any of these subunits could be involved in the acute locomotor response to ethanol

In NMRI outbred mice, two other nicotinic acetylcholine receptor antagonists have been tested. Dihydro-β-erythroidine is most selective for α4β2 nicotinic acetylcholine receptors, while methyllycaconitine is a selective antagonist at α7 nicotinic acetylcholine receptors. Neither of these drugs attenuated the locomotor stimulant effects of ethanol or blocked accumbal dopamine increase like mecamylamine, providing no evidence of a role for these receptor subtypes (Larsson et al., 2002). To identify which nicotinic acetylcholine receptors were involved in this response, Larsson and colleagues administered different conotoxins into the ventral tegmental area to see if they could attenuate ethanol-induced stimulation. α-conotoxin MII, which is specific for α3β2-, β3-, and α6-containing nicotinic acetylcholine receptors, attenuated ethanol-induced locomotor activation, but α-conotoxin PIA-analogue, which is specific for α6-containing nicotinic acetylcholine receptors, did not (Larsson et al., 2004; Jerlhag et al., 2006). These data suggest that α3β2- or β3-containing nicotinic acetylcholine receptors are involved in ethanol-induced locomotor activation. To my knowledge, the role of α3β4

nicotinic acetylcholine receptors, where mecamylamine is most potent (Papke et al., 2001), in ethanol-induced stimulation has not been specifically examined.

The evidence that nicotinic acetylcholine receptors are involved in the acute locomotor response to psychostimulants is mixed. In rats, pretreatment with mecamylamine, dihydro-β-erythroidine, methyllycaconitine, and 18-methoxycoronaridine (α3β4-specific) had no effect on the locomotor response to amphetamines (Szumlinski et al., 2000a; Schoffelmeer et al., 2002; Escubedo et al., 2005). In contrast, the effect of nicotinic acetylcholine receptors on cocaine stimulation is receptor subtype-dependent. Mice lacking the gene encoding the α4 subunit of the nicotinic acetylcholine receptor were more stimulated by cocaine than were wildtype mice (Marubio et al., 2003), but mice lacking the β2 subunit showed a normal response to an acute injection of this drug. The role of α3β4 nicotinic acetylcholine receptors is unclear. In an initial study, blocking α3β4 receptors with 18-methoxycoronaridine had effects on cocaine stimulation that were time dependent. When 18-methoxycoronaridine was administered 1 hour prior to cocaine it decreased cocaine stimulation, but when given 19 hours prior to cocaine it increased activation (Maisonneuve et al., 1997). In a later study, pretreatment with 18-methoxycoronaridine (19 h) had no effect on cocaine-induced stimulation (Szumlinski et al., 2000b). These data provide some evidence that nicotinic acetylcholine receptors may be involved in the acute locomotor response to ethanol and psychostimulants.

Dissertation Goals

There were three goals of this dissertation. The first goal was to establish whether nicotinic acetylcholine receptors are involved in sensitivity to the stimulant effects of

cocaine, ethanol, and methamphetamine. To accomplish this, strains or lines of mice that are highly sensitive to the stimulant effects of these drugs were examined. FAST mice were chosen because they were selectively bred for their increased sensitivity to an acute injection of ethanol (see above) and D2 mice were chosen because they exhibit robust behavioral activation following administration of cocaine, ethanol, and methamphetamine compared to other strains of mice. I tested whether pretreatment with mecamylamine would attenuate the behavioral activation produced by these drugs. Furthermore, when mecamylamine did attenuate locomotor stimulation, hexamethonium, which does not cross the blood brain barrier, was then tested to determine if the effect was due to central nicotinic acetylcholine receptors. In addition, since FAST mice are a specific model of genetic susceptibility to ethanol stimulation, the effect of additional nicotinic acetylcholine receptor antagonists (dihydro-β-erythroidine and methyllycaconitine) on ethanol-induced locomotor stimulation were examined in these mice (Chapter 2).

The second goal of this dissertation was to confirm the presence of a QTL on mouse chromosome 9 for cocaine-, ethanol-, and methamphetamine-induced stimulation. I first set out to confirm the presence of a QTL using reciprocal congenic strains. When a QTL was detected for the locomotor response to ethanol and methamphetamine, an attempt was made to fine map the location of the gene. These studies are reported in Chapters 3 and 4.

Finally, my third goal was to explore the viability of a single gene (the gene coding for the α3 subunit of the nicotinic acetylcholine receptor) as a viable candidate gene for ethanol-induced stimulation. To address this goal, since no specific pharmacological antagonists for α3-containing nicotinic acetylcholine receptors are

commercially available, molecular approaches were used. *Chrna3* expression in the FAST and SLOW lines was examined as well as in a congenic strain that captured the QTL for ethanol stimulation. After examining *Chrna3* expression in the FAST and SLOW lines (Chapter 2), it was determined that the database used to determine the location of *Chrna3* in 2003 was incorrect. Instead of mapping to distal chromosome 9, *Chrna3* maps to mid-chromosome 9 (54.8 Mb) in a cluster of nicotinic acetylcholine receptor subunit genes. In addition to the α3 subunit, the α5 and β4 nicotinic acetylcholine subunits also map to this region. Therefore, in the congenic strain that captured the QTL for ethanol-induced stimulation expression of all three of these genes was analyzed (Chapter 3). Finally, protein expression was analyzed in the congenic strain using a receptor binding assay. Cytisine-resistant epibatidine binding was quantified as a measure of α3-containing nicotinic acetylcholine receptors (Chapter 3 appendix). These data are included in the appendix because there are concerns about the specificity of the assay, leaving inconclusive results regarding the involvement of α3-containing nicotinic acetylcholine receptors in ethanol stimulation.

Chapter 2: A Role for Neuronal Nicotinic Acetylcholine Receptors in Ethanol-Induced Stimulation, but Not Cocaine- or Methamphetamine-Induced Stimulation

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Abstract

Rationale. Cocaine, ethanol, and methamphetamine are widely abused substances. These drugs share the ability to stimulate locomotor activity in mice. Locomotor stimulation is a putative endophenotype for drug abuse. Understanding the biological basis of the acute locomotor response to cocaine, ethanol, and methamphetamine may provide a greater understanding of drug and alcohol abuse. *Objectives.* In these studies I set out to determine if neuronal nicotinic acetylcholine receptors, in particular those containing an α3 subunit, were involved in the acute locomotor responses to these drugs. *Methods.* A panel of acetylcholine receptor antagonists was used to determine if nicotinic receptors were involved in ethanol-, and psychostimulant-induced locomotor stimulation. The involvement of these receptors was examined in strains of mice (FAST and DBA/2J) that are extremely sensitive to this effect. To determine if the α3 subunit of the acetylcholine receptor is involved in ethanol stimulation, relative gene expression was examined between mice selectively bred for high and low response to ethanol. *Results.* Mecamylamine, but not hexamethonium, attenuated the acute locomotor response to ethanol. Furthermore, the acetylcholine receptor antagonists dihydro-β-erythroidine and methyllycaconitine had no effect on this response. The α3 subunit of the acetylcholine receptor was not differentially expressed between mice bred for extreme differences in ethanol-induced stimulation. Mecamylamine had no effect on psychostimulant-induced locomotor activity. *Conclusions.* Neuronal nicotinic receptors are involved in ethanol-, but not psychostimulant-induced, locomotor stimulation. These studies rule out the involvement

of some nicotinic receptor subtypes, but more work is needed to determine the specific receptors types involved in this behavior.

Introduction

Cocaine, ethanol, and methamphetamine are commonly abused substances, and share the ability to cause locomotor stimulation in mice (e.g., Crabbe et al., 1983; Dudek et al., 1991; Crabbe et al., 1994; Grisel et al., 1997; Marley et al., 1998; Phillips et al., 1998; Downing et al., 2006). Locomotor stimulation has been suggested to be an important endophenotype for drug abuse (Gabbay, 2005). The use of endophenotypes to understand the complex biological basis of behavior has grown in recent years. In work to identify genes that underlie alcoholism, the use of endophenotypes has proven more successful than using the clinical diagnosis of alcohol dependence to map genes (Dick et al., 2006).

Genetic correlations have been observed between the acute locomotor response to ethanol and psychostimulants. FAST and SLOW mice were selectively bred for their differential sensitivity to the stimulant effects of ethanol (Crabbe et al., 1987; Phillips et al., 1991; Shen et al., 1995; Phillips et al., 2002b). FAST mice, which were bred for heightened sensitivity to the stimulant effects of ethanol, were more sensitive to the stimulant effects of cocaine and methamphetamine compared to SLOW mice (Bergstrom et al., 2003). An independent set of selected lines has been bred for high (HMACT) or low (LMACT) sensitivity to an acute injection of methamphetamine. Similar to the FAST and SLOW mouse lines, the HMACT line was more stimulated by ethanol and cocaine compared to the LMACT line (Kamens et al., 2005, 2006). Furthermore, lines of mice selectively bred for sensitivity to cocaine's stimulatory effects also differed in response to amphetamine, such that the lines that were more stimulated by cocaine were more stimulated by amphetamine (Marley et al., 1998). No data exists regarding

sensitivity to ethanol in these lines. These data provide support that common genes and neural mechanisms may underlie the acute locomotor responses to ethanol, cocaine, and methamphetamine.

The mesolimbic dopamine system has been implicated in drug reward and behavioral activation (Amalric and Koob, 1993; Pierce and Kalivas, 1997; Kalivas and Nakamura, 1999). Elevations in dopamine levels in the nucleus accumbens, one of the key structures in this pathway, have been observed following the administration of ethanol, cocaine, and methamphetamine at doses which stimulate locomotor activity (Imperato and Di Chiara, 1986; Di Chiara and Imperato, 1988; Carboni et al., 1989; Larsson et al., 2002; Izawa et al., 2006). The mesolimbic dopamine system is a final common pathway through which all three of these drugs produce stimulation; therefore, modulation of this pathway may be important for this behavior. Acetylcholine receptors are expressed in this pathway. Specifically, the α3 subunit of the nicotinic acetylcholine receptor is expressed in midbrain neurons; therefore, these receptors are well positioned to modulate dopamine neuron activity (Klink et al., 2001; Azam et al., 2002). Nicotinic receptors containing this subunit may be involved in drug-induced stimulation.

Pharmacologic data have implicated α3-containing nicotinic acetylcholine receptors in ethanol-, cocaine-, and methamphetamine-induced behaviors. However, other nicotinic receptor subunit genes are also expressed in dopamine circuits associated with drug-related stimulation. Larsson and colleagues (2002) used a panel of acetylcholine receptor antagonists to determine the role of these receptors in ethanol-induced locomotor stimulation. They showed that mecamylamine, a nonspecific nicotinic acetylcholine receptor antagonist, can attenuate ethanol-induced locomotor

stimulation in NMRI outbred mice. In contrast, dihydro-β-erythroidine, an α4β2 receptor antagonist, and methyllycaconitine, an α7 receptor antagonist, had no effect on ethanol stimulation (Larsson et al., 2002). These data provide evidence against α4β2 and α7 nicotinic receptors in this response. Further, when α-conotoxin MII, an antagonist of α3β2, β3, and α6 containing nicotinic receptors, was injected into the ventral tegmental area, ethanol-induced stimulation was decreased (Larsson et al., 2004). The involvement of α6-containing nicotinic receptors in this response was ruled out by using α-conotoxin PIA-analogue, which specifically blocks α6 receptors; α-conotoxin PIA-analogue did not alter the acute response to ethanol (Jerlhag et al., 2006). Together these data provide support for the involvement of α3β2 nicotinic receptors in ethanol-induced locomotor stimulation, but also leave open the possibility that β3-containing receptors are important.

Further support for the involvement of α3-containing receptors in the behavioral response to ethanol, cocaine, and methamphetamine comes from the use of 18-methoxycoronaridine. 18-methoxycoronaridine is a potent α3β4 nicotinic antagonist (Glick et al., 2002). 18-methoxycoronaridine does not appear to alter the acute locomotor response to methamphetamine or cocaine (Szumlinski et al., 2000a; 2000b), but has been shown to decrease self administration of psychostimulants and ethanol. Administration of 18-methoxycoronaridine has been shown to attenuate operant responding for cocaine and methamphetamine (Glick et al., 1996; Glick et al., 2000; Maisonneuve and Glick, 2003; Pace et al., 2004). Additionally, this drug was shown to decrease ethanol consumption and preference in a 2-bottle choice paradigm (Rezvani et al., 1997), but 18-methoxycoronaridine has not been used to study ethanol stimulation. These data provide

support for the hypothesis that α3-containing nicotinic acetylcholine receptors are involved in the behavioral responses to these drugs.

The goal of these experiments was to evaluate the role of α3-containing acetylcholine receptors in the acute locomotor responses to ethanol, cocaine, and methamphetamine. 18-methoxycoronaridine is not commercially available; therefore, in these studies a panel of nicotinic acetylcholine antagonists (mecamylamine, hexamethonium, dihydro-β-erythroidine, and methyllycaconitine) was used. While there is evidence for a role of neuronal nicotinic receptors in the acute response to ethanol in outbred mice (Blomqvist et al., 1992; Larsson et al., 2002), I set out to test if these receptors are involved in ethanol-induced stimulation in two genetic models of high ethanol sensitivity, the selectively bred FAST mice and DBA/2J (D2) mice, an inbred strain that by chance exhibits robust locomotor stimulation following ethanol (Dudek et al., 1991; Crabbe et al., 1994). The involvement of neuronal nicotinic receptors was examined in the acute locomotor response to ethanol in FAST and D2 mice. I predicted that mecamylamine would attenuate ethanol-induced stimulation, but that hexamethonium, a nonspecific nicotinic acetylcholine receptor antagonist that does not cross the blood brain barrier, would not.

A more detailed investigation into which type of nicotinic receptors may be involved in the acute locomotor response to ethanol was performed in FAST mice since they were selectively bred for this trait. I predicted that dihydro-β-erythroidine and methyllycaconitine would have no effect on ethanol stimulation. Since the hypothesis was that α3-containing nicotinic receptors are involved in ethanol stimulation, quantitative real time-PCR (qRT-PCR) was used to examine potential line differences in

α3 gene expression. I predicted that SLOW mice would have more α3 gene expression than FAST mice. This prediction was based on the gene for the α3 subunit of the nicotinic acetylcholine receptor being expressed in midbrain dopamine and γ-amino butyric acid (GABA) neurons (Klink et al., 2001; Azam et al., 2002). α3-containing acetylcholine receptors do not appear to be involved in striatal dopamine release when using a synaptosome preparation (Salminen et al., 2004), but activation of this type of receptor on GABA neurons in the ventral tegmental area may lead to decreased activation of dopaminergic neurons and locomotor activity.

Finally, I wanted to examine if the effects of nicotinic receptors on ethanol-induced stimulation generalize to psychostimulants. D2 mice are extremely sensitive to cocaine- and methamphetamine-induced locomotor stimulation (Grisel et al., 1997; Phillips et al., 1998). To test the role of nicotinic receptors in the acute locomotor response to these drugs the nonspecific antagonist mecamylamine was used. I predicted that mecamylamine would attenuate cocaine- and methamphetamine-induced stimulation.

Methods

Mice were maintained in the Portland Veterans Affairs Veterinary Medical Unit. Mice were housed 2-5 per cage in standard plastic cages with Bed-o cobs' (The Andersons, Maumee, OH) lining. Animals had food (Purina Laboratory Rodent Chow #5001; Purina Mills, St. Louis, MO) and water available *ad libitum*. Mice were kept in colony rooms on a 12h light-dark cycle (lights on at 0600 h) with the temperature maintained at 21 ± 2˚C. All procedures were approved by the Portland Veterans Affairs Medical Center Institutional Animal Care and Use Committee, and were consistent with the *Guide for the Care and Use of Laboratory Animals* (1996).

All mice were 50 – 101 days old and experimentally naïve at the start of testing. Mice from a single cage were distributed across groups, to avoid all mice from a single family/cage being assigned to a single drug group.

Subjects

FAST Mice

Male and female FAST-1, SLOW-1, FAST-2, and SLOW-2 mice were used for these experiments. The selection of the FAST and SLOW lines has been described in detail elsewhere (Crabbe et al., 1987; Phillips et al., 1991; Shen et al., 1995; Phillips et al., 2002b). Briefly, the FAST and SLOW mice were created through a selective breeding process starting from the heterogenous HS/Ibg stock (McClearn et al., 1970). Mice were bred for 37 generations for their high (FAST) or low (SLOW) sensitivity to the acute locomotor stimulant effects of ethanol. First litter offspring of each generation were tested for their response to ethanol (1.5 – 2 g/kg) for 4 min beginning 2 min after injection in circular open fields (LVE model PAC-001; Lehigh Valley, PA) on day 1. Testing was repeated after an injection of saline on day 2. The difference in locomotor activity was used as the selection criterion (day 1 ethanol response – day 2 saline response). The breeders of the FAST lines were chosen for their extremely high activity scores, while the breeders of the SLOW lines were chosen for their low scores. After 37 generations, selection was relaxed and breeders were arbitrarily chosen within each line. The lines continued to remain divergent in their response to ethanol after selection pressure had been relaxed (Phillips et al., 2002b). Two replicated (1 and 2) lines in each direction were concurrently bred and maintained as independent breeding populations. Mice used in the behavioral studies came from $S_{37}G_{75}$ - G_{81}, while mice for the qRT-PCR

experiment came from $S_{37}G_{84}$ - G_{85} (where S_{xx} refers to the number of selected generations and G_{yy} refers to total number of generations that have elapsed since selection began). For pharmacology experiments, only FAST mice were used since I was interested in testing the effect of nicotinic receptor antagonists on ethanol's stimulant response and SLOW mice show no locomotor activation or show locomotor depression following ethanol (Shen et al., 1995; Palmer et al., 2002; Phillips et al., 2002b). Only females were used for the hexamethonium experiment because they were more available at the time of testing and because sex was not found to interact with independent variables in the other experiments.

D2 Mice

Male D2 mice were either bred at the Portland Veterans Affairs Medical Center or obtained from The Jackson Laboratory (Bar Harbor, ME). When mice were obtained from The Jackson Laboratory they acclimated to the animal facility for at least 2 weeks prior to being tested. Mice bred in our facility were derived from D2 mice obtained from The Jackson Laboratory; new breeder pairs are obtained each year to avoid spontaneous mutations arising in our colony.

Drugs

Cocaine hydrochloride, dihydro-β-erythroidine hydrobromide, hexamethonium bromide, mecamylamine hydrochloride, methamphetamine hydrochloride, and methyllycaconitine citrate were purchased from Sigma (St. Louis, MO). Two hundred proof ethyl alcohol was obtained from Pharmco (Brookfield, CT). All nicotinic antagonists were dissolved in physiological saline (0.9% NaCl; Baxter Healthcare

Corporation, Deerfield, IL) to appropriate concentrations, while ethanol was diluted in saline to 20% v/v. The injection volume of all nicotinic antagonists was 10 ml/kg body weight.

Apparatus

Eight AccuScan automated activity monitors (Columbus, OH) were used to measure locomotor activity. The monitors have 8 photocell beams along two sides and detectors on the opposite sides. The activity monitors record the number of photocell breaks which is translated into distance traveled (in cm). Inside each monitor sat a 40 x 40 x 30 cm (l x w x h) clear acrylic test chamber. The chamber and monitor were encased by a black insulated acrylic chamber to separate the testing environment from the external environment. Inside the chamber was a fan to provide background noise and an 8-W fluorescent light for illumination. Because mice were tested between 0800 – 1600 h, the light was on for consistency with their normal light/dark cycle.

Procedure

Mice were moved to the testing room 45 – 60 min prior to the start of the experiment to allow time to acclimate. Animals were weighed and placed into individual holding cages while syringes were prepared (for up to 10 min prior to testing). FAST mice were first injected with one dose of one of four pretreatment drugs, dihydro-β-erythroidine (0, 0.5, 1, 1.5, or 2 mg/kg), hexamethonium (0, 2, 4, 6, or 8 mg/kg), mecamylamine (0, 1, 2, 3, 4, or 6 mg/kg), or methyllycaconitine (0, 1, 2, 3, or 4 mg/kg). Ten min later they received a second injection of either ethanol (2 g/kg) or saline. The 2 g/kg dose of ethanol was chosen because FAST-1 and FAST-2 mice show their greatest

stimulation response to this ethanol dose (Palmer et al., 2002), and it was the dose used for most selection generations. D2 mice were treated with mecamylamine (0, 1, 2, 3, or 4 mg/kg) or hexamethonium (0, 2, 4, 6, or 8 mg/kg) immediately prior to an injection of ethanol (1.5 g/kg), cocaine (10 mg/kg), or methamphetamine (2 mg/kg). These doses were chosen because they were known to produce robust behavioral activation in this strain (Dudek et al., 1991; Phillips et al., 1998; Kamens et al., 2005). The pretreatment times and doses of nicotinic antagonist drugs were based on prior work examining the effects of these drugs on behaviors (Blomqvist et al., 1992; Gommans et al., 2000; Larsson et al., 2002; Damaj et al., 2003) as well as pilot testing in these mice. Immediately following the second injection, animals were placed into the center of the activity monitor where locomotor activity was measured for 15- (ethanol) or 30-min (cocaine and methamphetamine) in 5-min epochs. At the end of the test session animals that received ethanol had a 20 µl blood sample taken from the retro-orbital sinus to determine blood ethanol concentrations (BEC). The blood was put into tubes containing 50 µl $ZnSO_4$ (5%) and placed on ice until processing at which time 50 µl $Ba(OH)_2$ (0.3 N) and 300 µl dH_2OH were added to the tube. Following centrifugation, supernatant was removed and BECs were determined by gas chromatography (Agilent 6890) following procedures standard in our laboratory (Boehm et al., 2000).

RNA isolation and qRT-PCR

Tissue and RNA preparation

Naïve male and female FAST and SLOW mice were cervically dislocated before being decapitated; brains were removed and immediately frozen in isopentane. Brains were stored frozen at -80˚C until they could be processed. RNA was extracted using a

guanidinium isothiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987, 2006). Briefly, brains were homogenized in RNA Stat-60 (Tel-Test, Inc. Friendswood, TX) using a polytron tissue homogenizer (Brinkmann, Westbury, NY). Chloroform (0.2 ml) was mixed with 1 ml of brain homogenate and was incubated at room temperature. The homogenate was centrifuged, and RNA from the aqueous phase was removed and put into a new tube. RNA was precipitated out with the addition of isopropanol (0.5 ml), and allowed to sit at room temperature for 10 min before centrifugation. The RNA pellet was then washed with 0.5 ml 75% ethanol and centrifuged. The RNA was allowed to air dry before being resuspended in 50 µl diethylpyrocarbonate (DEPC) treated water. The sample was cleaned of DNA contamination using the DNA-Free RNA Kit (Zymo Research, Orange, CA), using the manufacturer's specifications. Briefly, the RNA was incubated for 15 min at 37˚C with RNase-free DNase I and 10X DNase I buffer to degrade DNA from the sample. The RNA was mixed with a RNA binding buffer before being put onto a Zymo-Spin IC column. The column was washed twice and RNA was eluted with the addition of DEPC water directly onto the column. RNA quality was assessed with a spectrophotometer (Eppendorf, Hamburg, Germany; ratio 260 nm/280 nm = 1.8 - 2; Chomczynski and Sacchi, 2006) and confirmed by gel electrophoresis.

Reverse transcription and qRT-PCR

One microgram of total RNA was reverse transcribed using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). To each RNA sample, 10 µl 10X RT Buffer, 4 µl 25X dNTPs, 10 µl 10X Random Primers, and 5 µl MultiScribe RT

was added. The mixture was incubated at 25˚C for 10 min followed by 37˚C for 2 h in an iCycler (Bio-Rad, Hercules, CA).

Pre-designed TaqMan gene expression assays (Applied Biosystems, Foster City, CA) were obtained for the *Chrna3* (the α3 subunit of the acetylcholine receptor) gene and the control *Hprt1* (hypoxanthine guanine phosphoribosyl transferase 1) gene. *Hprt1* was used as the control gene because it has been validated as a reference gene in studies using mouse models of neurological diseases (e.g., Meldgaard et al., 2006). qRT-PCR reactions were run using an iCycler (Bio-Rad, Hercules, CA). Five µl of cDNA were added to 10 µl TaqMan Universal Master Mix, 1 µl of the gene specific primers, 0.4 µl fluorescein, and 3.6 µl DEPC water. The reaction began at 50˚C for 2 min followed by 95˚C for 10 min; the sample then went through 40 cycles of denaturing at 95˚C for 15 sec followed by 1 min at 60˚C that allowed for the primers to anneal and for amplification. All samples were run in triplicate and the average crossing threshold (C_t) was used as a measure of relative quantification for both the *Chrna3* and *Hprt1* genes. For analysis, the average C_t of the control *Hprt1* gene was subtracted from the C_t of the *Chrna3* gene. Relative expression, based on the $\Delta\Delta$ C_t method, was defined as 2 to the power of the negative average expression of the FAST mice minus each individual value (this was done independently for each replicate).

Analyses

Statistica (StatSoft, Tulsa, OK) was used for all statistical analyses. Data were analyzed using factorial analysis of variance (ANOVA) with the alpha level set at 0.05. Depending on the study, a number of independent variables were used including: replicate, line, sex, pretreatment drug dose, and challenge drug dose. Interactions

involving 3 or more factors were broken down using ANOVAs with fewer factors; two-way interactions were analyzed using simple effects and Newman-Keuls test for post-hoc comparisons.

Results

FAST Mice

Mecamylamine attenuated ethanol stimulation in both the FAST-1 and FAST-2 lines of mice (Figure 4). For all ethanol experiments, data from the last 10 min of the 15 min test are presented because the results were strongest during this time; however, similar results were obtained for all time periods examined. Data were analyzed using a 4-way ANOVA with replicate, sex, mecamylamine dose, and ethanol dose as factors. There was a main effect of replicate ($F_{1, 498}$=11.2, p<0.001) and a significant replicate X ethanol dose interaction ($F_{1, 498}$=85.8, p<0.001), therefore all further analyses were performed separately on the data from the FAST-1 and FAST-2 lines. Because there were no main effects or interactions with sex, male and female data were combined.

For FAST-1 mice there was a significant pretreatment X ethanol dose interaction ($F_{5, 272}$=3.7, p<0.01); pretreatment with mecamylamine decreased ethanol-induced stimulation, but did not affect saline activity. There was a significant simple main effect when data from groups that received an ethanol injection were included in the analysis; there was less stimulation in the groups that received 3, 4, or 6 mg/kg mecamylamine compared to the saline pretreated animals or animals that received a pretreatment of 1 mg/kg mecamylamine. Additionally, mice that were pretreated with 6 mg/kg mecamylamine prior to an ethanol injection showed reduced stimulation compared to

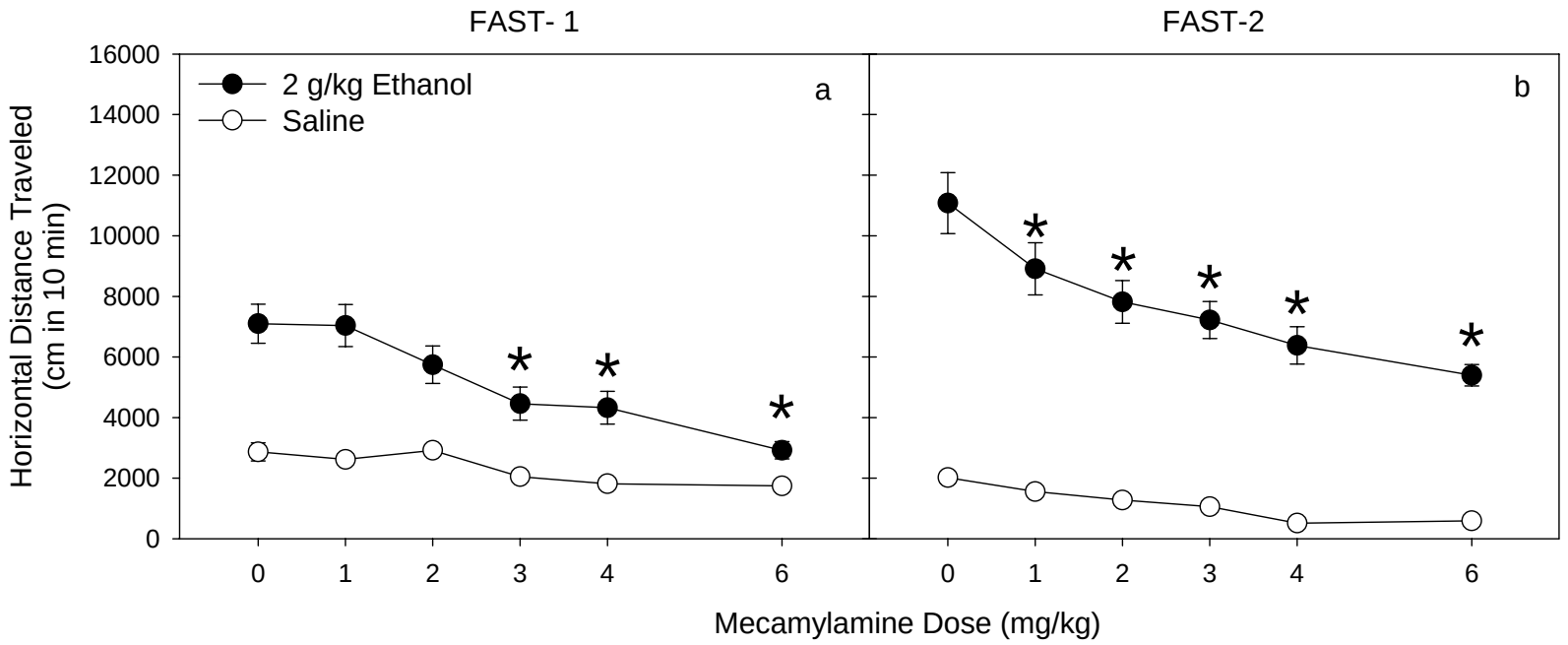

Figure 4. Mecamylamine attenuated ethanol-induced stimulation in (a**) FAST-1 and (**b**) FAST-2 mice.** Data (mean ± SEM) are from the last 10 min of a 15 min test. N = 20 - 26 per group. *Significantly different from the ethanol control group.

animals that received a 2 mg/kg mecamylamine pretreatment (p's < 0.01). Mecamylamine did not significantly decrease activity following saline, as indicated by non-significant simple main effect for the saline treated groups. BECs are missing for 17 animals due to blood processing errors. This resulted in data missing from 3 mice from each of the saline, 1, 2 and 6 mg/kg mecamylamine pretreatment groups and 4 data points missing from the 3 and 4 mg/kg mecamylamine pretreatment groups. Even with this data loss, the smallest number of blood sample data points in a single group was 21. There was no effect of pretreatment with mecamylamine on BECs 15-min post-ethanol injection in FAST-1 mice (mean ± SEM: 2.26 ± .07, 2.39 ± .08, 2.16 ± .08, 2.36 ± .06, 2.19 ± .09, 2.21 ± .07 for saline, 1, 2, 3, 4 and 6 mg/kg mecamylamine respectively).

A significant pretreatment X ethanol dose interaction was also observed in FAST-2 mice ($F_{5, 250}$=4.0, p<0.01). Ethanol-induced stimulation was reduced in all groups of animals receiving pretreatment with mecamylamine compared to groups that received a pretreatment with saline. Pretreatment with 6 mg/kg mecamylamine reduced ethanol stimulation to a greater extent than pretreatment with 2 mg/kg mecamylamine (p's < 0.05). Pretreatment with mecamylamine did not significantly alter saline activity; the simple main effect for the saline groups was not significant. Pretreatment with mecamylamine did not alter BECs in FAST-2 mice (mean ± SEM: 2.22 ± .09, 2.15 ± .09, 2.31 ± .06, 2.22 ± .08, 2.18 ± .08, 2.34 ± .06 for saline, 1, 2, 3, 4 and 6 mg/kg mecamylamine respectively).

None of the other nicotinic receptor antagonists tested significantly reduced ethanol-induced stimulation in FAST mice (Figures 5, 6, 7, see figure legends for additional statistics). In all studies, FAST mice were significantly more stimulated when

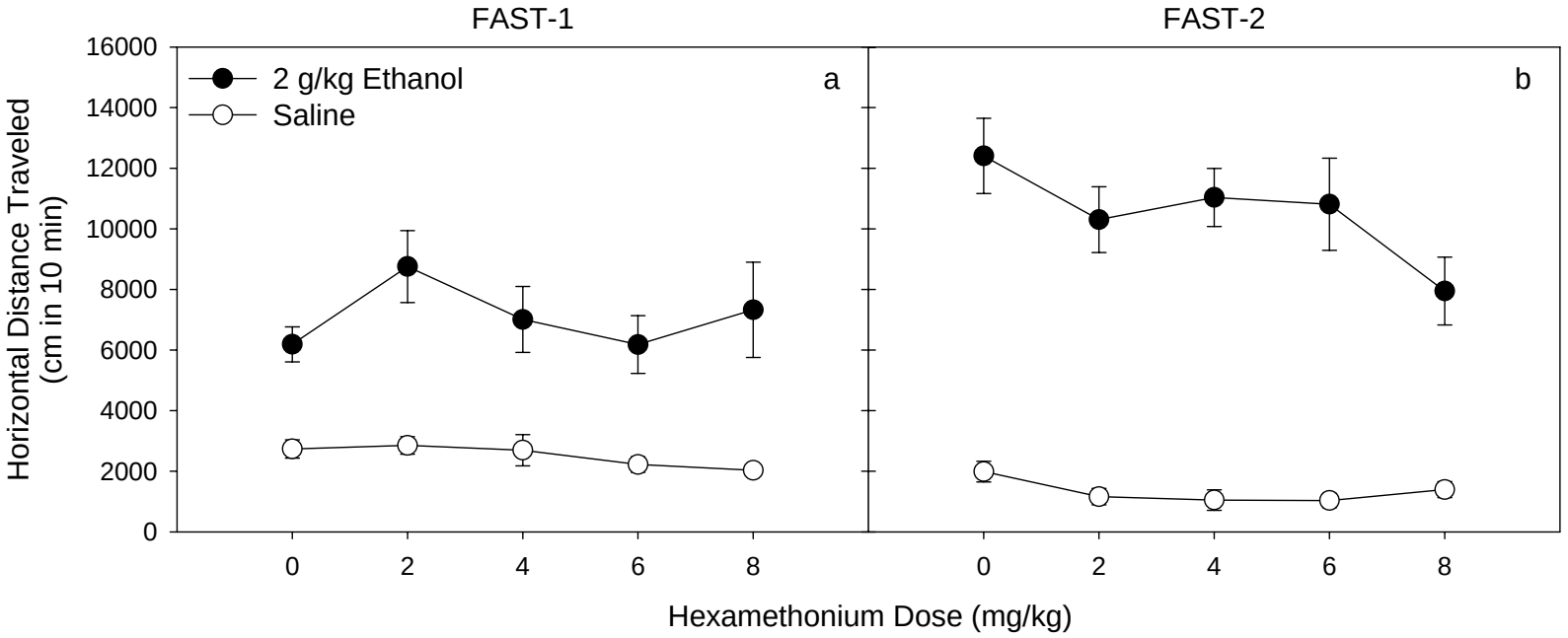

Figure 5. Hexamethonium did not affect ethanol (2 g/kg) stimulated activity in (a) FAST-1 or (b) FAST-2 mice. FAST-1 and FAST-2 mice were stimulated by ethanol ($F_{1, 62}=89.3$, $p<0.001$, $F_{1, 60}=250.1$, $p<0.001$, respectively), but ethanol dose did not interact with hexamethonium dose. N = 5 - 14 per group.

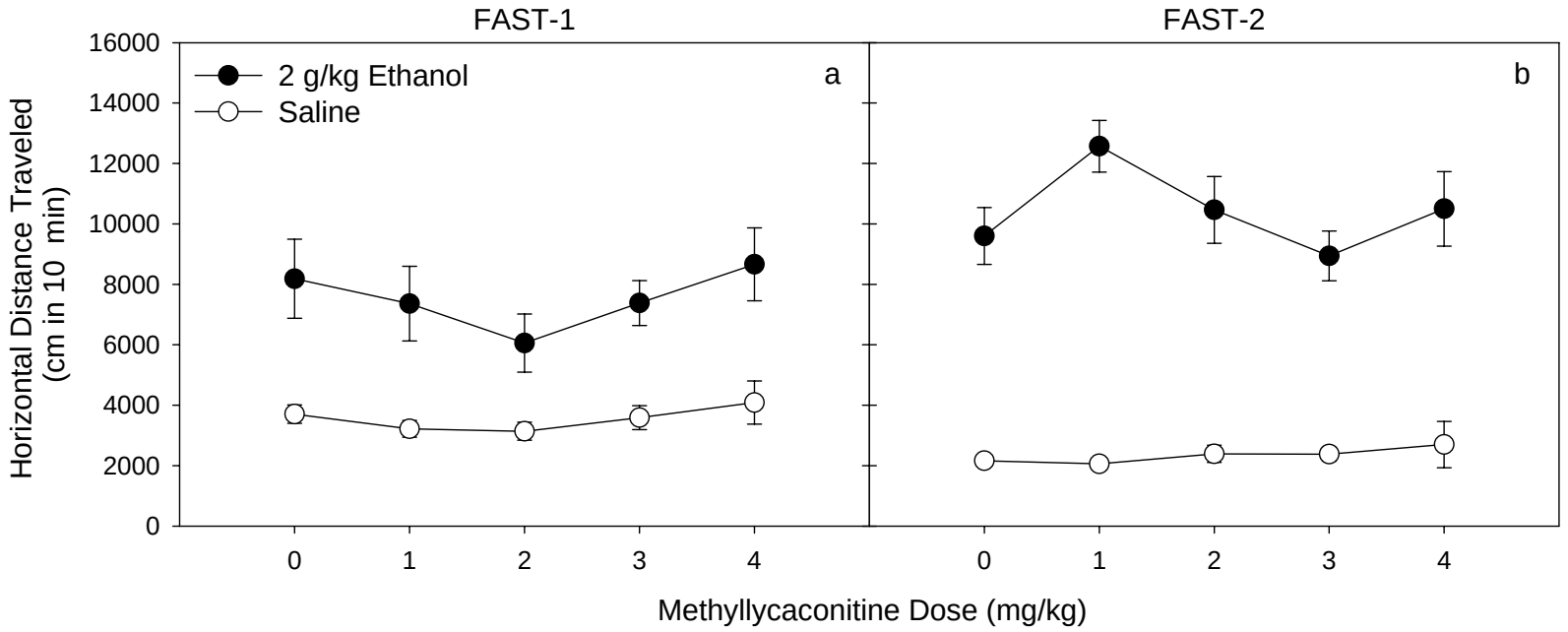

Figure 6. Methyllycaconitine did not affect ethanol (2 g/kg) stimulated activity in (a**) FAST-1 or (**b**) FAST-2 lines.** Shown is the mean (± SEM) of the last 10 min of a 15 min test. FAST-1 and FAST-2 mice were stimulated by ethanol ($F_{1, 101}$=54.7, p<0.001, $F_{1, 112}$=270.5, p<0.001, respectively), but ethanol dose did not interact with methyllycaconitine dose. N = 10 - 13 per group.

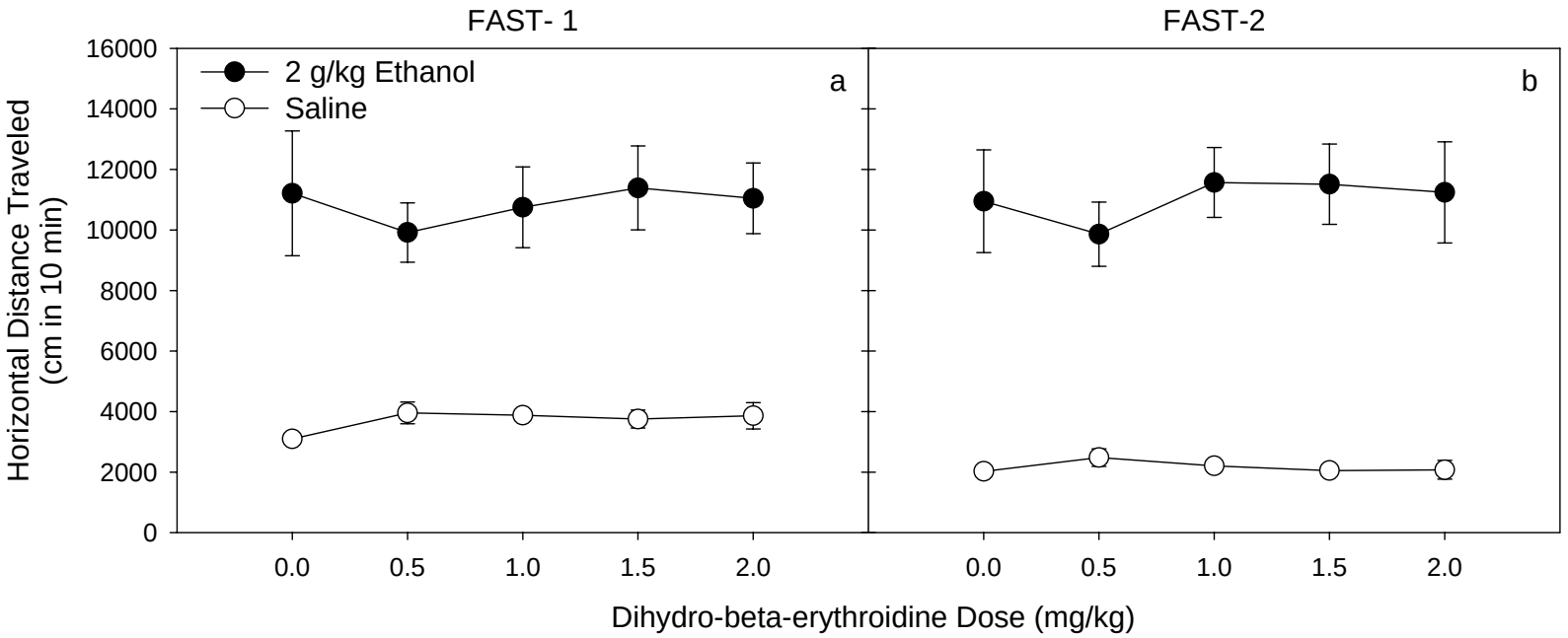

Figure 7. Dihydro-β-erythroidine did not affect ethanol (2 g/kg) stimulated activity in (a**) FAST-1 or (**b**) FAST-2 lines.** Shown is the mean (± SEM) of the last 10 min of a 15 min test. FAST-1 and FAST-2 mice were stimulated by ethanol ($F_{1, 90}$=123.8, p<0.001, $F_{1, 90}$=245.8, p<0.001, respectively), but this did not interact with dihydro-β-erythroidine dose. N = 9 - 12 per group.

given ethanol as compared to saline, as indicated by a main effect of ethanol dose. Pretreatment with hexamethonium, methyllycaconitine, or dihydro-β-erythroidine did not significantly alter the acute locomotor response to ethanol or saline. None of these nicotinic antagonists altered ethanol levels observed 15 min post-ethanol injection in either the FAST-1 or FAST-2 lines of mice (data not shown).

The *Chrna3* gene was not differentially expressed between the FAST and SLOW mice (Figure 8). Data were analyzed using a 4-way ANOVA with replicate, line, and sex as factors. There were no significant main effects or interactions.

D2 Mice

Ethanol

Similar to the results for FAST mice, mecamylamine completely abolished ethanol (1.5 g/kg) stimulation at the 3 and 4 mg/kg doses in D2 mice (Figure 9). Since there were no significant effects of sex in FAST mice, only male D2 mice were tested. A 2-way ANOVA provided evidence of a significant pretreatment X ethanol dose interaction ($F_{4, 79}$=3.6, p<0.05). There was a significant simple main effect when data from the groups of mice that were injected with ethanol were included in the analysis; ethanol-induced stimulation was decreased in mice that received all doses of mecamylamine compared to the saline pretreated animals. The simple main effect for the groups of mice receiving saline was not significant, indicating that mecamylamine did not significantly decrease basal locomotor activity. The saline and ethanol treatment groups differed when given saline pretreatment or pretreatment with 1 or 2 mg/kg mecamylamine, but not 3 or 4 mg/kg mecamylamine, providing evidence that these high

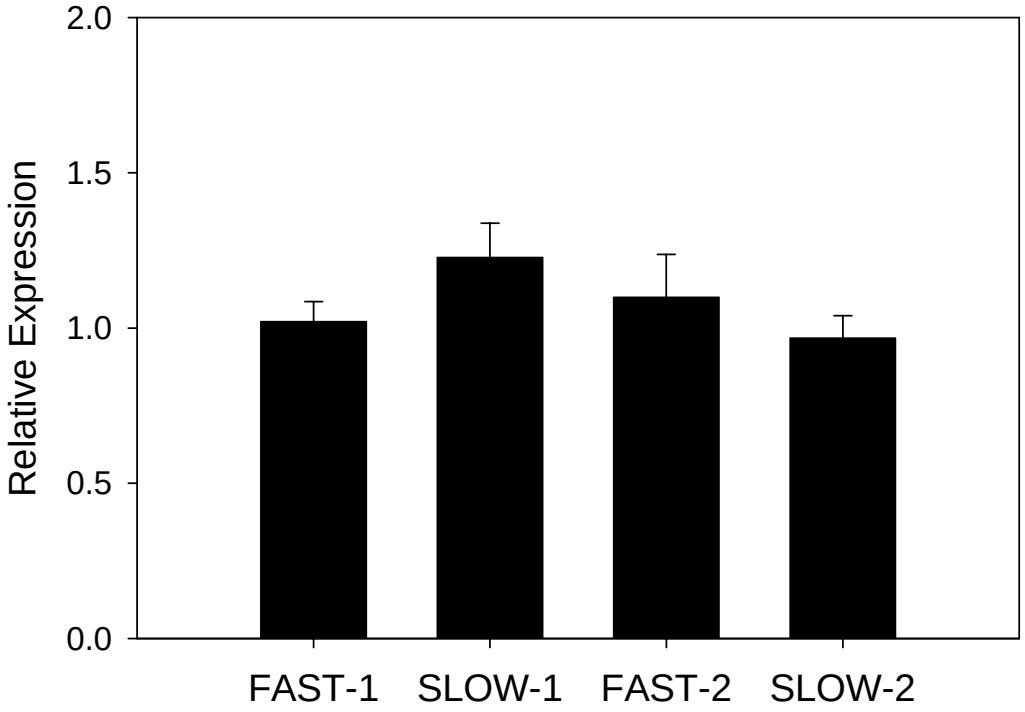

Figure 8. There were no differences in *Chrna3* gene expression among FAST-1, SLOW-1, FAST-2, and SLOW-2 mice. Mean (± SEM) relative *Chrna3* gene expression in the FAST and SLOW mice. N = 12 per group.

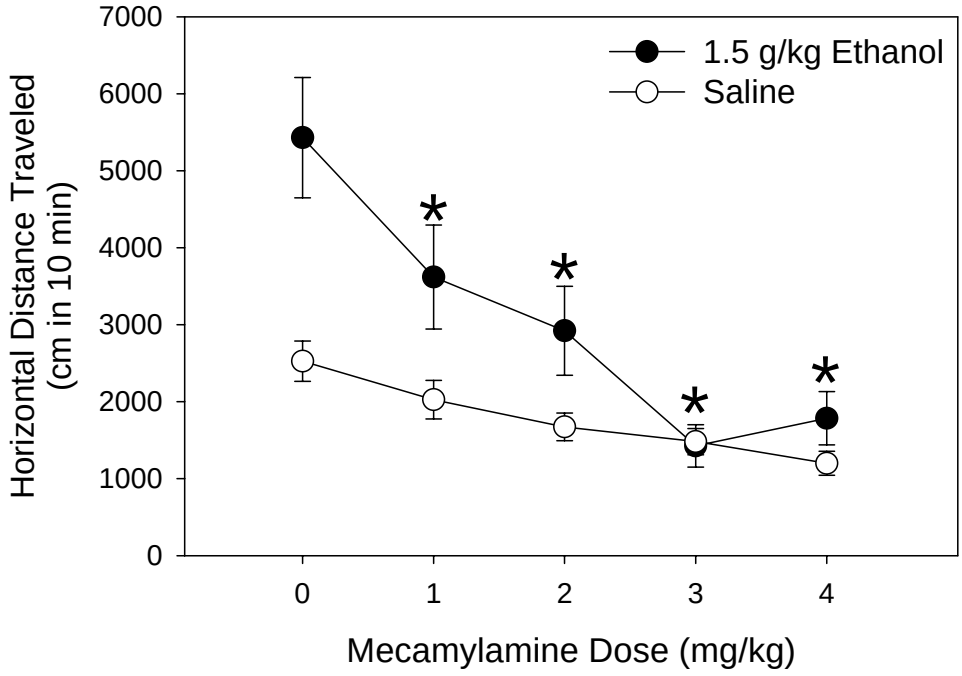

Figure 9. Mecamylamine attenuated ethanol-induced stimulation in D2 mice. Data (mean ± SEM) are from the last 10 min of a 15 min test. N = 8 - 9 per group.

*Significantly different from the ethanol control group.

doses of mecamylamine completely abolished ethanol-induced stimulation in D2 mice. Pretreatment with mecamylamine did not change BECs 15-min post-ethanol injection (mean ± SEM: 1.79 ± .06, 1.67 ± .12, 1.61 ± .09, 1.77 ± .05, 1.60 ± .14 for saline, 1, 2, 3 and 4 mg/kg mecamylamine respectively).

Hexamethonium did not attenuate ethanol-induced stimulation in D2 mice (Figure 10). There was a main effect of treatment; mice receiving ethanol were more stimulated than mice that received saline ($F_{1, 109}$=42.9, p<0.001), but there were no main effects or interactions with hexamethonium pretreatment. Likewise, pretreatment with hexamethonium did not alter BEC levels 15 min post-ethanol injection (data not shown).

Cocaine

Mecamylamine did not significantly attenuate cocaine-induced locomotor stimulation (Figure 11). To be consistent with the ethanol results data from min 5-15 of the 30 min test is presented. The results did not change when other time points were examined. There was a main effect of pretreatment ($F_{4, 126}$=7.1, p<0.001) and a main effect of cocaine dose ($F_{1, 126}$=300.3, p<0.001), but the pretreatment X cocaine dose interaction was not significant. The 2, 3, and 4 mg/kg doses of mecamylamine produced a nonspecific decrease in locomotor activity compared to the saline and 1 mg/kg dose groups, when data were collapsed on the cocaine dose factor. Cocaine enhanced locomotor activity compared to mice that received a saline injection.

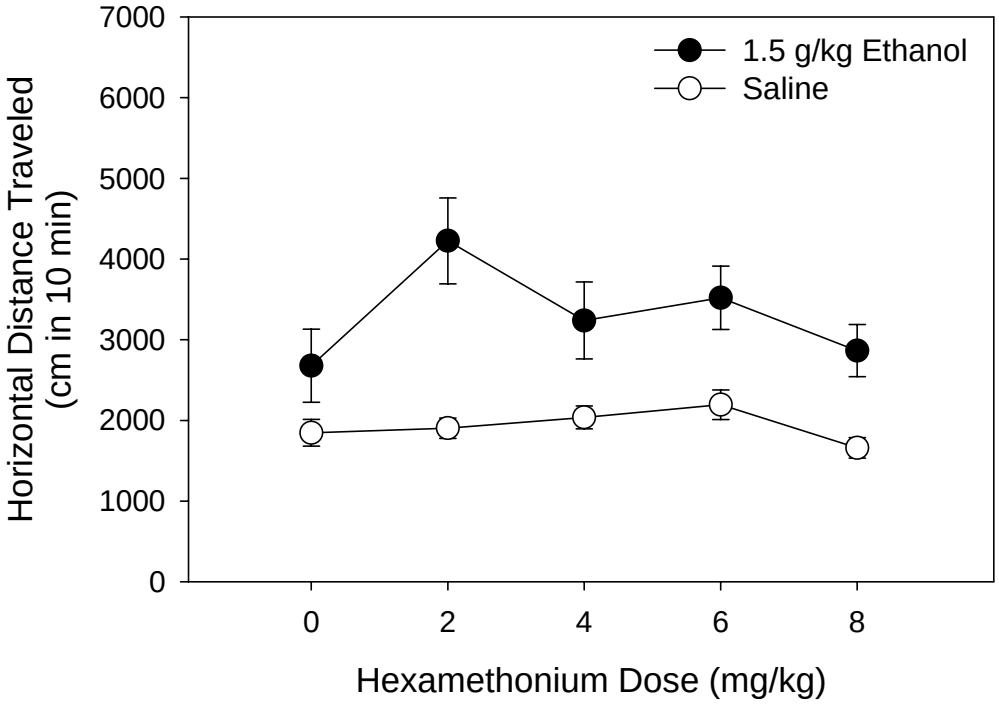

Figure 10. Hexamethonium did not affect ethanol (1.5 g/kg) stimulated activity in D2 mice. Shown is the mean (± SEM) of the last 10 min of a 15 min test. N = 11 - 12 per group.

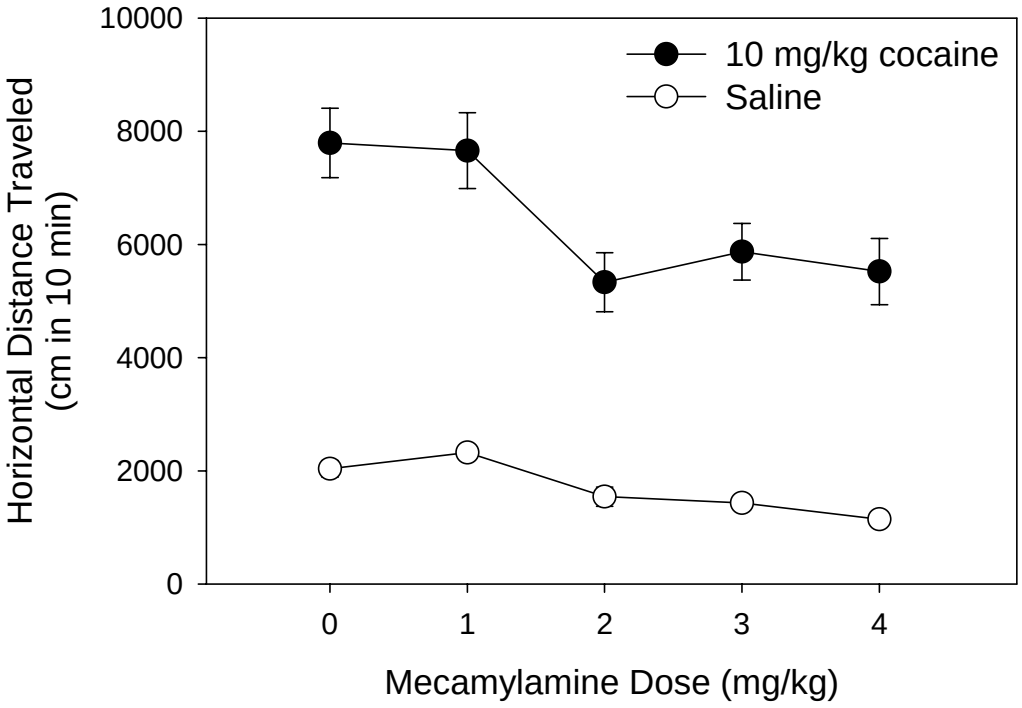

Figure 11. Mecamylamine did not affect cocaine (10 mg/kg) stimulated activity in D2 mice. Shown is the mean (± SEM) of min 5 – 15 of a 30 min test. N = 13 - 14 per group.

Methamphetamine

Pretreatment with mecamylamine did not significantly attenuate methamphetamine-induced stimulation (Figure 12) during min 5-15 of the 30 min test. Similar results were observed at all other time points. Methamphetamine (2 mg/kg) produced robust stimulation in D2 mice ($F_{1, 124}$=73.6, p<0.001), but there was no main effect of the pretreatment or a pretreatment X methamphetamine dose interaction.

Discussion

In the present studies I set out to determine if neuronal nicotinic acetylcholine receptors, particularly those containing an α3 subunit, were involved in the acute locomotor responses to ethanol, cocaine, and methamphetamine in mice. The nonspecific nicotinic receptor antagonist mecamylamine had no effect on cocaine- or methamphetamine-induced locomotor activity. However, mecamylamine was able to partially attenuate the acute locomotor response to ethanol in FAST mice and completely abolish this response in D2 mice. Additional work was done in FAST mice using a panel of acetylcholine receptor antagonists to examine which receptors were involved in this effect of mecamylamine. While the involvement of some nicotinic receptors was ruled out, more work is needed to determine if this effect is due specifically to α3-containing receptors.

Our inability to detect a significant effect of mecamylamine on methamphetamine-induced activity in D2 mice is consistent with prior literature. In rats, the acetylcholine receptor antagonists mecamylamine and dihydro-β-erythroidine had no effect on the acute locomotor response to amphetamine (Schoffelmeer et al., 2002). Similarly, 18-methoxycoronaridine and methyllycaconitine did not alter the acute

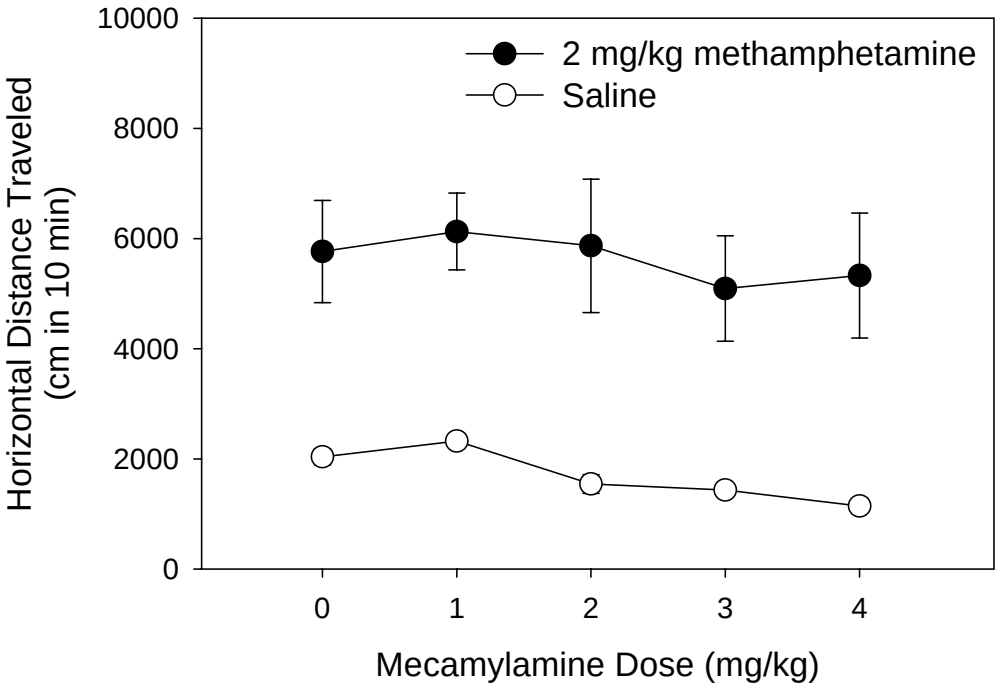

Figure 12. Mecamylamine did not affect methamphetamine (2 mg/kg) stimulated activity in D2 mice. Shown is the mean (± SEM) of min 5 – 15 of a 30 min test. N = 13 - 14 per group.

locomotor response to methamphetamine (Szumlinski et al., 2000a; Escubedo et al., 2005). These data support the idea that nicotinic receptors are not involved in the acute locomotor response to amphetamines.

The results involving nicotinic acetylcholine receptors in cocaine stimulation are less consistent. Although no effect of mecamylamine on cocaine-induced locomotor activity was observed in this study, other studies have implicated nicotinic receptors in this response. Mice lacking the α4 subunit showed a heightened locomotor response to an acute injection of cocaine (Marubio et al., 2003). Consistent with the present results, the acute locomotor response to cocaine in mice lacking the β2 subunit was indistinguishable from that of wildtype mice (Zachariou et al., 2001). Additionally, 18-methoxycoronaridine had no effect on cocaine stimulation (Szumlinski et al., 2000b).

In contrast to cocaine, the results obtained in the FAST and D2 mice regarding the involvement of neuronal nicotinic receptors in ethanol-induced stimulation are consistent with findings from NMRI outbred mice. That neuronal nicotinic receptors in the central nervous system have been implicated in this response in outbred, inbred, and selectively bred mice provides strong support for the involvement of these receptors in ethanol stimulation. This is supported by no effect of pretreatment with hexamethonium, but a reduction in ethanol-induced stimulation following an injection of mecamylamine (Blomqvist et al., 1992). Mecamylamine appears to decrease baseline locomotor activity, although in no case is this effect statistically significant; therefore, the significant effects in the ethanol studies only provide stronger evidence of the involvement of these receptors in ethanol-stimulated activity.

Data in NMRI and FAST mice begin to address which nicotinic acetylcholine receptors may be involved in this behavior. The acute locomotor response to ethanol in NMRI mice has been shown to be attenuated by mecamylamine, but not methyllycaconitine or dihydro-β-erythroidine (Larsson et al., 2002). In this paper, similar findings in FAST mice that were selectively bred for extreme ethanol-induced stimulation are reported. Since dihydro-β-erythroidine and methyllycaconitine do not influence this behavior this provides evidence against the involvement of α4β2 and α7 acetylcholine receptors, respectively. In NMRI outbred mice ethanol-induced stimulation has been shown to be attenuated by the α-conotoxin MII, providing evidence that α3β2, β3, or α6 containing nicotinic receptors may be involved in this response (Larsson et al., 2004).

Nicotinic acetylcholine receptors containing an α6 subunit are located in the mesolimibic dopamine pathway, and have recently been found to modulate dopamine release (Salminen et al., 2004). The localization and function of these receptors make them an interesting candidate as a modulator of this response, but there is evidence against the involvement of α6-containing receptors. In NMRI outbred mice the α6 specific conotoxin, α-conotoxin PIA-analogue, did not significantly alter the acute locomotor response to ethanol (Jerlhag et al., 2006).

The β3 subunit of the nicotinic acetylcholine receptor forms functional receptors when combined with other α/β pairs such as α3β4, α6β2, and α4β2 (Groot-Kormelink et al., 1998; Broadbent et al., 2006). This subunit has been shown to modulate nicotinic receptor kinetics, but doesn't appear to change agonist binding (Boorman et al., 2003). Mice carrying a null mutation of the β3 subunit have enhanced baseline locomotor

activity (Cui et al., 2003), but to my knowledge these mice have not been tested for drug-induced activity.

The role of α3-containing acetylcholine receptors in ethanol-induced stimulation remains to be determined. To my knowledge, 18-methoxycoronaridine has not been tested for ethanol stimulation, but does decrease ethanol drinking (Rezvani et al., 1997). *Chrna3* expression was similar in the FAST and SLOW mice, but this does not rule out the involvement of receptors containing this subunit. One possible reason why gene expression difference may not have been detected is that a whole brain preparation was used in this initial investigation. Gene expression assays using brain regions known to contain this subunit may provide different results. Further, work should be done to see if there is differential receptor density of α3-containing receptors in FAST and SLOW mice. The gene that codes for the α3 subunit of the acetylcholine receptor resides on chromosome 9 in a gene cluster with the α5 and β4 subunit genes. This region has been implicated as a quantitative trait locus (QTL) for the acute locomotor response to ethanol (Palmer et al., 2006). Thus, the *Chrna3* gene should be regarded as a candidate gene for this QTL; however, finer mapping of the region would help to establish plausibility.

A knockout mouse of the α3 gene has been created, but has severe impairments in growth and they die within weeks after birth (Xu et al., 1999); therefore, the homozygous mutants are not amenable for testing drug-induced behaviors. Although autoradiography studies have shown that heterozygous mice have similar acetylcholine receptor binding as wildtype mice (Whiteaker et al., 2002), these mice have been useful for behavioral studies with nicotine (Salas et al., 2004). Conotoxins are peptides which need to be centrally administered, since they cannot cross the blood brain barrier. A number of

conotoxins have been recently identified that may help elucidate whether α3-containing nicotinic receptors are involved in ethanol stimulation. These include: α-conotoxin Vc1.1, which is specific for α3-containing nicotinic receptors (Clark et al., 2006), as well as α-conotoxin OmIA and α-conotoxin AuIB which preferentially block α3β2 and α3β4 receptors, respectively (Talley et al., 2006). Since these data provide evidence of a role for neuronal nicotinic receptors in ethanol-induced stimulation, further studies using conotoxins are warranted. Brain site-specific administration of these conotoxins may provide information on the involvement of α3-containing receptors in ethanol stimulation as well as which brain regions are important in this response.

The acute locomotor response to drugs of abuse is an endophenotype for alcohol abuse (Gabbay, 2005). Heavy drinkers report more stimulation compared to light drinkers (Holdstock et al., 2000; King et al., 2002). Not only have neuronal nicotinic receptors been implicated in the acute locomotor response to ethanol in mice, but in humans mecamylamine decreased reported ratings of ethanol-induced stimulation (Young et al., 2005) and euphoria (Chi and de Wit, 2003). Together these data provide support for the idea that nicotinic receptors may be involved in the acute response to ethanol in both humans and animal models and may provide important information about the neural mechanisms involved in alcoholism.

Chapter 3: The Alpha 3 Subunit of the Nicotinic Acetylcholine Receptor is a Candidate Gene for Ethanol-Induced Locomotor Activation

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This manuscript is in publication format and will be submitted soon.

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Abstract

Alcohol and nicotine are co-abused, and data in both humans and animal models provide evidence that common genes may underlie responses to both drugs. Prior work supports the existence of a gene on mouse chromosome 9 that influences the acute locomotor response to ethanol in a region that contains genes encoding three nicotinic acetylcholine receptor subunits. In the current studies, congenic mice were used to confirm that an influential gene resides on chromosome 9. To more finely map the location of the gene, congenic F_2 mice were utilized. Finally, to examine if specific nicotinic acetylcholine receptor subunits are involved in the acute locomotor response to ethanol, gene expression was quantified in strains of mice that differ in response to this drug. Genes that are differentially expressed are more likely to be involved in the trait than those that are not differentially expressed. A gene on chromosome 9 that accounts for 20% of the phenotypic variation in the response to ethanol was confirmed, and the location of this gene was narrowed to a 46 cM region that contains the acetylcholine receptor genes. There was greater expression of the α3 subunit of the nicotinic acetylcholine receptor (*Chrna3*) in mice that were less sensitive to ethanol-induced stimulation compared to mice that were robustly stimulated by this drug. This gene expression difference was observed in both the whole brain and dissections of brain regions known to be important in locomotor activity. The other two nicotinic receptor subunits in the gene cluster (α5 and β4) were not differentially expressed. *Chrna3* is a candidate gene for the acute locomotor response to ethanol and should be examined further.

Introduction

Alcohol and nicotine co-abuse is commonplace (Istvan and Matarazzo, 1984; Talcott et al., 1998; Madden et al., 2000; Grant et al., 2004), and abuse of these drugs appears to be influenced by some of the same genes. In humans, common genetic factors account for as much as 40% of the covariance between alcohol use and cigarette use (Swan et al., 1997), but the genetic correlation between alcohol dependence and nicotine dependence has been shown to be as high as .68 (True et al., 1999; Tsuang et al., 2001). The responses to these two drugs in animal models of addiction-related traits also share some genetic co-determination. Mice selectively bred for their differential acute locomotor response to ethanol also differed in their acute locomotor response to nicotine, such that the mice more stimulated by ethanol were also more stimulated by nicotine (Bergstrom et al., 2003). Further, selective breeding of rats and mice for differences in sensitivity to the sedative-hypnotic effects of ethanol resulted in differences in response to nicotine (de Fiebre et al., 1987; de Fiebre et al., 1990; de Fiebre et al., 1991; Collins et al., 1993; de Fiebre et al., 2002). Together these findings provide evidence that common genes acting through common neural mechanisms may underlie certain responses to alcohol and nicotine and may be related to their co-abuse.

Individuals who report higher levels of ethanol stimulation following alcohol administration in a laboratory setting also drink more alcohol outside of the laboratory than those that report lower levels of stimulation (Holdstock et al., 2000; King et al., 2002; Young et al., 2005). Therefore, magnitude of the acute locomotor response to ethanol has been proposed to be an endophenotype for excessive alcohol use (Gabbay,

2005). Understanding the biological basis of the acute locomotor response to ethanol may lead to a greater understanding of alcohol abuse.

Quantitative trait locus (QTL) mapping is used to locate genes that contribute to variation in traits influenced by multiple genes, such as the acute locomotor response to ethanol (Dudek et al., 1991). QTL mapping has provided evidence that at least one gene on mouse chromosome 9 influences this trait (Cunningham, 1995; Demarest et al., 1999; Palmer et al., 2006). Genes encoding the α5 (*Chrna5*), α3 (*Chrna3*), and β4 (*Chrnb4*) subunits of the nicotinic acetylcholine receptor all reside in the mapped region of mid-chromosome 9. Pretreatment with the nonspecific nicotinic acetylcholine receptor antagonist mecamylamine attenuated ethanol-induced locomotor stimulation (Chapter 2; Blomqvist et al., 1992; Larsson et al., 2002), but the involvement of specific nicotinic receptor subunit genes has not been clearly identified.

The nicotinic acetylcholine receptor subunit genes were considered as candidates for the ethanol stimulation QTL. To explore their possible involvement, I used congenic strains of mice, a congenic F_2 population of mice for finer mapping, and quantitative real time-PCR (qRT-PCR) gene expression analysis. These analyses allowed me first to confirm the ethanol stimulation QTL on mouse chromosome 9, and subsequently to provide supporting evidence for one of the subunit genes as the stronger candidate for influencing the acute response to ethanol.

Methods

Subjects

All mice tested in these experiments were produced by breeder pairs at the Portland Veterans Affairs Medical Center. Congenic mice were obtained from Dr. John

K. Belknap, whereas C57BL/6J (B6) and DBA/2J (D2) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6 and D2 breeder pairs were refreshed yearly from The Jackson Laboratory to minimize drift.

Congenic strains are a unique kind of inbred strain used to map QTL regions (Bennett, 2000). These strains contain alleles from two different inbred strains. A chromosomal segment from one inbred strain (the donor strain) is introgressed onto another inbred strain (the background strain) through homologous recombination. Reciprocal congenic strains are a valuable tool where in one case one strain (e.g., B6) is the donor strain while another strain (e.g., D2) is the background strain and another congenic strain exists where D2 is the donor strain and B6 is the background strain. If the congenic and background strains differ for a trait of interest, this provides evidence that a gene (or genes) in the introgressed region accounts for part of the trait variance (i.e., the QTL has been captured within the introgressed region).

Congenic Mice

In these studies three chromosome 9 congenic strains were used. Chromosome 9 D2.B6 (D9Mit90,18; 32.2 – 120.1 Mb), chromosome 9 B6.D2 - short (D9Mit90,182; 32.2 – 101.4 Mb), and chromosome 9 B6.D2 - long (D9Mit90,18; 32.2 – 120.1 Mb) congenics. The indicated DNA markers (e.g., D9Mit90 and D9Mit18) define the start and end of the introgressed region. The congenic strain notation (e.g., D2.B6) defines the background strain (in this case D2) and the strain which provided the donor alleles (B6).

D2 X Chromosome 9 D2.B6 (D9Mit90,18) Congenic F_2

The congenic F_2 mice were derived by crossing male chromosome 9 D2.B6 (D9Mit90,18) congenic mice to female D2 mice. The F_1 offspring from this cross, which were heterozygous for the introgressed region, were then interbred to produce the F_2 mice used in the current study.

Animal Husbandry

Mice were iso-sexually housed 2-5 per cage in standard shoebox size cages (internal dimensions: 28.5 cm long X 17.5 cm wide X 12 cm high). Animals housed together were usually littermates, but in some cases animals close in age (< 5 days difference), but from a different litter of the same strain were housed together to avoid singly housing animals. Mice had *ad libitum* access to rodent chow (Purina Laboratory Rodent Chow #5001; Purina Mills, St. Louis, MO) and tap water. The lighting in the animal rooms was maintained on a 12-h light/dark cycle (lights on at 0600).

At the start of the experiment all animals were between 51 and 114 days of age and mice of both sexes were included in these studies. In each study, the strains tested were approximately age balanced. All procedures were approved by the Portland Veterans Affairs Medical Center's Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health's Principles of laboratory animal care (1985).

Drugs

Ethyl alcohol (200 proof) was obtained from Pharmco (Brookfield, CT), and diluted in physiological saline (Baxter Healthcare Corporation, Deerfield, IL) to 20% v/v. Injection volumes were adjusted for body weight to achieve doses of 1.5 or 2 g/kg.

Testing Apparatus

AccuScan automated activity monitors (40 X 40 X 30 cm, Columbus, OH) were used to assess locomotor activity via 8 infrared beams, located 2 cm above the chamber floor, along 2 sides of the monitors that were detected by sensors located on the opposite 2 sides of the monitors. Beam breaks were recorded by a computer and converted into horizontal distance traveled (in cm). The activity monitors were housed in custom-made (Flair Plastics, Portland, OR) black acrylic test chambers designed to separate the monitors from the test room environment. Each chamber was lined with sound attenuating foam and housed an 8-W fluorescent light and a fan to provide background noise. Mice were tested during the light phase of the light/dark cycle, between 0800 and 1600 h, with the fluorescent lights on in the chambers.

Experiment 1

In this experiment I set out to confirm the presence of a QTL for ethanol-induced locomotor stimulation in mice congenic for a region of chromosome 9. Chromosome 9 D2.B6 congenic, chromosome 9 B6.D2 – short congenic, B6 and D2 mice were moved from the colony room to the procedure room 45 - 60 min prior to the initiation of behavioral testing. The 3-day testing procedure used in these studies is identical to that which was originally used to map this QTL (Phillips et al., 1995; Palmer et al., 2006).

Animals were injected with saline on days 1 and 2, and ethanol (2 g/kg) on day 3. Immediately after the injection on each day, mice were place into the activity monitors and horizontal distance traveled was measured for 15 min, in 5-min epochs.

The acute locomotor response to ethanol was defined as the day 3 ethanol response minus the day 2 habituated baseline response (Day 3 – Day 2). Subtracting day 2 allows for a measure of the acute locomotor response to ethanol while accounting for differences in habituated locomotor activity. Data from the first 5 min of the test session were used as the primary dependent variable consistent with the original QTL mapping experiment (Phillips et al., 1995). This time period captured pure stimulation, whereas longer test periods permit locomotor depression in response to ethanol to develop in some mouse strains (Phillips et al., 1995).

Based on the predicted amount of trait variation accounted for by this QTL (14 - 34%), a group size of 15 - 40 was predicted to be needed to confirm its presence in the introgressed region (Belknap and Atkins, 2001). Twenty-six to 49 mice were tested per sex per strain; in eight cohorts. In a representative number of animals from some cohorts, blood samples were obtained to determine if behavioral differences between the strains were due to differences in blood ethanol concentrations (BEC). A 20 µl blood sample was obtained from these mice from the retro-orbital sinus and processed following procedures standard in our laboratory (Boehm et al., 2000). BECs were determined by gas chromatography (Agilent 6890). Mice were humanely euthanized by carbon dioxide asphyxiation immediately after testing or blood sampling on day 3.

All other behavioral testing used similar procedures. Differences in procedure from this experiment are noted below.

Experiment 2

A QTL capture was confirmed in the D2.B6, but not B6.D2 – short congenic. Ethanol produces biphasic effects on locomotor activity in mice that are strain dependent (Dudek et al., 1991; Crabbe et al., 1994; Dudek et al., 1994). While 2 g/kg ethanol in the D2 strain is stimulatory, in the B6 strain this dose is more likely to be sedative (i.e., reduces locomotor activity as compared to baseline; Dudek et al., 1991; Crabbe et al., 1994). The increased ethanol stimulation conferred by the D2 genotype on mouse chromosome 9 may not be able to overcome this alternate effect on the B6 background. To reduce the possibility of enlisting sedative mechanisms by ethanol, a lower ethanol dose was used to test the chromosome 9 B6.D2 - short congenic and B6 control mice in this experiment. B6.D2 – short and B6 mice were tested for their locomotor response to 1.5 g/kg ethanol. Thirty to 32 mice (half of each sex) were tested per genotype using the same behavioral procedure as in experiment 1, except that mice were given 1.5 g/kg ethanol on day 3. All mice had blood taken after testing on day 3 for BEC analysis.

Experiment 3

Experiments 1 and 2 used a pre-existing B6 background congenic (B6.D2 – short) that possessed an introgressed D2 segment that was 18.7 Mb shorter than the congenic segment in the D2.B6 strain (32.2 – 101.4 Mb as compared to 32.2 – 120.1 Mb, respectively). A QTL capture was confirmed in the D2.B6, but not B6.D2 – short congenic even with the lower 1.5 g/kg dose of ethanol. It was possible that the QTL was not captured in the B6.D2 – short congenic because the gene that accounts for variation in the acute locomotor response to ethanol resides between 101.4 and 120.1 Mb. Thus, a new B6 background congenic with a longer 32.2 – 120.1 Mb introgressed region (B6.D2

– long) was created. Forty – 41 chromosome 9 B6.D2 - long congenic and B6 control mice were tested for ethanol (2 g/kg)-induced stimulation with the procedure described above. In this study the original 2 g/kg ethanol dose was used because there was no evidence from experiment 2 that the mice were more responsive to 1.5 g/kg ethanol (compared to 2 g/kg in experiment 1). Furthermore, all strains of mice were stimulated by 2 g/kg ethanol in experiment 1. A blood sample was taken from all animals following testing on day 3 to examine BEC.

Experiment 4

All 260 congenic F_2 mice had a small portion of the tail (approximately 3 mm) taken for DNA analysis at least two weeks prior to being tested for their acute locomotor response to ethanol (2 g/kg), using the procedures already described. Of the 260 congenic F_2 animals behaviorally tested, those that had a behavioral response in the top or bottom 12.5% of the phenotypic distribution were genotyped and used for QTL analyses. Genotyping this portion of the population provides the majority of linkage information available from the whole population (Lander and Botstein, 1989; Darvasi and Soller, 1992).

Experiment 5

The QTL for ethanol-induced locomotor stimulation on mouse chromosome 9 was captured on the D2, but not B6, background. This led to an exploration of the possibility that one or more of the nicotinic acetylcholine receptor subunit genes might play a role in the differential ethanol response. Expression of the three nicotinic receptor subunit genes was quantified in the chromosome 9 D2.B6 congenic and D2 control mice.

Differences in gene expression have been suggested to be important for complex traits (Glazier et al., 2002; Korstanje and Paigen, 2002) therefore, if differences in expression are detected these genes should be regarded as candidates for this response. In this initial analysis, whole brain tissue from naïve male and female mice (N = 6 per D2.B6 and D2 strain) was used for qRT-PCR analysis of the *Chrna5*, *Chrna3*, and *Chrnb4* genes.

Experiment 6

In experiment 5, only *Chrna3* was differentially expressed between the D2 and D2.B6 congenic mice. To examine if *Chrna3* was also differentially expressed in brain regions known to be important in the locomotor response to drugs of abuse, the striatum and ventral midbrain were dissected for qRT-PCR. These regions were chosen because they are part of the motive circuit (Kalivas and Volkow, 2005; Nestler, 2005) and have been shown to express the α3 gene (Azam et al., 2002; Zoli et al., 2002). These brain regions were dissected from naïve male and female chromosome 9 D2.B6 congenic and D2 mice (N = 10 - 12), and *Chrna3* expression was again determined using qRT-PCR.

DNA Isolation and Genotyping

Genomic DNA was extracted from tail tissue using the Puregene Tissue and Mouse Tail Kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Briefly, cells were placed overnight in cell lysis solution and proteinase K. RNA was removed with the addition of RNase and then proteins were precipitated with a protein precipitation solution. Finally, DNA was precipitated with isopropanol, washed, and re-hydrated in TE buffer (10 mM Tris pH 7.5, 1 mM EDTA).

Six microsatellite markers spaced approximately every 18 Mb throughout the introgressed congenic region were genotyped in the F_2 mice. To each DNA sample, 1.2 µl $MgCl_2$ (25 mM), 1.5 µl buffer, 0.15 µl AmpliTaq Gold (5 U/ µl; Applied Biosystems, Foster City, CA), 2.5 µl dNTPs (1.25 mM; Promega, Madison, WI), 7.15 µl ddH_20, and 0.3 µl forward and reverse primers (6.6 µM; Research Genetics, Inc, Huntsville, AL) were added. The reaction was then amplified in a PerkinElmer PCR machine under the following conditions (95˚C for 10 min, 80˚C for 5 min, 40 cycles of 94˚C for 30 sec, 53˚C for 30 sec, and 72˚C for 30 sec, followed by 72˚C for 7 min and finally holding at 4˚C). Amplified PCR products were detected by ethidium bromide staining on 3% agarose gels.

Tissue Extraction

Whole Brain

Mice were cervically dislocated, decapitated, and whole brains were removed. Brains were immediately frozen by submersion in cold isopentane, put into RNase-free tubes, and stored at -80˚C until processing.

Microdissections

Whole brains from chromosome 9 D2.B6 congenic and D2 control mice were removed and placed on an ice cold platform. Coronal slices were made at approximately + 1.75 and + .25 bregma using a razor blade. The olfactory tubercles served as a landmark to ensure the same brain section was obtained for each mouse. The section was laid flat on the cold platform with the rostral (+ 1.75 bregma) portion of the section facing up. The striatum (caudate putamen) was visualized and dissected from remaining

tissue. The ventral midbrain was dissected from the brains of the same animals. The cerebral cortices were pealed away and two coronal slices were made at – 3.25 and - 4.25 bregma; visualization of the superior colliculus guided these cuts. The slice was placed on the platform, the section was cut at the periaqueductal gray, and the dorsal part was discarded. The cerebral peduncles were then removed from the bottom of the slice. The striatum sample and ventral midbrain section containing the ventral tegmental area, substantia nigra, and interpeduncular nuclei, were placed into RNase-free tubes on dry ice before being transferred to a -80˚C freezer for storage until processing.

RNA Extraction

RNA was extracted using methods appropriate for either whole brain samples or for microdissected tissue.

Whole Brain

RNA was extracted using the guanidinium isothiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987, 2006) following procedures standard in our laboratory (Chapter 2). Briefly, RNA Stat-60 (Tel-Test, Inc. Friendswood, TX) was used to extract RNA which was then cleaned of DNA contamination. RNA quality was assessed with a A260 nm/A280 nm ratio by spectrophotometer (1.8 - 2 was considered good quality; Eppendorf, Hamburg, Germany; Chomczynski and Sacchi, 2006) and confirmed by electrophoresis on a 1% agarose gel.

Microdissections

RNA from the microdissections was extracted using the Absolutely RNA Microprep Kit (Stratagene; La Jolla, CA) and the manufacturer's protocol. Briefly, β-

mercaptoethanol/lysis buffer mixture (100 µl) was added to each sample before vortexing and manual homogenization. 70% ethanol (100 µl) was then added to the mixture prior to vortexing (5 sec) and transfer to an RNA-binding spin cup. Following centrifugation (max speed, 60 sec), 600 µl of a low-salt wash buffer was added to the spin cup before another round of centrifugation (60 sec at max speed). The filtrate was then removed, and the column was dried by a 2 min spin at max speed. To remove DNA contamination, a 30 µl mixture of RNase-free DNase I and DNase Digestion Buffer was added to the spin cup matrix and samples were incubated at 37ºC for 15 min. Samples were then washed first with 500 µl of High-Salt Wash Buffer, then 600 µl of Low-Salt Wash Buffer, and finally 300 µl of Low-Salt Wash Buffer, followed by centrifugation (60 sec to 2 min) after each wash. To elute the RNA from the spin cup matrix, 30 µl of the Elution Buffer was added directly to the matrix and the sample was centrifuged at max speed. The quality of the microdissection RNA samples were examined using the same criterion as the whole brain RNA samples.

qRT-PCR

Quantitative RT-PCR was performed in a 2-step reaction using standard procedures in our laboratory (Chapter 2). Briefly, total RNA was reverse transcribed using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) and the manufacturer's specifications. mRNA gene expression of the *Chrna5*, *Chrna3*, and *Chrnb4* acetylcholine receptor subunit genes, and of the control Hprt1 (hypoxanthine guanine phosphoribosyl transferase 1) gene were analyzed using pre-designed TaqMan gene expression assays (Applied Biosystems, Foster City, CA). 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 *Chrna5*, *Chrna3*, *Chrnb4*, and *Hprt1* genes was determined. For each sample the C_t for *Hprt1* was subtracted from the expression of the acetylcholine receptor subunits. Relative expression (calculated as 2 to the negative power of the average expression of D2 mice minus each individual value) was used as the primary dependent variable.

Statistics

Behavioral and gene expression data from each congenic strain were compared independently to data from the relevant background strain. Data were analyzed with 2-way factorial analysis of variance (ANOVA). Strain and sex were used as independent variables. Interactions were analyzed with simple main effects, while main effects were followed up by Newman-Keuls post hoc comparisons. χ^2 analysis was used to determine if the B6 allele was dominant in the congenic F_2 study by examining if the frequency of heterozygous animals differed between the high and low phenotypic extremes. Statistica (StatSoft, Tulsa, OK) was used for all statistical analyses with an alpha level set at 0.05.

Effect Size

The percentage of variation accounted for by the QTL was calculated for each congenic strain that was significantly different from its control strain (i.e., each one that captured the QTL). The QTL effect size (R^2) was calculated from a 1-way ANOVA using the equation $R^2 = F/(F + dfw)$ (dfw = degrees of freedom within; Rosenthal, 1994). To estimate the R^2 from the congenic F_2 experiment the maximum logarithm of the odds (LOD) score was converted to χ^2. The equation $R^2 = \chi^2/(n + \chi^2)$ was then used to estimate the effect size (Rosenthal, 1994). To make this effect size comparable with that from the

congenic studies this value was multiplied by 2, since in the congenic studies only the 2 homozygous classes are represented.

QTL Analysis

R/qtl was used to analyze the genotypic and phenotypic data from the congenic F_2 study (Broman et al., 2003). Animals from the top and bottom 12.5% of the phenotypic distribution were analyzed with the 'scanone' command. This command identifies QTL using the expectation maximization algorithm. The results of this experiment are presented relative to the cM map locations on chromosome 9. The reason this data is presented in this format, compared to in Mb locations used in the rest of this dissertation, is because cM is a unit of recombination frequency between two loci used by R/qtl, compared to Mb locations which are physical map locations. QTL interval mapping takes into account the frequency of crossovers (i.e., recombinations) between two loci.

Results

Experiment 1: Confirmation of a QTL for ethanol-induced locomotor stimulation in a D2, but not B6, background congenic

Chromosome 9 D2.B6 congenic and D2 mice were differentially sensitive to an acute injection of ethanol, providing evidence of a QTL on chromosome 9 for ethanol-induced locomotor stimulation (Figure 13). Data from 3 animals were excluded from the analysis due to computer malfunction, illness, or misplaced injection. A 2-way ANOVA with strain and sex as independent variables revealed a significant main effect of strain ($F_{1, 128}$=34.4, $p<0.001$) and sex ($F_{1, 128}$=7.7, $p<0.01$), but no interaction of the two factors. This QTL accounted for 20.4% of the variability in ethanol-induced stimulation. Female

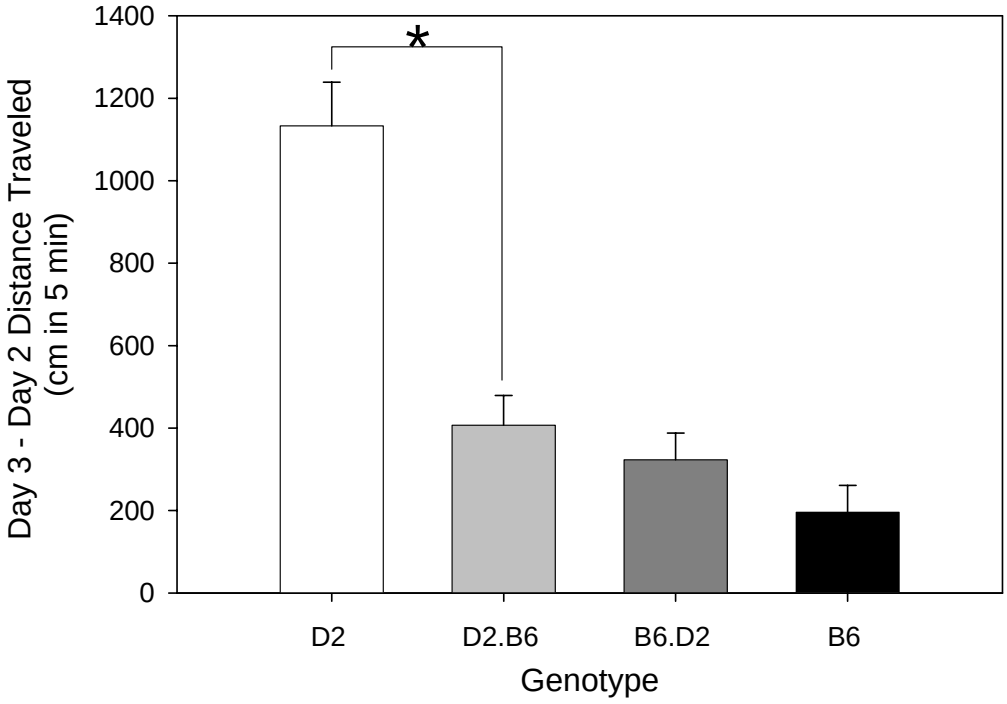

Figure 13. Capture of the chromosome 9 QTL for the locomotor response to ethanol is dependent on congenic background strain. Shown is the acute locomotor response to ethanol (2 g/kg) in chromosome 9 D2.B6 (D9Mit90,18) congenic, chromosome 9 B6.D2 - short (D9Mit90,182) congenic, D2, and B6 strains. Data are for the first 5 min (mean ± SEM) of the 15-min test session. *$p < 0.05$.

mice were more stimulated than male mice (1009.8 ± 137.1 vs. 592.9 ± 71.9, respectively), but there was no indication that the QTL was sex-specific. A subset of the animals tested for their acute response to ethanol (20 – 21 per strain) was used to assess BEC. There was no significant strain difference in BEC from samples taken at the conclusion of the behavioral test (15-min after the 2 g/kg ethanol injection), but there was a significant main effect of sex ($F_{1, 37}$=6.0, p<0.05). Female mice had higher BECs than male mice (2.15 ± .04, 1.98 ± .02, respectively).

The chromosome 9 B6.D2 - short congenic and B6 control mice were equally sensitive to the locomotor stimulant effect of ethanol (Figure 13). Data from one mouse were excluded due to a computer malfunction. A 2-way ANOVA revealed a significant main effect of sex ($F_{1, 184}$=21.3, p<0.01), but not other significant results. Female mice were more stimulated than male mice (463.3 ± 66.4, 61.8 ± 57.4, respectively) and had a significantly higher mean BEC ($F_{1, 43}$=4.3, p<0.05; 2.03 ± .04, 1.88 ± .06, respectively; n = 22 – 25 per strain). There was no significant difference in BEC between the 2 strains.

Experiment 2: The B6 background congenic does not capture a QTL for the acute locomotor response to a 1.5 g/kg injection of ethanol

Chromosome 9 B6.D2 - short congenic and B6 mice were also equally sensitive to 1.5 g/kg ethanol, as indicated by no significant main effect or interaction with strain (Figure 14). Similar to results for the 2 g/kg ethanol dose, female mice were significantly more stimulated than male mice ($F_{1, 58}$=9.2, p<0.01; 351.9 ± 96.6, 26.3 ± 61.3, respectively). A 2-way ANOVA with stain and sex as independent variables detected no significant main effects or interactions for BEC.

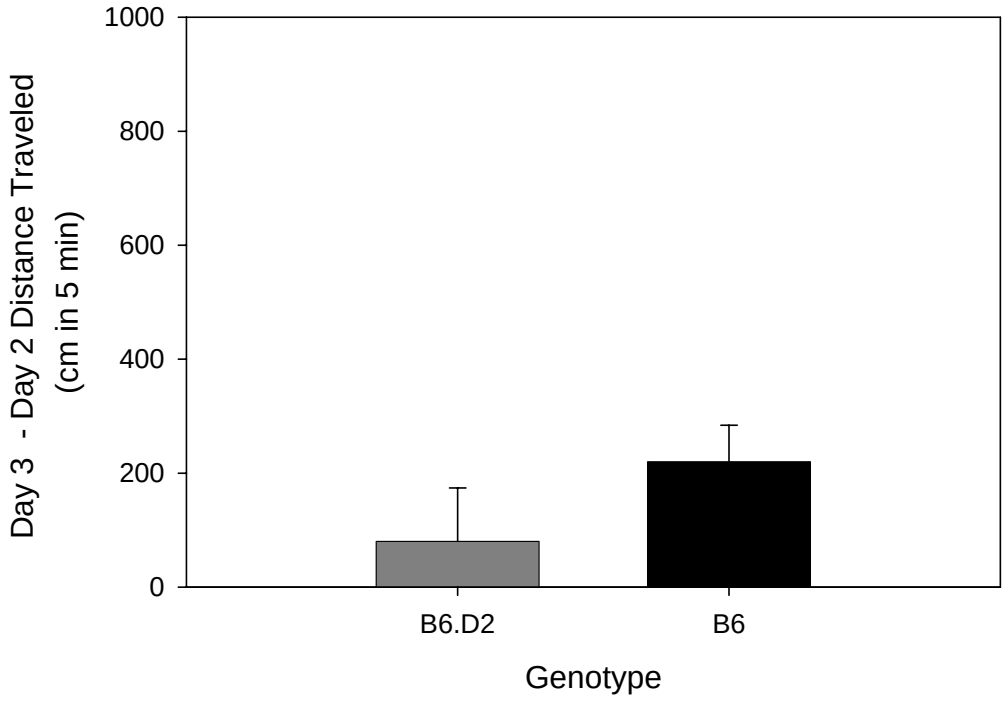

Figure 14. The chromosome 9 ethanol stimulation QTL is not captured in a B6 background congenic. Shown is the acute locomotor response to ethanol (1.5 g/kg) in chromosome 9 B6.D2 - short (D9Mit90,182) congenic and B6 strains. Data are for the first 5 min (mean ± SEM) of the 15-min test session.

Experiment 3: There is no evidence of a QTL for ethanol stimulation on distal chromosome 9 when tested on the B6 background

When the chromosome 9 B6.D2 – long congenic was tested for ethanol (2 g/kg)-induced locomotor stimulation, no difference in response compared to B6 control mice was observed (Figure 15). A 2-way ANOVA revealed a significant main effect of sex ($F_{1, 77}$=12.2, $p<0.001$) that did not interact with strain. Female mice were more stimulated by ethanol than male mice (430.1 ± 102.3,-86.0 ± 104.1, respectively). When BEC values were examined there was a significant strain X sex interaction ($F_{1, 77}$=4.6, $p<0.05$) due to higher BEC only in male congenic compared to male B6 mice ($p < .05$; 2.34 ± .07, 2.18 ± .08, respectively).

Experiment 4: The QTL for ethanol-induced locomotor stimulation resides between 23 and 69 cM

The congenic F_2 mice provided some resolution of the QTL region. One animal appeared to be suffering from hydrocephalus, therefore its data were excluded. When tested for the acute locomotor response to ethanol, the 259 congenic F_2 mice showed a normal phenotypic distribution (data not shown). When the top and bottom 12.5% of the phenotypic population were analyzed using R/qtl there was significant evidence of a QTL for ethanol stimulation on mouse chromosome 9 that accounts for 10% of the phenotypic variation in this trait. The peak LOD score was at 59 cM with a 1 LOD support interval ranging from 23 – 69 cM (Figure 16). This narrows the location of the QTL from a 62 cM region to a 46 cM region, which still contains the 3 genes encoding the nicotinic

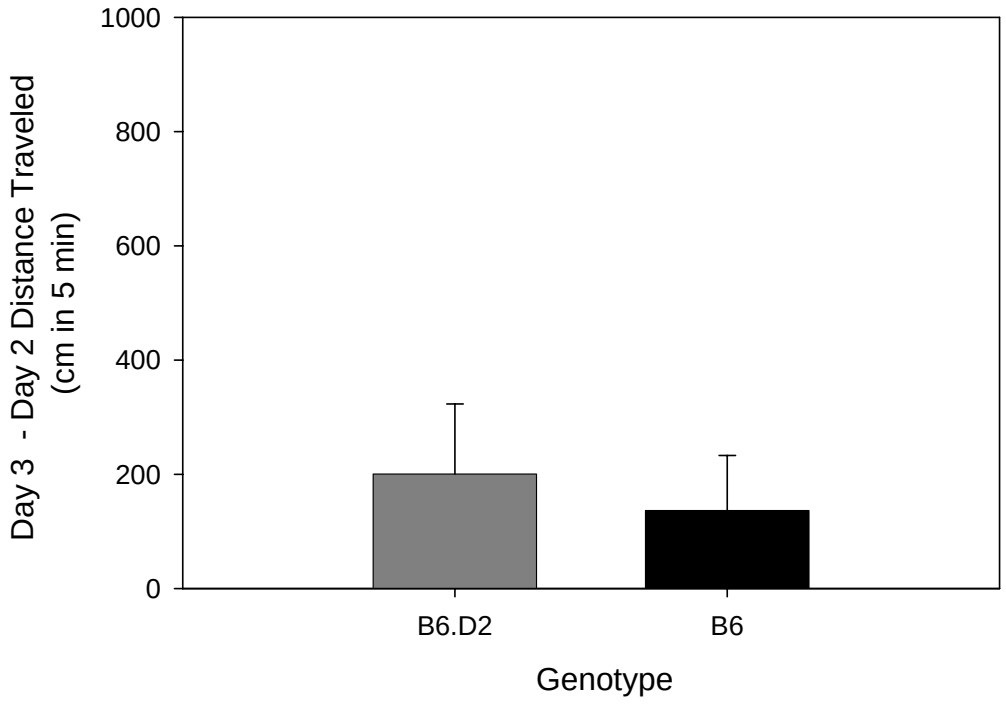

Figure 15. A longer segment congenic does not capture the chromosome 9 ethanol stimulation QTL on the B6 background. Shown is the acute locomotor response to ethanol (2 g/kg) in chromosome 9 B6.D2 - long (D9Mit90,18) congenic and B6 strains. Data from the first 5 min (mean ± SEM) of the 15-min test session.

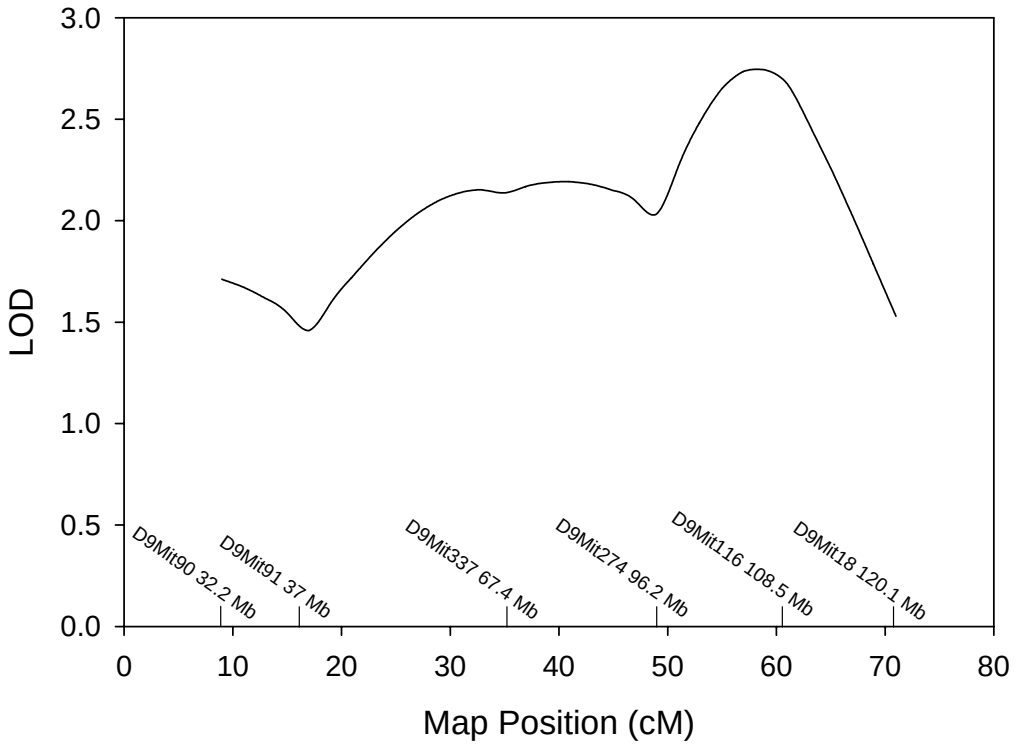

Figure 16. QTL mapping in a congenic F_2 population reduces the QTL interval from a 62 to a 46 cM region. The peak LOD score for the chromosome 9 QTL is at 59 cM with a 1-LOD interval of 23 – 69 cM. Shown is the LOD score representing the most likely location of the QTL for ethanol (2 g/kg)-induced stimulation on chromosome 9 based on interval mapping in the Chromosome 9 D2.B6 (D9Mit90,18) congenic X D2 F_2 mice. The genomic makers used and their map locations (in Mb) are displayed above the X axis.

acetylcholine receptor subunits. There was no evidence of dominance of the B6 allele when the extreme high and low scoring animals were analyzed by a χ^2 analysis (D9Mit90 $\chi^2(1, N = 64) = 0.58$, $p>0.05$; D9Mit91 $\chi^2(1, N = 64) = 0.06$, $p>0.05$; D9Mit337 $\chi^2(1, N = 64) = 0.06$, $p>0.05$; D9Mit274 $\chi^2(1, N = 64) = 0.25$, $p>0.05$; D9Mit116 $\chi^2(1, N = 64) = 0.57$, $p>0.05$; D9Mit18 $\chi^2(1, N = 64) = 0.56$, $p>0.05$).

Experiment 5: The α3 subunit of the nicotinic acetylcholine receptor is a candidate gene for ethanol stimulation

The α3, but not α5 or β4, subunit gene of the acetylcholine receptor was differentially expressed between the chromosome 9 D2.B6 congenic and D2 mice (Figure 17). Congenic mice had significantly more *Chrna3* gene expression than D2 mice ($F_{1, 8}=29.1$, $p<0.001$), independent of sex.

Experiment 6: Strains of mice that differ in response to an acute injection of ethanol also differ in *Chrna3* expression in brain regions involved in this response

Chromosome 9 D2.B6 congenic mice had significantly more *Chrna3* expression than D2 mice in both the ventral midbrain ($F_{1, 18}=35.0$, $p<0.001$) and striatum ($F_{1, 18}=5.9$, $p<0.05$), independent of sex (Figure 18).

Discussion

The current studies confirmed the presence of a gene on mouse chromosome 9 that accounts for approximately 20% of the variation in the acute locomotor response to ethanol. These studies also provided evidence that the gene expressing the α3 subunit of the nicotinic acetylcholine receptor is a stronger candidate for the chromosome 9 QTL

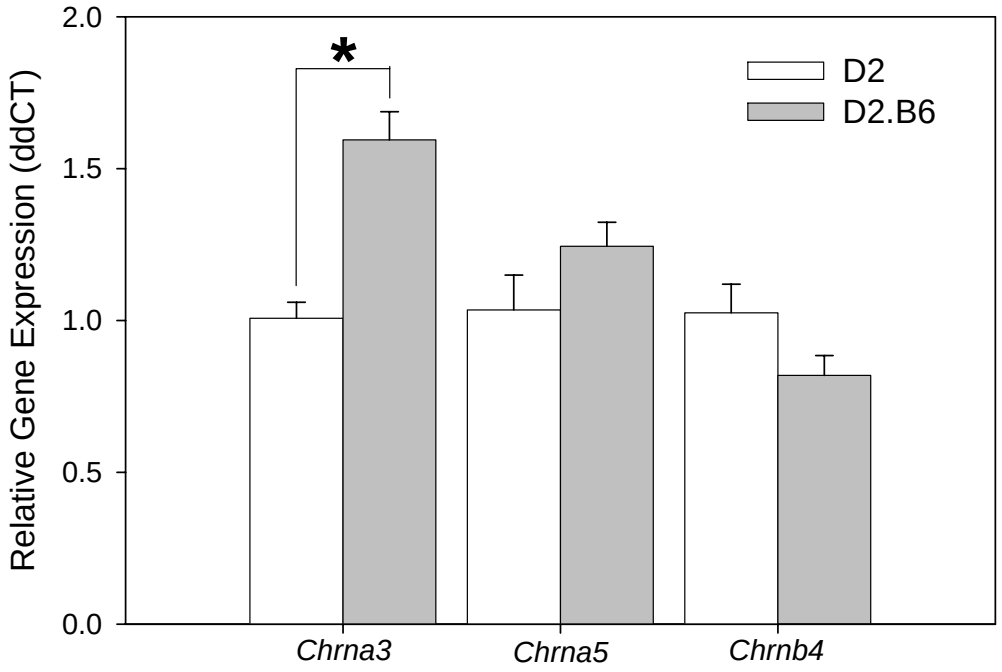

Figure 17. Greater whole brain *Chrna3,* but not *Chrna5* or *Chrnb4,* expression corresponds with reduced locomotor response to ethanol. Shown is mean ± SEM relative *Chrna3*, *Chrna5*, and *Chrnb4* expression in the chromosome 9 D2.B6 (D9Mit90,18) congenic and D2 strains of mice. *$p < 0.001$.

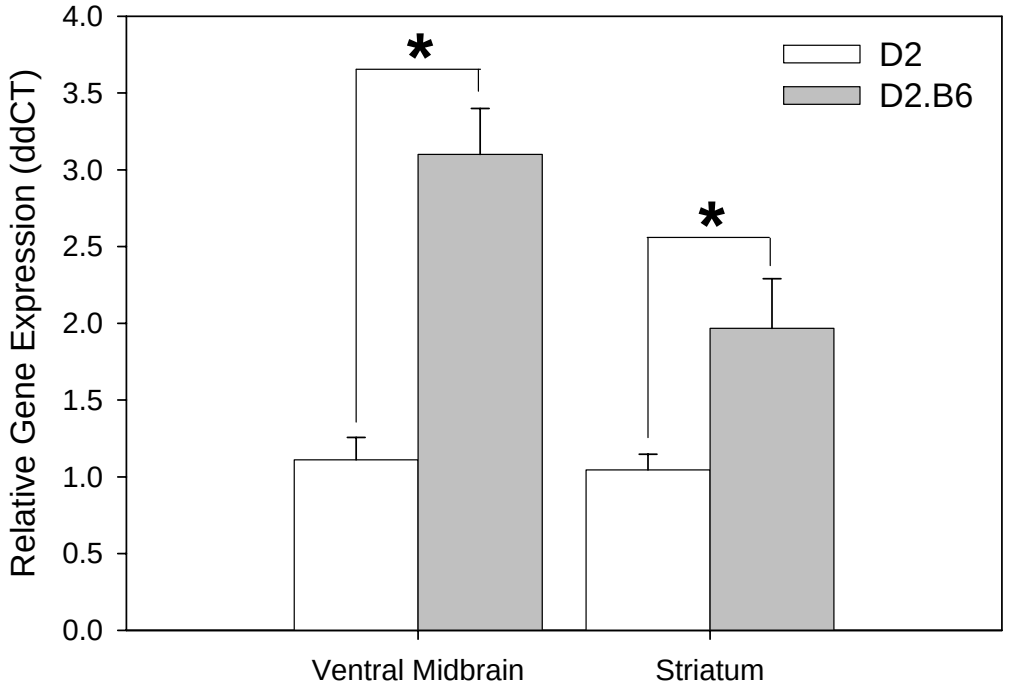

Figure 18. Greater ventral midbrain and striatum *Chrna3* expression corresponds with reduced locomotor response to ethanol. Shown is mean ± SEM relative *Chrna3* expression in the chromosome 9 D2.B6 (D9Mit90,18) congenic and D2 strains of mice. *$p < 0.05$.

than are the α5 and β4 subunits genes based on expression profiles in mice that differ greatly in the stimulant response to ethanol.

Prior mapping data had provided suggestive evidence of a QTL for ethanol-induced stimulation on chromosome 9 (Cunningham, 1995; Demarest et al., 1999; Palmer et al., 2006). The presence of a QTL in this region in a D2 background congenic was confirmed at a p-value which exceeds Lander and Kruglyak's (1995) threshold for a significant QTL. I was not able to detect the QTL for ethanol stimulation using two B6 background congenics. There are at least three possible reasons why the QTL could not be detected on this background. First, because B6 mice are particularly sensitive to the inhibitory effects of ethanol on locomotor behavior, the 2 g/kg dose of ethanol may have been too high, and a single gene in this region that influences the locomotor stimulant response (present in the B6.D2 – short congenic) could not completely overcome sedative mechanisms. These sedative effects are likely mediated by at least some non-overlapping neurobiological systems, compared to the stimulant effect (Phillips et al., 2002b). Even when the B6.D2 - short congenic was tested at a lower ethanol dose (1.5 g/kg), the QTL for ethanol stimulation on chromosome 9 QTL could not be confirmed on this background.

The second reason for lack of confirmation applies to the B6.D2 - short congenic. This congenic had a shorter introgressed region than the D2 background congenic that did capture the QTL. However, when a comparable congenic to the D2.B6 was created and tested, the QTL for ethanol stimulation was not detected. These data do not rule out that a gene influencing ethanol stimulation resides in the distal region, which leads to a third possible explanation.

A third reason why I may not have been able to detect the QTL for ethanol stimulation on the B6 background is that this gene epistatically interacts with other loci elsewhere in the genome. In other words, D2 alleles at other loci may have to be present in combination with the chromosome 9 D2 allele to see a phenotypic effect. Similar epistatic interactions (i.e., background effects) have been observed for ethanol consumption. For example, the effect of the 5-hydroxytryptamine $(5\text{-HT})_{1B}$ receptor on ethanol consumption appears to be dependent on epistatic interactions with other loci (Phillips et al., 1999; Phillips and Belknap, 2002). There are currently no existing data that allow for examination of this hypothesis for the ethanol stimulation trait. However, crossing the B6 background congenic to other congenics known to capture a QTL for ethanol-induced locomotor stimulation may provide support of this hypothesis. For example, a QTL for ethanol stimulation has recently been confirmed on mouse chromosome 2 in a B6 background congenic (Palmer et al., 2006). Creating double B6.D2 congenics (i.e., mice carrying introgressed D2 segments on both chromosome 2 and 9) may begin to address the possibility of epistatic interactions, or more simple additive effects of trait relevant genes.

To my knowledge, this is the first attempt to fine map a QTL using a congenic F_2 population. I was able to narrow the location of the gene from a 62 cM region to a 46 cM region. The effect size of this QTL is large for a behavioral QTL, but in the range of effect sizes of QTL that have been successfully fine mapped (Flint et al., 2005). Additional strategies, such as the use of interval-specific congenic strains (ISCS), may further fine map the location of the gene on chromosome 9 that influences ethanol stimulation (Darvasi, 1997). The Portland Alcohol Research Center has a panel of ISCS

for chromosome 9 that breaks up the relevant region and has been efficacious for more finely mapping a methamphetamine locomotor stimulation trait (Chapter 4). However, it is a B6 background strain panel, so not likely to be useful for identification of the ethanol stimulation quantitative trait gene.

Nicotinic acetylcholine receptors have been implicated in the acute locomotor response to ethanol (Chapter 2; Blomqvist et al., 1992; Larsson et al., 2002). Of particular importance to this study is that the nonspecific acetylcholine receptor antagonist mecamylamine attenuated ethanol-induced stimulation in D2 mice (Chapter 2). While mecamylamine is regarded as nonspecific, some suggest that this drug is most potent at inhibiting α3β4 acetylcholine receptors (Papke et al., 2001). Further evidence for the involvement of α3-containing acetylcholine receptors comes from work done in NMRI outbred mice. Whereas, mecamylamine attenuated ethanol stimulation in this mouse strain, the involvement of α4β2 and α7 nicotinic acetylcholine receptors was excluded because the cholinergic antagonists, dihydro-β-erythroidine (α4β2-specific) and methyllycaconitine (α7-specific) had no effect on ethanol-induced stimulation (Larsson et al., 2002). Additionally, α-conotoxin MII (α3β2-, β3-, and α6-specific), but not α-conotoxin PIA-analogue (α6-specific), attenuated ethanol-induced locomotor stimulation, providing evidence that α3β2- or β3-containing nicotinic acetylcholine receptors are involved in this response (Larsson et al., 2004; Jerlhag et al., 2006).

18-Methoxycoronaridine appears to be specific for α3β4 acetylcholine receptors, but is not commercially available. To my knowledge, the effect of 18-methoxycoronaridine on ethanol-induced stimulation has not been tested, but this drug was shown to decrease ethanol consumption and preference (Rezvani et al., 1997). To

date, there are no known coding sequence polymorphisms in α3 gene between B6 and D2 mice (www.genenetwork.org). Homozygous α3 knockout mice have growth impairments and die within weeks after birth (Xu et al., 1999), however mice heterozygous for the null mutation may be useful. Studies examining nicotinic receptor densities in these mice have revealed similar receptor levels as wildtype animals (Whiteaker et al., 2002), but mice lacking a single copy of *Chrna3* are less sensitive to nicotine-induced seizures than wildtype mice (Salas et al., 2004). These data suggest that testing heterozygous mice may be useful in examining the role of α3-containing receptors in ethanol-induced stimulation. Intra-cerebral administration of conotoxins that are specific for α3-containing nicotinic acetylcholine receptors may also help to elucidate the role of these receptors in ethanol stimulation with regard to neuroanatomical location (Clark et al., 2006; Talley et al., 2006).

In this study differences in *Chrna3* expression between chromosome 9 D2.B6 congenic and D2 control mice were observed, but further work is needed to determine if these strains have differences in α3 protein levels. The currently assays used to measure α3 protein levels appear to have some inadequacies. Cytisine-resistant epibatidine binding has been used to measure α3-containing receptors (Perry et al., 2002), but there is accumulating evidence that this assay is not detecting exclusively α3-containing receptors (Parker et al., 1998; Kuryatov et al., 2000; Xiao and Kellar, 2004; Marks et al., 2006). Rather, these data suggest that cytisine-resistant epibatidine binding is measuring acetylcholine receptors that contain an α3 subunits, as well as some which do not. Further, there appear to be no well validated antibody based approaches to examine the

α3 subunit (please see appendix following this chapter for my results using the epibatidine binding assay).

The α3 subunit gene is expressed in both midbrain dopamine and γ-amino butyric acid (GABA) neurons (Klink et al., 2001; Azam et al., 2002). Mouse synaptosome preparations have provided evidence that receptors containing the α3 subunit are not directly involved in striatal dopamine release (Salminen et al., 2004), but acetylcholine receptors containing this subunit located on dopamine cell bodies could modulate dopamine release. Mecamylamine administered directly into the ventral tegmental area decreases both ethanol-induced locomotor stimulation and elevations in dopamine levels in the nucleus accumbens which occur in response to ethanol (Blomqvist et al., 1997). Because the ventral tegmental area is a major site of dopamine cell bodies, these data suggest the hypothesis that nicotinic acetylcholine receptors containing an α3 subunit on dopamine cell bodies may modulate these responses to ethanol. The expression differences observed in the chromosome 9 D2.B6 congenic and control mice are also consistent with this hypothesis. D2 control mice had less *Chrna3* expression than D2.B6 congenic mice. The lower level of *Chrna3* expression in the D2 mice may be the result of greater endogenous acetylcholine levels. This would be consistent with the work of Schwartz and Kellar (1983) who showed that treatment with a cholinesterase inhibitor, which had the effect of increasing acetylcholine levels, decreased nicotinic receptor levels. An alternative hypothesis also exists. Presynaptic acetylcholine receptors on glutamate terminals (originating from the laterodorsal tegmental nucleus, pedunculopontine nucleus, or prefrontal cortex) may have a similar effect because activation of these receptors would also increase activation of VTA dopamine neurons.

Although there is no direct evidence that α3-containing acetylcholine receptors exist on glutamatergic terminals located in the VTA, there is evidence nicotinic acetylcholine receptors modulate glutamate transmission in other brain regions (Vidal and Changeux, 1993; Guo et al., 1998)

In humans, administration of ethanol in the laboratory can produce self-reported ratings of stimulation and euphoria, an effect which is decreased by pretreatment with mecamylamine (Chi and de Wit, 2003; Young et al., 2005). Similarly, mecamylamine has been shown to attenuate intravenous (i.v.) nicotine-induced euphoria (Lundahl et al., 2000). Together these data suggest that nicotinic receptors may be involved in the acute response to both ethanol and nicotine and may serve as a common neural mechanism for sensitivity to these drugs and their co-abuse. Improvements in the detection of nicotinic acetylcholine receptors constructed from specific subunits will lead to a better understanding of the role of these receptors in addictive-related processes.

Appendix

Preamble

Receptor binding experiments can provide information regarding the density of receptors in the brain, which is a measure of protein abundance. Since differences in *Chrna3* gene expression in the chromosome 9 D2.B6 (D9Mit90,18) congenic and D2 control mice were observed (see results of Chapter 3) a published α3 subunit binding assay was utilized (Perry et al., 2002) to determine if these mice had different levels of the α3 subunit of the nicotinic acetylcholine receptor. There was greater *Chrna3* gene expression in the chromosome 9 D2.B6 congenic mice compared to D2 control mice, therefore I hypothesized that congenic mice would have more α3-containing acetylcholine receptors as indicated by greater cytisine-resistant [^{125}I]-epibatidine binding than D2 mice.

These data are being presented in an appendix rather than in Chapter 3 of the dissertation because I believe that there are a number of problems with the cytisine-resistant epibatidine binding assay. The issues associated with this assay are described in the discussion of this section.

Methods

Animal housing conditions and husbandry were consistent with those already described in this dissertation. Brains were harvested from 52 to 80 day old experimentally naïve chromosome 9 D2.B6 (D9Mit90,18) congenic and D2 control mice. Mice were cervically dislocated and quickly decapitated. Brains were removed and placed on an ice cold platform for microdissections. The olfactory bulbs were cut where the bulb connects to the olfactory tract. The striatum and ventral midbrain were dissected

as described in the methods of Chapter 3. For the olfactory bulb and striatal samples, tissue from two animals was pooled to obtain enough protein; tissue from three animals was needed to obtain enough tissue for ventral midbrain analysis.

Materials

[^{125}I]-epibatidine (specific activity 2200 Ci/mmol) was purchased from PerkinElmer (Boston, MA). (-)-Nicotine hydrogen tartrate, cytisine, sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and polyethyleneimine were obtained from Sigma Aldrich (St. Louis, MO). The Protease Inhibitor Cocktail Set III was purchased from EMD Bioscience (San Diego, CA).

Membrane Preparation

Membranes were isolated using the procedure described by Whiteaker et al. (2000). Protease inhibitor (1 µl/3 ml buffer) was added to ice cold hypotonic buffer (14.4 mM NaCl; 0.2 mM KCl; 0.2 mM $CaCl_2$; 0.1 mM $MgSO_4$; 2 mM HEPES; pH 7.5) prior to homogenization. Tissue was thawed and homogenized in the hypotonic buffer using a glass-Teflon tissue grinder. The homogenate was centrifuged at 20,000 x g for 15 min at 4˚C to isolate the particulate fractions. The samples were then resuspended in fresh buffer and incubated at 22˚C for 10 min. This process of centrifugation/resuspension was repeated 3 more times to remove endogenous acetylcholine from the tissue. Following the last resuspension, an aliquot was taken for protein quantification using a Peirce BCA protein assay (Rockford, IL). The remaining sample was centrifuged and stored in pellet form at -80˚C.

Verification of Assay Conditions

[^{125}I]-Epibatidine Saturation Binding

Saturation binding of [^{125}I]-epibatidine to membranes was performed as previously described by Whiteaker and colleagues (2000). Briefly, reactions (total volume = 50 µl) were run in triplicate or quadruplicate (depending on protein availability) in 96-well polystyrene plates. Brain homogenates were prepared in binding buffer (144 mM NaCl; 1.5 mM KCl; 2 mM $CaCl_2$; 1 mM $MgSO_4$; 20 mM HEPES; pH = 7.5 with a protease inhibitor cocktail) to appropriate concentrations (30 µg). Samples were incubated at 22˚C for 2 h in the presence of 4 - 800 pM [^{125}I]-epibatidine. Nonspecific binding was determined in the presence of (-)-nicotine hydrogen tartrate (1 mM). Reactions were terminated by filtering samples onto a polyethyleneimine (0.05%)-soaked glass fibre filter (Wallac, Turku, Finland) using a harvester (Tomtec, Hamden, CT). Filters were washed with ice-cold binding buffer before being air dried. Scintillation fluid (Betaplate Scint; PerkinElmer, Boston, MA) was added to the filters and radioactivity was counted using a microbeta scintillation counter (1450 MicroBeta Trilux; PerkinElmer, Boston, MA). Free [^{125}I]-epibatidine was estimated by subtracting the amount of bound epibatidine from the total amount of epibatidine added to the assay. From saturation binding curves B_{max} and K_d values were obtained.

[^{125}I]-Epibatidine Inhibition by Cytisine

For competition curves, increasing concentrations of cytisine (0.053 – 3000 nM) were added to 200 pM [^{125}I]-epibatidine using the procedures described above. Estimates of IC_{50} values were determined from the binding curves. From the IC_{50} values, inhibition constants (K_i) values were determined using the equation described by Cheng & Prusoff

(1973), $K_i = IC_{50}/(1 + (L/ K_d))$, where L refers to the amount of [^{125}I]-epibatidine in the assay and the K_d is the dissociation constant of epibatidine.

Cytisine-Resistant [^{125}I]-Epibatidine Binding

To determine if the chromosome 9 D2.B6 (D9Mit90,18) congenic and D2 control mice differ in amount of α3 receptor binding, cytisine-resistant [^{125}I]-epibatidine binding was used (Whiteaker et al., 2000; 2002). Binding assays were performed as above. Cytisine (20 nM) was used to assess cytisine-resistant epibatidine binding, while nonspecific binding was assessed with (-)-nicotine hydrogen tartrate (1 mM) in the presence of 200 pM [^{125}I]-epibatidine. To determine cytisine-resistant epibatidine binding, counts per minute from the nonspecific binding samples were subtracted from the cytisine-resistant epibatidine binding. Counts per minute of the cytisine-resistant binding wells were converted into fmol using the specific activity of epibatidine and the efficiency of the counter (39%), this value was then divided by the amount of protein in the assay to get fmol/mg protein (the dependent variable).

Statistics

Epibatidine saturation binding and inhibition by cytisine were analyzed in GraphPad Prizm 4 (San Diego, CA). This program was used to generate the saturation binding parameters (B_{max} and K_d) and IC_{50} values from the cytisine competition curves. Statistical analyses were performed using Statistica (StatSoft, Tulsa, OK, USA). Data were analyzed using a 2-way analysis of variance (ANOVA) with the independent variables sex and strain. Significant interactions were followed up by simple main effects analyses, while significant main effects were further characterized by Newman-Keuls mean comparisons. The alpha level was set at 0.05.

Results

Verification of Assay Conditions

[^{125}I]-Epibatidine Saturation Binding

Extensive pilot data verified the parameters of the ligand binding procedures used in the literature (Whiteaker et al., 2000; 2002). Briefly, time course experiments verified that incubation at 22˚C for 2 h allowed for binding to come to equilibrium. Equilibrium was reached by 60 min and remained constant until 2 h (data not shown). Protein curves indicated that a linear increase in [^{125}I]-epibatidine binding up to 45 µg of protein was observed (data not shown). Saturation binding curves provided an estimate of B_{max} and K_d. The estimate of B_{max} was 39.8 (± 2.1) fmol/mg protein and K_d was estimated to be 71 pM (95% confidence interval: 42 – 119 pM; Figure 19). These values are similar to that found by Whiteaker and colleagues (Whiteaker et al., 2000). Furthermore, the saturation binding data were used to create a Scatchard plot which provided evidence of a single site fit.

[^{125}I]-Epibatidine Inhibition by Cytisine

Competition curves provided evidence that epibatidine binds to two populations of receptors, one sensitive to inhibition by low doses of cytisine and the other resistant, in specific brain regions (Figure 20), but not in whole brain preparations (data not shown). Consistent with prior studies, two receptor binding populations were detected in the olfactory bulbs and ventral midbrain. A two-site binding model was not consistently detected in the striatum; therefore this brain region was not used in further studies.

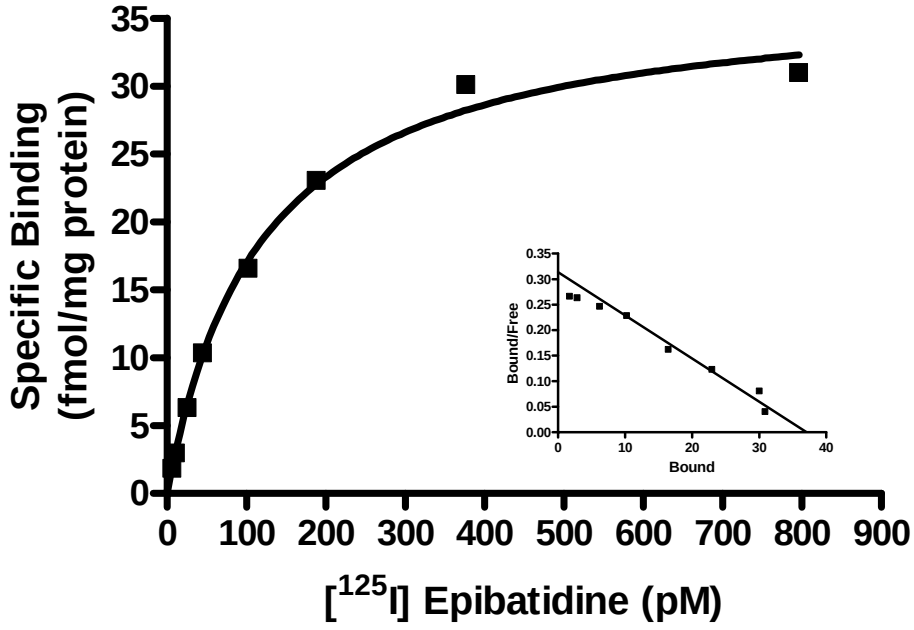

Figure 19. [^{125}I]-epibatidine saturation binding in olfactory bulb tissue from chromosome 9 D2.B6 congenic and D2 control animals. The assay was repeated 3 times in this tissue and 7 times in whole brain tissue; results were similar for both tissue types. A representative binding curve is shown. The mean B_{max} was 39.8 (± 2.1) fmol/mg protein and K_d was 71 pM (95% confidence interval: 42 – 119 pM). The inset is a Scatchard plot corresponding to the saturation data, the linearity of the plot indicates a single binding site for [^{125}I]-epibatidine.

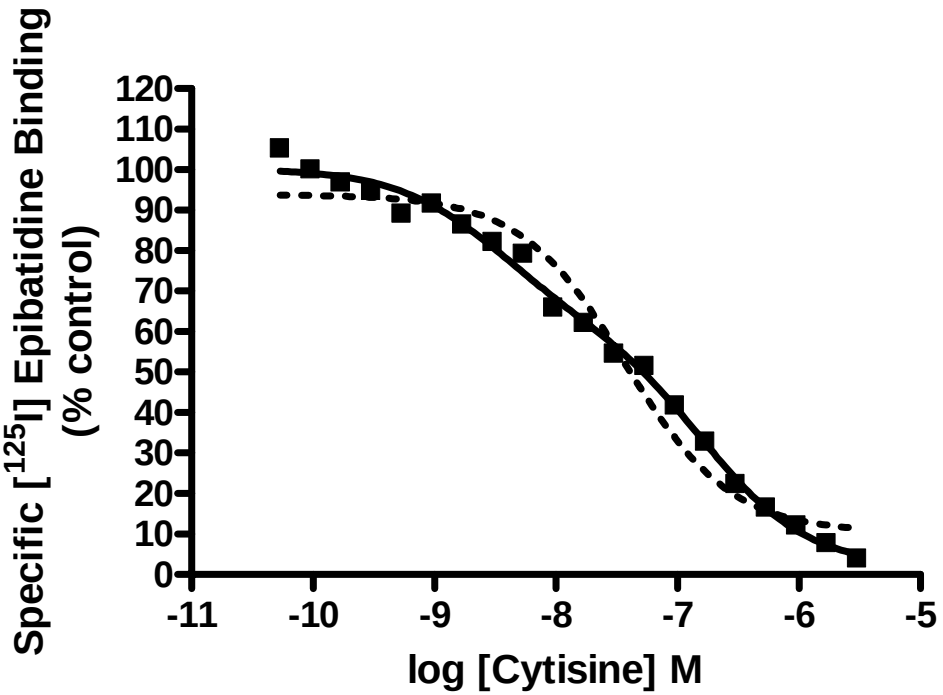

Figure 20. Cytisine inhibition of [^{125}I]-epibatidine binding in olfactory bulb tissue from chromosome 9 D2.B6 congenic and D2 control animals. The assay was repeated 3 times in tissue from the olfactory bulb and 2 times in tissue from the ventral midbrain; results were similar for both brain regions. Total binding was typically 2200 cpm, nonspecific binding was 24 cpm, and cytisine (20 nM) resistant epibatidine binding was 400 cpm. Deviation in binding from a sigmoidal curve (dotted line) provides evidence of a 2-site model with a population of receptors sensitive to inhibition by low doses of cytisine and a second population which is not.

Data obtained from these inhibition experiments provided an estimate of the IC_{50} values for the epibatidine binding sites with high and low affinity for cytisine. The IC_{50} value corresponding to cytisine-sensitive epibatidine sites (i.e., high affinity) was 2.07 nM (95% confidence interval: 0.87 – 4.94 nM), while the IC_{50} value of cytisine-resistant sites (i.e., low affinity) was 220 nM (130 – 360 nM). Approximately 48% of the binding sites were resistant to cytisine-inhibition, a value that is consistent with prior studies (Marks et al., 1998). K_i values were determined using the equation described by Cheng & Prusoff (1973) with the K_d estimate of 71 pM. The K_i values observed for the sensitive [0.34 nM (0.14 – 0.81 nM)] and resistant [35 nM (22 - 57 nM)] populations of receptors were consistent with that of prior binding studies in B6 mice (Marks et al., 1998; Whiteaker et al., 2000). Based on these data, 20 nM cytisine was chosen for the cytisine-resistant [^{125}I]-epibatidine binding study to compare α3 receptor densities in congenic and control mice.

Cytisine-Resistant [^{125}I]-Epibatidine Binding

The chromosome 9 D2.B6 (D9Mit90,18) and D2 control mice did not differ in cytisine-resistant [^{125}I]-epibatidine (Figure 21). Binding (fmol/mg protein) data from each brain region were analyzed separately using a 2-way ANOVA with sex and strain as independent variables. There were no significant main effects or interactions in data from either brain region.

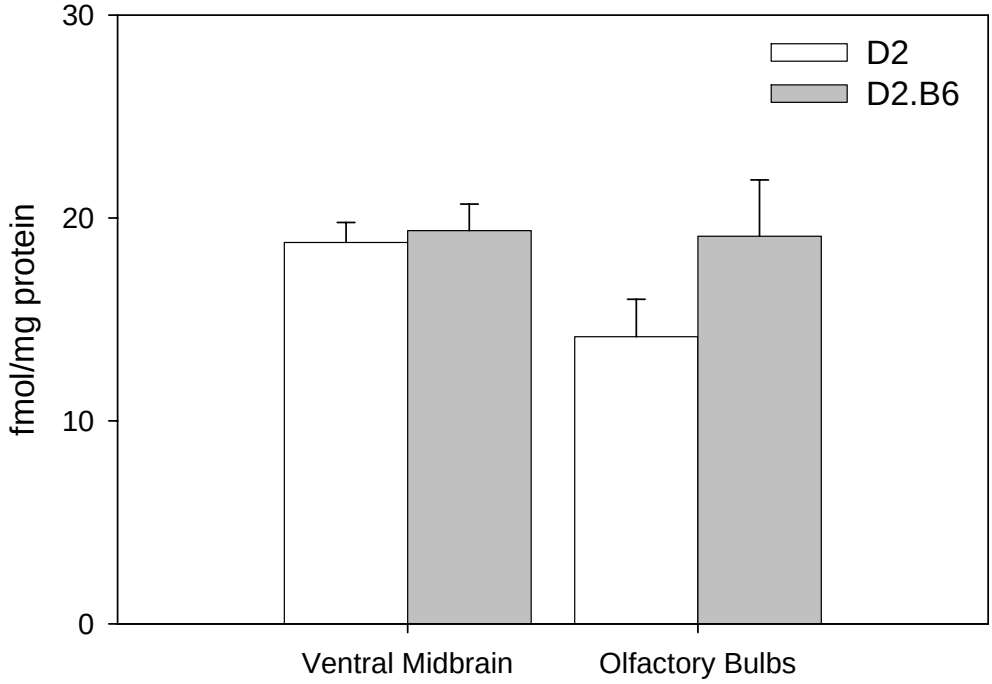

Figure 21. Strains of mice that differ in response to an acute injection of ethanol have similar densities of α3-containing acetylcholine receptors in the ventral midbrain and olfactory bulbs. Mean ± SEM fmol cytisine-resistant [^{125}I]-epibatidine receptor binding/ mg protein in the chromosome 9 D2.B6 and D2 strains of mice. N = 8 - 10.

Discussion

Cytisine-resistant epibatidine binding was used to measure α3-containing nicotinic acetylcholine receptors (Perry et al., 2002), but accumulating data provide evidence that this assay does not solely measure acetylcholine receptors containing this subunit. Receptor binding methods that examine nicotinic acetylcholine receptors containing an α3 subunit have a history of methodologic problems. One of the first ligands thought to bind specifically to α3β2 receptors was α-Conotoxin MII (Cartier et al., 1996). Since the original publication describing the use of this ligand to examine α3β2 nicotinic receptors, a number of problems with this assay have been discovered. The first problem was discovered when receptor autoradiography was performed using this ligand in mice lacking the α3 receptor; high levels of α-Conotoxin MII binding were observed (Whiteaker et al., 2002), providing evidence that this toxin does not bind solely to receptors containing this subunit. Additional data in support of this finding came when α-Conotoxin MII binding was found to be decreased in α6 and β3 knockout mice (Champtiaux et al., 2002; Cui et al., 2003), indicating that this toxin binds to α6- and β3-containing nicotinic acetylcholine receptors, in addition to α3β2 receptors.

Following the discovery that α-Conotoxin MII was not specific for α3β2 receptors, the cytisine-resistant epibatidine binding assay was developed as a tool to examine these receptors. The basic premise to this assay is that epibatidine binds to α4β2, α3β2, and α3β4 receptors (Perry et al., 2002) and that cytisine binds with high affinity to α4β2 receptors (Zhang and Steinbach, 2003). Therefore, cytisine-resistant epibatidine binding should quantify α3-containing nicotinic receptors (Perry et al., 2002). Unfortunately, there is accumulating data that the assumptions regarding this assay are

not valid. For example, epibatidine has been shown to bind to nicotinic acetylcholine receptors other than those containing the α3, α4, β2 and β4 subunits. Specifically, epibatidine has been shown to bind to receptors containing α2 and α6 subunits (Parker et al., 1998; Kuryatov et al., 2000; Xiao and Kellar, 2004). The current thought on the cytisine-sensitive epibatidine binding is that these receptors require a β2, while there are two distinct populations of cytisine-resistant sites, one population requiring β2 and the other requiring the presence of a β4 subunit (Marks et al., 2006). While these receptors may contain the α3 subunit, there are likely some receptors that do not have this subunit.

The most prominent other way to examine protein levels is through antibody-based approaches (i.e., western blot or immunohistochemistry). These assays require an antibody that specifically binds to the protein of interest. Emerging evidence suggests that the antibodies currently available for acetylcholine receptor subunits are inadequate. Data from α7 knockout mice have shown that three of the most commonly used antibodies had a similar amount of binding in α7 knockout and wildtype animals, providing evidence that these antibodies are not specific for this receptor subunit (Herber et al., 2004). Recently, similar data have been observed with other nicotinic receptor subunits including α3 (Moser et al., 2007). Furthermore, the high degree of sequence similarity between the α3 and α6 nicotinic acetylcholine receptor subunits (Le Novere and Changeux, 1995) adds further difficulty in developing an antibody specific for the α3 subunit. Therefore, approaches using antibodies need to be rigorously validated and the available α3 antibodies may not be adequate.

Because of the issues associated with cytisine-resistant epibatidine binding as an estimate of α3-containing nicotinic receptors, it cannot be concluded that there are no

differences in α3 protein in the chromosome 9 D2.B6 congenic and D2 control animals. What can be concluded is that there are no differences between the chromosome 9 D2.B6 congenic and D2 control mice in the density of the population of receptors quantified by this assay. There is a small, nonsignificant, difference in cytisine-resistant epibatidine binding in the olfactory bulb samples between these two strains. At a mean difference of 5 fmol/mg protein approximately 35 olfactory bulb samples per strain would be needed to detect a significant difference of this magnitude. While it is likely that this population includes receptors containing an α3 subunit, the assay is not exclusively measuring receptors containing this subunit. The dissociation between α3 gene expression and protein levels observed in these mice is addressed in the general discussion. To date there are no validated assays to measure α3 protein. The development of new assays to detect α3-containing nicotinic receptors will be needed to more fully address this question, but antibody-based approaches may have as many problems as binding experiments.

Chapter 4: Mapping Quantitative Trait Loci for Psychostimulant-Induced Locomotor Activation

Helen M. Kamens & Tamara J. Phillips

This chapter is written in a manuscript format for consistency with the other chapters, but is not intended for publication. These experiments will be combined with other data not a part of my dissertation research for publication purposes.

ACKNOWLEDGEMENTS

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Abstract

The magnitude of acute locomotor response to psychostimulants may predict one's likelihood of further drug use. Therefore, understanding the biological basis of this response may provide a greater understanding of drug abuse. Cocaine and methamphetamine are known to have psychomotor activating properties which are influenced by genetics. A number of quantitative trait loci (QTL) have been mapped for the locomotor response to an acute injection of these drugs. A QTL for both cocaine and methamphetamine stimulation has been mapped to mouse chromosome 9. To confirm the presence of this QTL, reciprocal chromosome 9 congenic strains of mice were tested. The presence of the QTL for methamphetamine stimulation was confirmed, but only weak evidence of a QTL for cocaine stimulation was observed. To more finely map the QTL for methamphetamine stimulation a panel of nine chromosome 9 interval-specific congenic strains (ISCS) was tested. Four of the ISCS captured the methamphetamine stimulation QTL, narrowing the location of the gene(s) from a 69.13 Mb region to a 24.16 Mb region. Approximately 20% of the phenotypic variation in the response to methamphetamine is accounted for by this QTL. Additional work is needed to more finely map this QTL and identify the quantitative trait gene (QTG).

Introduction

Genes and environment are known to be important factors in determining drug abuse. Twin studies provide evidence of a genetic contribution to determining one's likelihood to use or abuse cocaine and amphetamines (van den Bree et al., 1998; Kendler et al., 2000; Kendler et al., 2003). Data from a drug behavioral preference procedure in humans has provided evidence that the acute locomotor activating and euphoric effects of psychostimulants may predict one's likelihood of further drug use (de Wit et al., 1986; Gabbay, 2003). In these studies, subjects had 4 sampling sessions on 4 different days where they were given a pill containing either amphetamine or placebo. The subjects were not told what drug they had received, but were instructed to remember the color of the pill (the amphetamine and placebo pills were different colors). The subjects then had 3 - 5 choice tests on the different days where they chose the color of the pill they would like to ingest that day. The group of subjects that chose amphetamine on all choice tests had been more stimulated by amphetamine during the sampling phase compared to the group of subjects that did not choose amphetamine on any of the choice tests (de Wit et al., 1986; Gabbay, 2003). These data support the idea that understanding the biological basis of the acute locomotor response to psychostimulants may help understand the basis of drug abuse.

A number of studies have examined inbred mouse strain differences in the locomotor response to amphetamine (Moisset and Welch, 1973; Oliverio et al., 1973; Anisman et al., 1975; Remington and Anisman, 1976; Moisset, 1977; Kitahama and Valatx, 1979; Hamburger-Bar et al., 1986; Wenger, 1989; Zocchi et al., 1998; Ralph et al., 2001) and cocaine (Ruth et al., 1988; Jones et al., 1993; Tolliver and Carney, 1994;

Womer et al., 1994; Henricks et al., 1997; Miner, 1997; Marley et al., 1998; Rocha et al., 1998; Downing et al., 2003a), providing evidence that genetics may be important in psychostimulant-induced activation. Additional studies provide stronger evidence. The strain distribution pattern of the acute locomotor response to amphetamine among seven recombinant inbred (RI) strains derived from the C57BL/6By (B6; this abbreviation will be used for any C57BL/6 subline in this paper) and BALB/cBy strains provided evidence that multiple genes influence this response (Oliverio et al., 1973). Two selective breeding experiments have provided additional evidence that the locomotor response to psychostimulants is polygenic. In 1998, a selection commenced for cocaine sensitivity (CAHI) and insensitivity (CALO). The CAHI and CALO lines were derived from genetically heterogeneous (HS/Ibg) mice (McClearn et al., 1970), based on locomotor stimulation observed following cocaine (10 mg/kg; Marley et al., 1998). Two replicate CAHI and CALO lines were bred for 12 generations for sensitivity to the locomotor stimulant effects of cocaine. After the first selection generation, the CAHI and CALO differed in the acute locomotor response to cocaine, and these lines continued to diverge through selection generation 9. At the end of the 12 generations of selective breeding, cocaine-induced stimulation was 4-fold greater in the 2 CAHI lines than in the 2 CALO lines. A second set of selected lines was bred for the acute locomotor response to methamphetamine (2 mg/kg; Kamens et al., 2005). The HMACT and LMACT lines were selectively bred from a B6 X DBA/2J (D2) F_2 population for increased or decreased sensitivity to methamphetamine, respectively. These lines differed in response to an acute injection of methamphetamine after the first selection generation, and continued to progressively diverge further over the three additional selection generations. That lines

of mice can be selectively bred for the locomotor response to psychostimulants provides evidence of additive genetic influence on these traits.

Selected lines of mice can also be used to examine if genes have pleiotropic influence on the selected trait and other behaviors (Crabbe et al., 1990). In other words, a single gene may influence both traits. The previously described selection experiments tested the hypothesis that the acute locomotor responses to cocaine and amphetamine were genetically correlated (i.e., common genetic influence). The CAHI and CALO lines (collapsed on replicate) were tested for their acute locomotor response to amphetamine. CAHI mice were significantly more stimulated by a range of doses of amphetamine (1 – 5 mg/kg) than CALO mice. A genetic correlation between the locomotor responses to cocaine and methamphetamine stimulation was also observed in the HMACT and LMACT lines (Kamens et al., 2005). The HMACT line was more stimulated by cocaine (10 – 30 mg/kg) compared to the LMACT line. Together these data suggest that a common gene (or genes) underlies a portion of the acute locomotor response to cocaine and amphetamine.

A quantitative trait locus (QTL) refers to a region of a chromosome that contains a gene that influences a complex trait like the locomotor response to psychostimulants. A number of QTL have been mapped for psychostimulant-induced activation. QTL that are common between cocaine and amphetamines may contain a gene that influences both traits. Most QTL mapping data in mice for drug-related traits come from studies that used populations of mice derived from the B6 and D2 inbred strains. RI mice, derived from the these strains (BXD RI), have provided suggestive evidence of a QTL on chromosome 9 for cocaine- (Tolliver et al., 1994; Miner and Marley, 1995; Phillips et al.,

1998) and methamphetamine-induced locomotor activation (Grisel et al., 1997). Additional evidence for the methamphetamine activity QTL comes from a comparison of the B6 and D2 allele frequencies in the HMACT and LMACT lines (Palmer et al., 2005). A QTL for cocaine (Gill and Boyle, 2003) and amphetamine stimulation (Torkamanzehi et al., 2006) on chromosome 9 has also been observed in crosses of mice derived from the A/J and B6 inbred strains.

In the current study I first set out to directly confirm the presence of a QTL on mouse chromosome 9 for the acute locomotor responses to cocaine and methamphetamine. Reciprocal chromosome 9 congenic mice derived from the B6 and D2 progenitor strains (for a detailed discussion of these topics, please refer to the introduction to this dissertation) were tested. I predicted that there would be a QTL on chromosome 9 that accounts for variation in the acute locomotor responses to cocaine and methamphetamine, and that mice possessing D2 alleles in this region would be more stimulated by these drugs than mice with B6 alleles. This hypothesis was based on the aforementioned QTL mapping data. In these studies, having D2 alleles on chromosome 9 was associated with increased cocaine and methamphetamine stimulation (Tolliver et al., 1994; Miner and Marley, 1995; Phillips et al., 1998; Palmer et al., 2005). After obtaining strong evidence of the QTL for methamphetamine-induced locomotor stimulation, the QTL was then more finely mapped using interval-specific congenic strains (ISCS; see the introduction for details on this method).

Methods

Subjects

All mice were the offspring of pairs of mice bred at the Department of Veterans Affairs Medical Center in Portland, OR. Breeder pairs for the B6 strain were purchased from The Jackson Laboratory (Bar Harbor, ME); new breeder pairs were obtained once a year to minimize genetic drift. Congenic strains are derived from 2 inbred strains. A region of the genome from one inbred strain is introgressed onto the background of another inbred strain through homologous recombination. If the congenic strain and the pure background strain differ in their behavioral response, this provides evidence that a gene in the introgressed region accounts for part of the variation in the response (i.e., captures the QTL). The chromosome 9 B6.D2 (D9Mit90,182; with the introgressed region defined by the proximal marker D9Mit90 and the distal marker D9Mit182) and chromosome 9 D2.B6 (D9Mit90,18) congenic strains were obtained from Dr. John K. Belknap. The first strain in the name (e.g., B6.D2) refers to the background strain (in this case B6) and the second strain refers to the inbred strain from which the donor alleles for the introgressed region originated (D2). These strains had been backcrossed to the background strain for at least ten generations when breeder pairs were obtained, thus are considered to be inbred strains. While in our laboratory, breeder pairs from the chromosome 9 D2.B6 congenic strain produced few offspring. At this time Dr. Belknap was no longer breeding these animals, so to avoid loss of this strain male chromosome 9 D2.B6 mice were backcrossed to female D2 mice. This resulted in the production of mice that were heterozygous throughout the introgressed congenic region, but homozygous D2 at all other loci. These F_1 offspring were intercrossed to produce F_2

animals, some of which were identical to the chromosome 9 D2.B6 congenic or D2 mice, but others had recombinants of varying lengths throughout the introgressed B6 region. These animals were genotyped for 4 microsatellite markers in the introgressed B6 region to identify mice that were genetically identical to the original chromosome 9 D2.B6 congenic. Animals that were genotyped as B6 homozygous at the markers that were originally used to define the region (D9Mit90 and D9Mit18), as well as 2 additional markers within the introgressed region, were interbred to produce new chromosome 9 D2.B6 (D9Mit90,18) congenic mice. At the same time, animals genotyped as homozygous D2 throughout the congenic region were used to create the D2 control strain.

The chromosome 9 B6.D2 ISCS panel was created by backcrossing male chromosome 9 B6.D2 (D9Mit90,182) mice to female B6 mice. Heterozygous F_1 offspring were backcrossed to B6 mice; the resulting first generation backcross animals were genotyped for DNA markers throughout the original D2 introgressed region. Recombinant mice, where a crossover had occurred in the original introgressed region, were backcrossed to B6 to produce additional recombinant mice with varying lengths of introgressed D2 regions. Chromosome 9 B6.D2 ISCS mice were produced by brother/sister matings to produce mice homozygous for the new introgressed regions (see Figure 25a for genotyping information for the chromosome 9 ISCS mice). At this time, a B6 control strain was also produced that no longer carried a D2 introgressed region. ISCS on the D2 background were not produced.

Animal Husbandry

Mice of the same sex were group housed 2-5 per cage. In most cases animals were littermates, but in some cases, animals close in age (< 5 days different), but from a different litter of the same strain were housed together to avoid single housing. Animals were housed in standard shoebox size polycarbonate cages with corncob bedding, and had ad libitum access to food (Purina Laboratory Rodent Chow #5001; Purina Mills, St. Louis, MO) and tap water. The colony room was maintained at 21 ± 2˚C on a 12 hour light/dark cycle (lights on a 0600).

Drugs

Cocaine hydrochloride and methamphetamine hydrochloride were obtained from Sigma (St. Louis, MO) and were dissolved in physiological saline (0.9% NaCl; Baxter Healthcare Corporation, Deerfield, IL). Drugs were injected into the peritoneal cavity (i.p.) at a volume of 10 ml/kg.

Testing Apparatus

Mice were tested for locomotor activity in automated activity monitors (40 X 40 X 30 cm, AccuScan, Columbus, OH). These monitors were housed in black acrylic test chambers which were lined with sound-attenuating foam. Inside the chamber a fan provided background noise and a fluorescent light (8-W) illuminated the chamber. Each monitor had 8 infrared beams along 2 sides located 2 cm above the chamber floor; along the other 2 sides were detectors. A computer recorded when these beams were broken and converted consecutive beam interruptions into distance traveled (cm), which was used as the dependent variable.

Testing Procedure

Animals began testing between 50 and 105 days of age. To allow animals time to acclimate to the testing room, all animals were moved from the colony room 45 – 60 min prior to the initiation of testing. The procedures used in these experiments were identical to those which were used to initially map QTL for the acute locomotor responses to cocaine and methamphetamine (Phillips et al., 1998; Palmer et al., 2005). Briefly, animals were weighed and placed in individual holding cages, identical to their home cages, for up to 10 min to allow time to prepare syringes for injection. On days 1 and 2, each animal received an injection of saline before being placed into the center of the locomotor activity chamber. This allowed mice to habituate to the testing environment on day 1, and allowed for a measurement of habituated baseline locomotor activity on day 2. On day 3, animals received an injection of 10 mg/kg cocaine or 2 mg/kg methamphetamine before being place into the activity monitor. On all days, locomotor activity was monitored for 15 min in 5-min epochs. Following testing on day 3, all animals were euthanized by carbon dioxide asphyxiation.

The chromosome 9 B6.D2, chromosome 9 D2.B6, B6, and D2 strains were first tested for their sensitivity to an acute injection of cocaine or methamphetamine. Since the congenic results indicated capture of a QTL for methamphetamine stimulation in the chromosome 9 B6.D2 congenic strain (see results), a panel of nine chromosome 9 B6.D2 ISCS was tested to attain higher resolution mapping of the region that contains the gene for methamphetamine stimulation. The chromosome 9 B6.D2 ISCS panel was not tested for cocaine stimulation since only weak evidence of a QTL for this drug was obtained (see results). To obtain a large enough sample size, mice had to be tested in a number of

passes. Two to 4 passes were needed for studies involving the chromosome 9 B6.D2 and D2.B6 congenics; approximately equal numbers of congenic and control animals were tested in each pass. For the ISCS study, animals were tested in 13 passes over a 12 month period. Two to 6 strains were tested per pass depending upon availability.

All experiments were approved by the Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health's Principles of laboratory animal care (1985).

Statistics

The acute locomotor response was defined as the day 3 drug response minus the day 2 habituated baseline (Day 3 - Day 2) for each individual animal. Day 3 – Day 2 was used as the dependent variable since this was the trait used in the original QTL mapping experiments, and it provides a measure of the acute locomotor response to drugs that takes into account differences in baseline locomotor activity (Phillips et al., 1998; Palmer et al., 2005). Data for each congenic strain were compared independently to data for its background strain. Data were analyzed using a 2-way analysis of variance (ANOVA). Both independent variables had two levels: sex (male vs. female) and strain (congenic vs. control). Significant main effects and interactions were followed up with Newman-Keuls mean comparisons and simple main effects analyses. Statistical analyses were performed using Statistica (StatSoft, Tulsa, OK, USA) with the alpha level set at 0.05.

Effect Size

The percentage of phenotypic variation accounted for by the chromosome 9 introgressed genotype was calculated for each ISCS that was significantly different from

the control strain (i.e., each one that captured the QTL). R^2 (the QTL effect size) was calculated from a 1-way ANOVA using the equation $R^2 = F/(F + dfw)$ (dfw = degrees of freedom within; Rosenthal, 1994).

Results

Cocaine

The chromosome 9 B6.D2 congenic and B6 control mice were differentially sensitive to cocaine-induced locomotor activity, but this effect was sex dependent (Figure 22). Data from one female chromosome 9 B6.D2 congenic mouse were excluded from the analysis because the mouse lost weight over the three days of testing and appeared to be suffering from malocclusion. A 2-way ANOVA revealed a significant sex X strain interaction ($F_{1, 159} = 12.2$, $p < 0.001$). Consistent with the QTL mapping studies (Phillips et al., 1998), female chromosome 9 B6.D2 congenic mice were more stimulated by cocaine compared to female B6 control mice ($p < 0.01$). In contrast, the QTL effect in males was in the opposite direction, male chromosome 9 B6.D2 congenic mice were less stimulated by cocaine than male B6 control mice ($p < 0.05$). This interaction appeared to be due to a significant difference between male and female B6 mice, such that males were more sensitive to cocaine-induced stimulation compared to female mice ($p < 0.001$). Male and female chromosome 9 D2.B6 congenic mice were equally sensitive.

There was no difference in sensitivity to the locomotor stimulant effect of cocaine between the chromosome 9 D2.B6 congenic and D2 control mice (Figure 23). Data from one female chromosome 9 D2.B6 congenic mouse were excluded from the analysis because of a computer malfunction on day 3. A 2-way ANOVA with strain and sex as independent variables revealed no significant main effects or interactions.

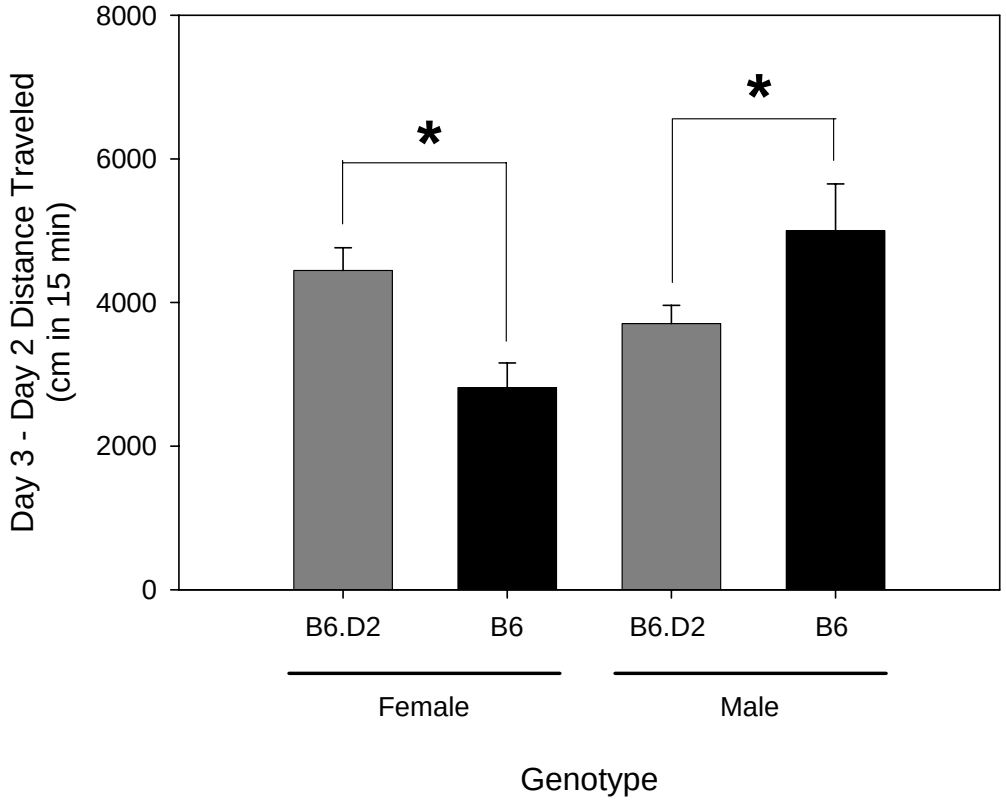


Figure 22. The locomotor response to cocaine in the chromosome 9 B6.D2 congenic and B6 strains is dependent on sex. The acute locomotor response to cocaine (10 mg/kg) in chromosome 9 B6.D2 congenic and B6 strains. Shown are the data (mean ± SEM) from the 15 min test session. N = 40 - 41 per strain and sex. *$p < 0.05$.

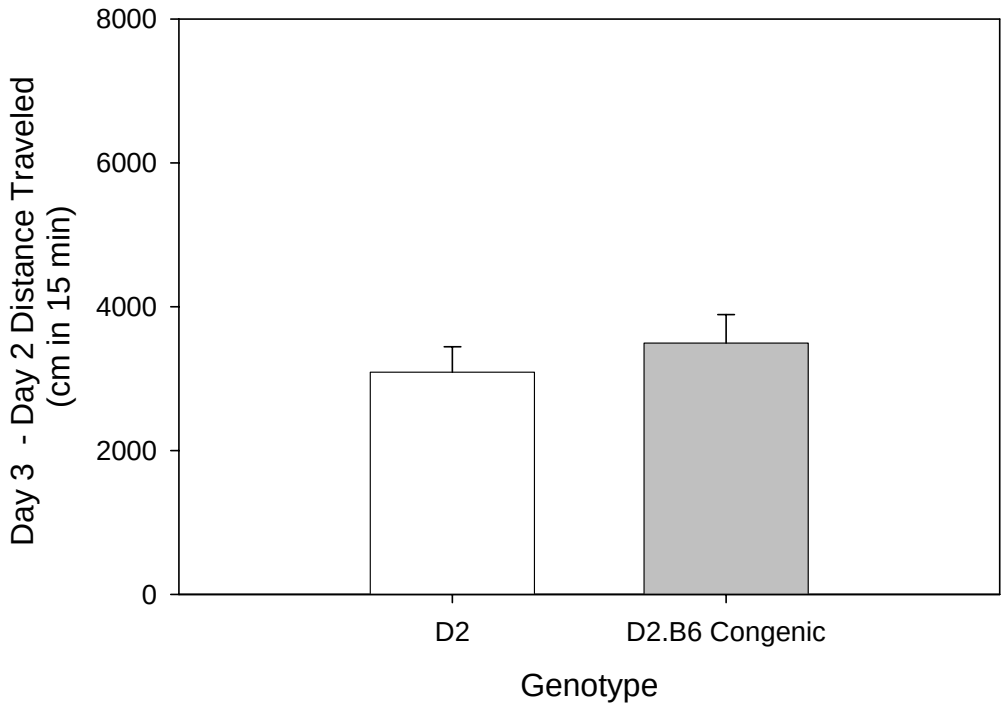


Figure 23. The chromosome 9 D2 background congenic strain provides no evidence of a QTL on chromosome 9 for cocaine-induced locomotor activity. The acute locomotor response to cocaine (10 mg/kg) in chromosome 9 D2.B6 congenic and D2 strains. Data were collapsed on sex because no significant main effects or interactions with this factor were detected. Shown are the data (mean ± SEM) from the 15 min test session. N = 82 - 84 per strain.

Methamphetamine

Congenics

When the chromosome 9 congenic strains were compared to their appropriate control strains, mice with D2 alleles on chromosome 9 were more stimulated by methamphetamine (2 mg/kg) compared to animals with B6 alleles in this region (Figure 24). When the chromosome 9 B6.D2 mice were compared to B6 control mice there was a significant main effect of strain ($F_{1, 82} = 13.2$, $p < 0.001$) that did not interact with sex. Chromosome 9 B6.D2 congenic mice displayed a markedly elevated acute locomotor response to methamphetamine compared to B6 control mice. Similarly, in the chromosome 9 D2.B6 congenic and D2 control strains of mice there was a significant main effect of strain that did not interact with sex. D2 mice were significantly more stimulated than chromosome 9 D2.B6 congenic mice ($F_{1, 91} = 12.7$, $p < 0.001$). These data provide strong evidence for an acute methamphetamine stimulation QTL on chromosome 9 in both reciprocal congenic strains.

ISCS

Genotyping results for the DNA markers that define the ISCS D2 introgressed regions and the behavioral responses to methamphetamine in these strains are found in Figure 25. Four of the nine ISCS had a significantly elevated acute response to methamphetamine compared to B6 control mice. Data from two male ISCS 2 animals were excluded from the analysis because there was a computer issue on day 2. Data from five animals were excluded from the analysis because their data were extreme ($> \pm 2.5$ SD from the strain mean). The five animals that were excluded came from five different

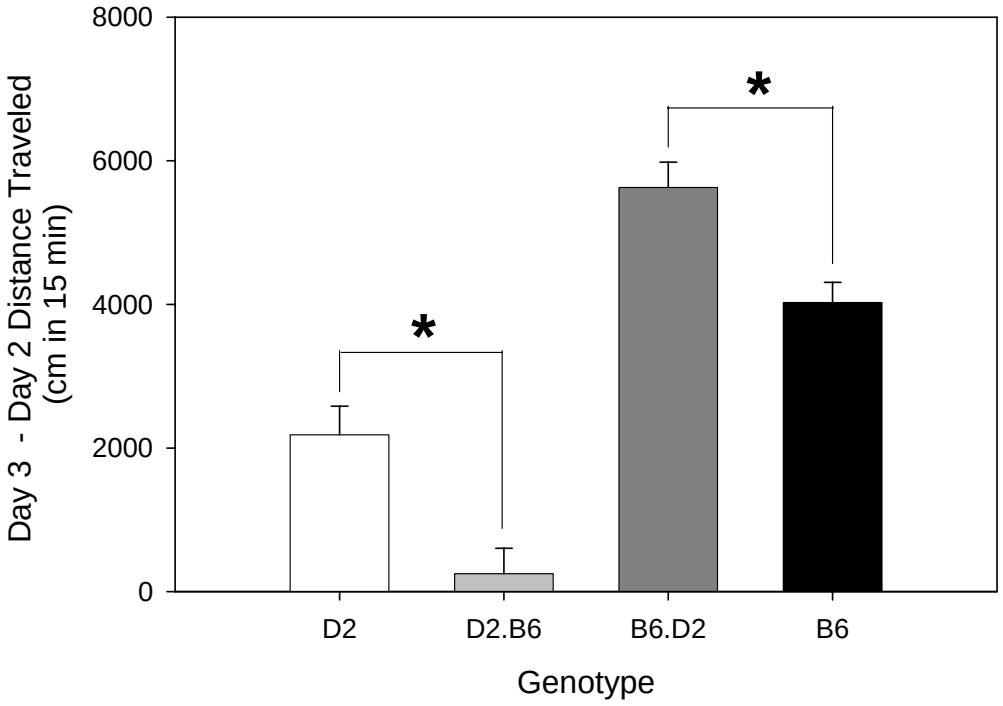


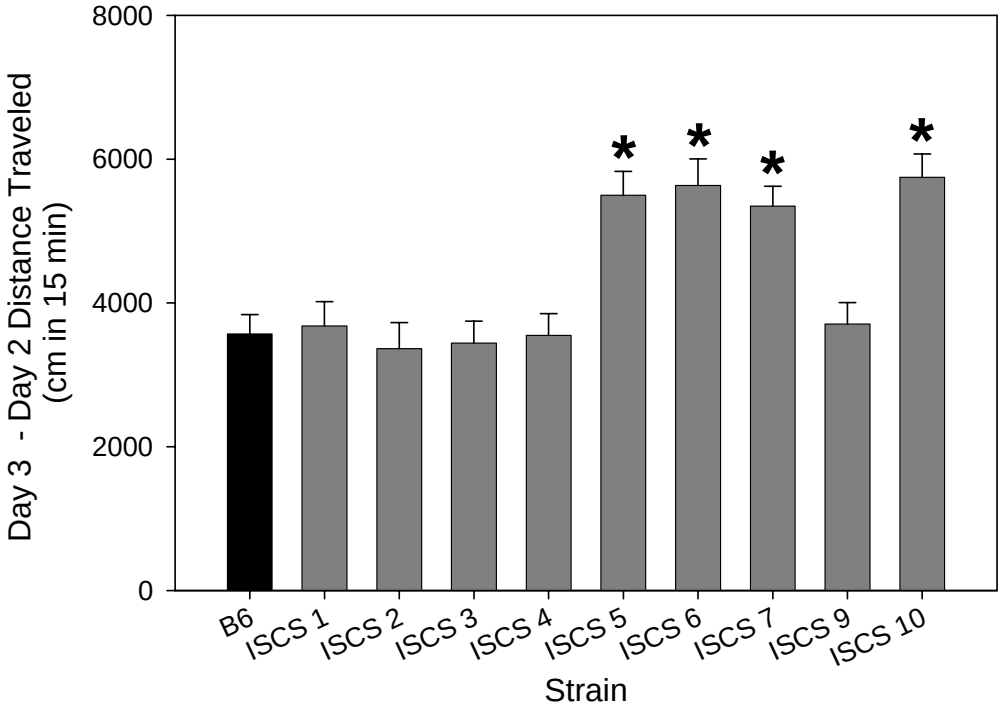
Figure 24. The QTL for the acute locomotor response to methamphetamine is captured in the chromosome 9 D2.B6 and B6.D2 congenic mouse strains. The acute locomotor response to methamphetamine (2 mg/kg) in D2, chromosome 9 D2.B6 congenic, chromosome 9 B6.D2 congenic, and B6 strains. Data were collapsed on sex because no significant main effects or interactions with this factor were detected. Shown are the data (mean ± SEM) from the 15 min test session. N = 42 - 50 per strain. *$p < 0.001$

Figure 25. Fine mapping of a QTL for methamphetamine stimulation on mouse chromosome 9 to a 24.16 Mb region. Genotyping and the acute locomotor response to methamphetamine (2 mg/kg) in chromosome 9 B6.D2 ISCS and B6 mice. (a) Genotyping of the chromosome 9 ISCS panel of mice. Dark black lines represent the more finely mapped QTL region. D2 = D2 homozygous, HET = D2/B6 heterozygous, B6 = B6 homozygous, * = lines that capture the QTL (b) Shown are the acute locomotor response data (mean ± SEM) from the 15 min test session. Data were collapsed on sex. N = 39 - 41 per strain. *$p < 0.05$ compared to the B6 strain

Figure 25a

	DNA Marker																	
	D9Mit90	D9Mit297	D9Mit206	D9Mit129	D9Mit192	D9Mit93	D9Mit71	D9Mit96	D9Mit4	D9Mit142	D9Mit48	D9Mit31	D9Mit271	D9Mit269	D9Mit10	D9Mit50	D9Mit274	D9Mit115
Ensembl (Mb)	32.25	33.81	40.25	43.63	45.37	45.98	49.95	50.5	51.87	56.08	57.69	63.58	86.97	87.74	89.77	94.25	96.2	101.46
ISCS 1	D2	D2	D2	D2	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6
ISCS 2	D2	D2	D2	D2	D2	D2	D2	D2	D2	B6	B6	B6	B6	B6	B6	B6	B6	B6
ISCS 3	B6	D2	D2	D2	D2	D2	D2	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6
ISCS 4	B6	B6	B6	B6	B6	D2	D2	D2	D2	B6	B6	B6	B6	B6	B6	B6	B6	B6
ISCS 5 *	B6	B6	B6	D2	D2	D2	D2	D2	D2	D2	D2	D2	D2	D2	D2	D2	B6	B6
ISCS 6 *	B6	B6	B6	B6	B6	B6	B6	D2	D2	D2	D2	D2	D2	D2	D2	D2	D2	D2
ISCS 7 *	B6	B6	B6	B6	B6	B6	B6	B6	B6	D2	D2	D2	D2	D2	D2	D2	D2	D2
ISCS 9	B6	D2	D2	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6
ISCS 10 *	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	B6	D2	D2	HET	B6	B6	B6	B6

Figure 25b


8000
6000
4000
2000
0
Day 3 - Day 2 Distance Traveled
(cm in 15 min)
*
*
*
*
B6
ISCS 1
ISCS 2
ISCS 3
ISCS 4
ISCS 5
ISCS 6
ISCS 7
ISCS 9
ISCS 10
Strain

strains: B6 control, ISCS 3, 5, 7, and 9. When the B6 control strain was compared to the ISCS 1, 2, 3, 4, and 9 strains, no significant main effects or interactions were observed. ISCS 5, 6, and 7 were all significantly more stimulated by methamphetamine compared to B6 control mice, as evidenced by a significant main effect of strain that did not interact with sex ($F_{1, 76} = 19.9$, $p < 0.001$, $F_{1, 76} = 21.0$, $p < 0.001$, $F_{1, 77} = 20.6$, $p < 0.001$, respectively). When ISCS 10 was compared to B6 control mice a significant sex X strain interaction was observed ($F_{1, 76} = 5.1$, $p < 0.05$). Both male and female ISCS 10 mice were significantly more stimulated by methamphetamine compared to male and female control mice, but this difference was larger in female mice than male mice ($p < 0.05$; male ISCS 10: 4962.4 ± 349.4, male control: 3709.8 ± 435.4, female ISCS 10: 6533.5 ± 496.6, female control: 3444.0 ± 331.0). Since ISCS 5, 6, 7, and 10 capture the QTL for methamphetamine stimulation, these data narrow the location of the QTL for methamphetamine stimulation from a 69.13 Mb region to a 24.16 Mb region between D9Mit31 and D9Mit269 (Figure 25a). The effect size for the chromosome 9 QTL for methamphetamine stimulation ranged from 0.208 – 0.256. Thus, approximately 20 – 25% of the variability in the acute locomotor response to methamphetamine can be accounted for by genotype in this region of mouse chromosome 9.

Discussion

Gene mapping in crosses of mice derived from the B6 and D2 progenitor strains had provided suggestive evidence of a QTL on mouse chromosome 9 for the acute locomotor responses to cocaine and methamphetamine (Tolliver et al., 1994; Miner and Marley, 1995; Grisel et al., 1997; Phillips et al., 1998; Palmer et al., 2005). In this paper, the presence of a QTL for the acute response to methamphetamine on mouse

chromosome 9 was confirmed, but there was only weak evidence of a QTL for cocaine stimulation. Further, the location of the QTL for methamphetamine stimulation was narrowed to a 24.16 Mb region using ISCS.

The evidence of a QTL for sensitivity to methamphetamine's stimulatory effects was quite strong. Both the chromosome 9 B6.D2 and D2.B6 congenic strains captured this QTL. Data from studies in the reciprocal chromosome 9 congenic strains, suggest that mice with D2 alleles on chromosome 9 were more stimulated by methamphetamine than mice with B6 alleles in the same region. The p-value associated with the capture of the chromosome 9 QTL in the ISCS strains exceeds the Lander and Kruglyak (1995) threshold for a significant QTL. Combining the results of this study with the existing QTL mapping data would only increase the significance level associated with this QTL even further. A gene in the mid-chromosome 9 region accounted for 20 - 25% of the phenotypic variation in methamphetamine stimulation. Extrapolating to a congenic F_2, this estimate would be 10 – 13%. A QTL with an effect size this large is on the extreme end of what is normally mapped for behavioral traits, but is consistent with the size of QTL that have been successfully fine mapped (Flint et al., 2005).

While QTL mapping results in the reciprocal chromosome 9 congenic strains provided evidence that mice with D2 alleles were more stimulated by methamphetamine than mice with B6 alleles in this region, it is interesting to note that the inbred B6 strain appears more stimulated by 2 mg/kg methamphetamine than the D2 strain (see Figure 24). The response data of the B6 and D2 inbred strains were not statistically compared because the purpose of this study was to determine if there was a QTL for the acute locomotor response to methamphetamine on chromosome 9. Therefore, the appropriate

comparison was between the congenic and relevant background strain. Furthermore, the 2 reciprocal congenic strains, and their background control strains, were run in independent experiments. A consistent pattern of strain differences in response to amphetamines between the D2 and B6 strains has not been observed. In some studies, the D2 strain is more stimulated by amphetamine than the B6 strain (Anisman et al., 1975; Remington and Anisman, 1976), but in another study the reverse was observed (Zocchi et al., 1998). The acute locomotor response to methamphetamine is influenced by the combined effect of many genes. Each of these genes may have individual effects, increasing or decreasing the acute locomotor response to methamphetamine, and they may interact. On chromosome 9, these data suggest that having D2 alleles, as compared to B6 alleles in the same region, increases methamphetamine-induced stimulation, when all other genetic loci are identical between the strains.

In contrast, these data provide only weak evidence for a QTL for cocaine-induced locomotor activation on mouse chromosome 9. A sex-specific QTL effect in the B6 background congenic was observed, but there was no difference in sensitivity to cocaine in either male or female D2.B6 congenics when compared to D2 control mice. Furthermore, in the study where a sex-specific QTL was observed the effect was opposite for the two sexes, and likely accounted for by differences in sensitivity to cocaine between male and female B6 control mice. There is no consistent sex difference in response to an acute injection of cocaine. One study showed that males are more sensitive to cocaine than females (Morse et al., 1993), but another study showed the opposite result (Downing et al., 2003a). The direction of effect observed in the female B6.D2 congenic and B6 control mice was consistent with a prior QTL mapping study

(Phillips et al., 1998). Future work using female mice from the chromosome 9 B6.D2 ISCS panel may provide finer mapping of this QTL. In contrast, the direction of effect in the male B6 background congenics was opposite to what has previously been observed (Miner and Marley, 1995). However, the original QTL mapping work in male BXD RI animals was done using a procedure different from that used in this study. In that study mice were habituated to the locomotor chamber for 30-min, then they received a 10 mg/kg cocaine injection and were place immediately back into the chamber (Miner and Marley, 1995). It is possible that this difference in procedure could account for the differences in the direction of effect observed in this study. Together these data provide weak evidence of a QTL for the acute locomotor response to cocaine on mouse chromosome 9 that is sex-specific.

Genetic correlations have been observed between the acute locomotor responses to amphetamines and cocaine (Marley et al., 1998; Kamens et al., 2005). These correlations have been observed in selected lines derived from a cross of B6 and D2 mice (Kamens et al., 2005) as well as in lines selectively bred from HS/Ibg mice (derived from the crossing of eight inbred strains including the B6 and D2 inbred strains; Marley et al., 1998). These data suggest that a common gene (or genes) in mice derived from the B6 and D2 inbred strains has pleiotropic influence on both amphetamine- and cocaine-induced activity. The possibility that a gene in the introgressed region of the chromosome 9 congenic has pleiotropic effects on these behaviors still exists, but may be sex-specific. Other QTL have been mapped on a number of different chromosomes for the acute locomotor responses to cocaine and methamphetamine. A gene(s) in one of these other QTL regions may have pleiotropic effects on both traits and account for the

observed genetic correlations (Tolliver et al., 1994; Miner and Marley, 1995; Grisel et al., 1997; Phillips et al., 1998; Palmer et al., 2005).

Higher resolution mapping of the QTL for methamphetamine stimulation was attained using ISCS. The ISCS used in these studies were obtained through the backcrossing of the chromosome 9 B6.D2 congenic strain to the background B6 strain. Mice with overlapping donor regions were identified by genotyping microsatellite markers. The markers shown in Figure 25a are only a subset of those used to genotype these mice. ISCS 10 was originally bred using D9Mit144 (63.78 Mb) as the DNA marker that defined the proximal end of the D2 introgressed region and D9Mit319 (77.07 Mb) as the marker which defined the distal boundary. The markers D9Mit48 (57.69 Mb) and D9Mit274 (96.2 Mb) were used to show that the introgressed region ended because they were genotyped as homozygous B6. This left a 6.09 Mb proximal and 19.13 Mb distal region flanking the introgressed region with an unknown genotype. While it is common to have an unknown region when utilizing congenic strains (i.e., Fehr et al., 2002), additional genotyping in this region was performed to better define where the D2 introgressed region began and ended to definitively map the QTL region. This additional genotyping provided evidence that a crossover had occurred in one of the initial homozygous breeders and thus there is a small heterozygous region (see Figure 25a – marker D9Mit269). The interpretation of the results from this study does not change with the information that there is a small heterozygous region (< 3 Mb) at the end of ISCS 10. If the gene that accounts for variation in methamphetamine stimulation resides in the heterozygous region it would provide evidence that the D2 allele is dominant, since there is complete capture of the QTL in this strain. There is currently no other existing data

regarding the genetic architecture of this QTL to allow speculation concerning dominance.

The addition of new ISCS that have introgressed regions covering the 24.16 Mb segment implicated in this study will be needed for additional fine mapping of this QTL. New ISCS are currently being created from backcrossing ISCS 5 and 10 animals to B6 mice. The ISCS 10 strain was chosen because it possesses the smallest congenic region that captures the QTL. ISCS 5 was chosen because it captures the whole QTL region, has a history of being a productive breeder, and allows for additional congenics to be created not in the QTL region to confirm the exclusion of more proximal chromosome 9 regions. The creation of new ISCS will help narrow the location of the QTL and thus decrease the number of candidate genes in the region.

The 24 Mb QTL region identified in this study contains approximately 280 genes (www.genome.uscs.edu). Prior studies which used ISCS mapping to fine map QTL regions have filtered genes for functional polymorphisms and whether or not the gene is expressed in the brain to identify the most promising candidates (Fehr et al., 2002; Ferraro et al., 2004; Shirley et al., 2004). This approach worked to identify either the quantitative trait gene (QTG) or a small number of candidate genes because the QTL was fine mapped to a much smaller region, containing fewer genes, than what has been achieved for the methamphetamine stimulation QTL (< 4.1 Mb compared to 24.16 Mb). This is an approach that will be used when higher resolution mapping of the chromosome 9 QTL for methamphetamine sensitivity has been obtained, but at the current time there are too many genes in the QTL region to be amenable to this strategy.

A complementary approach to identify candidate genes is to determine which genes that map to the QTL region are differentially expressed in other strains or genotypes relevant to this trait. This approach has been successful at identifying candidate genes for methamphetamine-induced locomotor activation. For example, differential expression of casein kinase 1 epsilon (*Csnk1e*) between the HMACT and LMACT lines of mice pointed to this as a candidate gene (Palmer et al., 2005). Further, a polymorphism in this gene was found to alter the acute response to amphetamine in human subjects (Veenstra-VanderWeele et al., 2006). Gene expression differences were examined in the HMACT and LMACT lines and chromosome 9 B6.D2 congenic and B6 mice (Palmer et al., 2005; Mulligan et al., 2006). Since the HMACT and LMACT lines were derived from a cross of the B6 and D2 strains, differences in these lines are directly relevant to the QTL mapped in the congenics. Mice from the 4th generation of selective breeding (at which time there was a 5-fold difference in sensitivity to methamphetamine stimulation) were used for gene expression analysis. Naïve mice were tested since baseline gene expression differences between the two lines are likely to account for differences in the response to methamphetamine. Three genes were found to be differentially expressed in nucleus accumbens tissue from the HMACT and LMACT lines of mice that map to chromosome 9 between D9Mit31 and D9Mit269 (the markers that define the 24 Mb QTL region). These three genes: *Arpp19* (cyclic AMP phosphoprotein), *Car12* (carbonic anhydrase 12), and *Spg21* (spastic paraplegia 21 homolog) are thus candidate genes for this response.

An additional candidate gene comes from a study examining whole brain basal gene expression differences between the chromosome 9 B6.D2 congenic and B6 control

mice (Mulligan et al., 2006). Comparisons of these strains are relevant since this congenic captures the QTL for methamphetamine stimulation and was used to create the chromosome 9 ISCS used for finer mapping. One gene was differentially expressed in whole brain samples between these two strains ($q < 0.05$) that maps to the QTL region. This gene, *Eef1a1* (eukaryotic translation elongation factor 1 alpha 1) should also be considered as a candidate gene.

At least three of these genes (*Car12*, *Eef1a1*, and *Arpp19*) are expressed in the brain and have functions that could be important for the response to methamphetamine. Carbonic anhydrases are important in a number of cell actions including: pH regulation, cell proliferation, acidification, signal transduction, ion transport, and balance of water and electrolytes (Sly and Hu, 1995; Tureci et al., 1998; Kallio et al., 2006). There are 16 different carbonic anhydrases that differ in regional and cellular distributions. Carbonic anhydrase 12, the isoform that was differentially expressed between the HMACT and LMACT lines, is expressed in the brain and is localized to the cell membrane (Ivanov et al., 2001; Kallio et al., 2006). Further evidence for the possible role of this gene in methamphetamine stimulation comes from WebQTL (www.genenetwork.org). Whole brain expression of *Car12* in BXD RI mice is significantly correlated with the locomotor response to an acute injection of methamphetamine (16 mg/kg, $r = .436$, $p < 0.05$; this correlation is derived from WebQTL, but excludes the outlier BXD RI 24 strain).

Unlike *Car12*, level of *Eef1a1* expression is not correlated with the acute locomotor response to methamphetamine in BXD RI mice (analysis of WebQTL data suggests there is a significant correlation, but when BXD RI 24 is removed because of its extreme values, the correlations between *Eef1a1* and methamphetamine expression is not

significant). *Eef1a1* is expressed in neurons, and as implied by its name (eukaryotic translation elongation factor 1 alpha 1) is involved in translation and the synthesis of new peptides (Petrushenko et al., 2002; McClatchy et al., 2006). Recently, other functions for this gene have been identified. For example, *Eef1a1* appears to be involved in the regulation of the number of M4 muscarinic acetylcholine receptors in the membrane (McClatchy et al., 2006). This may be particularly relevant to the locomotor response to methamphetamine because mice lacking the M4 muscarinic receptor had significantly more dopamine efflux in the nucleus accumbens following amphetamine administration than wildtype animals (Tzavara et al., 2004).

The location and function of *Arpp19* also make this gene of interest for methamphetamine stimulation. *Arpp19* is expressed throughout the central nervous system and is an intracellular signaling molecule (Girault et al., 1990). When neurotransmitters increase cAMP, cAMP-dependent protein kinases are activated. *Arpp19* is one substrate for cAMP-dependent protein kinases. Dopamine receptors activate this pathway and dopamine increases *Arpp19* phosphorylation (Dulubova et al., 2001). Doses of methamphetamine that stimulate locomotor activity increase dopamine in the nucleus accumbens (Izawa et al., 2006), indicating one way *Arpp19* may be involved in the response to methamphetamine. Expression of *Arpp19* in whole brain samples from BXD RI mice is significantly correlated with the acute response to methamphetamine and in particular, with the precise trait studied here (2 mg/kg; Phillips et al., unpublished data; $r = .483$, $p < 0.05$). Thus, the evidence is strongest for *Arpp19* as a candidate gene for the acute locomotor response to methamphetamine, since expression of this gene is correlated with the behavioral response for which this QTL was mapped.

In contrast to *Arpp19*, *Car12*, or *Eef1a1*, much less is known about localization and function of *Spg21* (aka, acidic cluster protein 33, ACP33). In one study, *Spg21* was shown to interact with the cell surface glycoprotein CD4 and mediate T cell activation (Zeitlmann et al., 2001). It is not known if *Spg21* interacts with other cellular proteins or if it has other functions on its own. Whole brain expression of *Spg21* was not significantly correlated with methamphetamine-induced locomotor activity in BXD RI mice.

It is possible that more than one gene in the QTL region may influence this trait. Of the 4 candidate genes listed, *Spg21* and *Car21* are less than 1.5 Mb apart, while the others are at least 3.4 Mb apart. With the addition of more ISCS, it may still be hard to separate some of these genes because, at least in the case of *Spg21* and *Car21*, they are tightly linked. The possibility exists that if multiple genes in this 24 Mb region additively influence this trait, the ability to detect this trait when new ISCS with additional recombinations in the region are tested may be lost.

In summary, a QTL for methamphetamine-induced locomotor activation was definitively mapped to a ~24 Mb region of mouse chromosome 9. A gene (or genes) in this region accounts for 20 - 25% of the variation in the stimulatory effects of methamphetamine. Existing gene expression data have allowed some speculation regarding candidate genes in this region. Additional work is underway to map this QTL to a smaller region, identify the QTG, and devise strategies for exploring its function.

Chapter 5: General Discussion

There were three goals for this dissertation research. The first goal was to determine if neuronal nicotinic acetylcholine receptors were involved in ethanol- and psychostimulant-induced locomotor activation. The second goal of this dissertation research was to confirm the presence of a quantitative trait locus (QTL) for the locomotor response to ethanol, cocaine, and methamphetamine on mouse chromosome 9. When a QTL was confirmed, steps were taken to more finely map the region. Finally, the last goal was to examined if the genes in the nicotinic acetylcholine receptor subunit gene cluster on chromosome 9 were viable candidates for ethanol-induced stimulation. In this discussion I will address the data separately for methamphetamine, cocaine, and ethanol. For each drug both the QTL and pharmacologic data will be discussed. Ethanol was saved for last because mechanism was studied most extensively for this drug.

Methamphetamine Stimulation

In Chapter 4 of this dissertation I present strong evidence of a QTL for methamphetamine-induced locomotor stimulation on mouse chromosome 9. Both the chromosome 9 D2.B6 and B6.D2 congenic strains captured this QTL. Furthermore, when a panel of chromosome 9 interval-specific congenic strains (ISCS) were tested for methamphetamine stimulation the QTL was narrowed to a 24.16 Mb region containing approximately 280 genes. Of these 280 genes, candidates could theoretically be identified by examining cellular expression patterns and sequence differences. However, it would be advantageous to more finely map the QTL and reduce the number of genes to be examined before engaging in expression and sequence analysis.

The nicotinic acetylcholine receptor antagonist mecamylamine did not attenuate methamphetamine-induced locomotor activation (Chapter 2). Other studies have also examined the role of these receptors in the acute response to amphetamines. Pretreatment with the nicotinic acetylcholine receptor antagonists mecamylamine, dihydro-β-erythroidine, 18-methoxycoronaridine, and methyllycaconitine had no effect on amphetamine stimulation (Szumlinski et al., 2000a; Schoffelmeer et al., 2002; Escubedo et al., 2005). Together with my data, it appears that nicotinic acetylcholine receptors are not involved in the locomotor response to amphetamines, and that genes other than those in the nicotinic acetylcholine receptor gene cluster should be considered as candidates for the QTL. In fact, the QTL mapping data for methamphetamine stimulation are consistent with the pharmacology data. Specifically, there was no evidence for the involvement of nicotinic acetylcholine receptors in methamphetamine activation based on the pharmacology studies, and the ISCS mapping data narrowed the QTL for this trait to between 63.58 and 87.74 Mb on chromosome 9 (Chapter 4), a region that eliminates the cluster of acetylcholine receptors on this chromosome (at 54 Mb) from further consideration.

Although it does not appear that nicotinic acetylcholine receptors are involved in amphetamine stimulation, nicotinic receptors have been implicated in other amphetamine behaviors. Although mecamylamine and dihydro-β-erythroidine had no effect on the acute stereotypic effect of amphetamine, these drugs were able to block sensitization to the stereotypic effects of repeated amphetamine administration (Karler et al., 1996). Mecamylamine also has been shown to block the development of sensitization as measured by an increase in locomotor activity (Schoffelmeer et al., 2002).

Methyllycaconitine, the α7 specific nicotinic antagonist, blocked methamphetamine-induced climbing (Escubedo et al., 2005). Finally, 18-methoxycoronaridine attenuated methamphetamine self-administration (Glick et al., 2000; Glick et al., 2002; Maisonneuve and Glick, 2003; Pace et al., 2004). These data provide evidence that although nicotinic acetylcholine receptors do not appear to be involved in the acute locomotor stimulant response to amphetamines, they may be involved in other amphetamine behaviors.

Cocaine Stimulation

Weak evidence of a QTL for cocaine stimulation on mouse chromosome 9 was found. There was no evidence of a QTL for cocaine activation using the D2 background congenic, but there was evidence of a sex-specific QTL for cocaine stimulation on the B6 background congenic (Chapter 4). Female chromosome 9 B6.D2 congenic mice were more stimulated by cocaine than B6 control mice. These data are consistent with the QTL mapped for cocaine stimulation in female BXD RI mice (Phillips et al., 1998); male mice were not included in this QTL mapping study. In contrast, male B6.D2 congenic mice were less stimulated by cocaine than B6 mice. The direction of effect observed in male congenic mice was opposite to the original QTL mapping for cocaine stimulation in male BXD RI mice (Miner and Marley, 1995). It is possible that this may be a spurious finding.

Pharmacologically I was unable to antagonize the stimulant effects of cocaine with mecamylamine (Chapter 2), although prior research has implicated nicotinic acetylcholine receptors in response to this drug. For example, α4 knockout mice were more stimulated by cocaine than wildtype mice (Marubio et al., 2003). There is mixed

data regarding the role of α3β4 nicotinic acetylcholine receptors in this response. In an early study, 1 hour pretreatment with 18-methoxycoronaridine attenuated cocaine-induced stimulation (Maisonneuve et al., 1997), but when administered 19 hours prior to the cocaine challenge 18-methoxycoronaridine enhanced or had no effect on this behavioral response (Maisonneuve et al., 1997; Szumlinski et al., 2000b). Due to the relatively weak evidence for a chromosome 9 QTL and the negative pharmacological data, this cocaine trait was not pursued further.

Acetylcholine receptors have been implicated in other cocaine behaviors. Mecamylamine has been shown to attenuate cocaine conditioned place preference (Zachariou et al., 2001), self-administration (Levin et al., 2000; Blokhina et al., 2005), escalation of cocaine self-administration (Hansen and Mark, 2007), and the development of cocaine-induced behavioral sensitization (Schoffelmeer et al., 2002). α3β4 receptors also appear to be involved in cocaine self-administration, since 18-methoxycoronaridine decreased this behavior (Glick et al., 1996). Furthermore, cocaine conditioned place preference was decreased in β2 knockout mice compared to wildtype controls (Zachariou et al., 2001).

There was only weak evidence of a QTL for cocaine-induced locomotor activation on mouse chromosome 9, but there was strong evidence of a QTL for methamphetamine stimulation in this region. These data suggest that a gene on mouse chromosome 9 likely does not influence both of these traits, but previous data has provided evidence that a common gene(s) influences both traits. A comparison of the BXD RI strain means for the acute response to cocaine and methamphetamine provides evidence that 44% of the variation in these traits is due to common genetic mechanisms

(R^2 = 0.44; Phillips et al., unpublished data; Phillips et al., 1998). Furthermore, data from the HMACT and LMACT lines of mice has provided evidence of a genetic correlations between these traits (Kamens et al., 2005). When the effect size was determined for the response to methamphetamine and cocaine in the methamphetamine selected lines the difference was approximately half (R^2 = 0.69 vs. 0.37, respectively; derived from a 1-way ANOVA based on the equation presented in Chapter 3). These data suggest that approximately 44 – 54 % of the genetic variance is shared between these two traits. These data also provide evidence that not all of the genes that influence methamphetamine stimulation also influence cocaine stimulation, an observation consistent with the data presented in this dissertation. A gene or genes, likely not on chromosome 9, influences the acute response to both psychostimulants.

Cocaine blocks the dopamine, norepinephrine, and serotonin (5-HT) transporters (Eshleman et al., 1999). The blockade of the dopamine transporter causes an increase in extracelluar dopamine levels (Mortensen and Amara, 2003; Elliott and Beveridge, 2005). Amphetamines act primarily as a substrate for the dopamine transporter, although they also act as substrates for the norepinephrine and 5-HT transporters (Rothman and Baumann, 2003; Sulzer et al., 2005). Amphetamine is taken up into the cell via the dopamine transporter. Once inside the cell, amphetamine interferes with the pH gradient of the synaptic vesicles disrupting dopamine accumulation (Sulzer and Rayport, 1990; Sulzer et al., 1995). Disruption of vesicular dopamine storage causes an increase of dopamine in the extracelluar space, similar to cocaine, due to dopamine release from the cell through a reversal of the dopamine transporter (Kahlig et al., 2005). The behavioral effects of amphetamine appear to be modulated by normal functioning of monoamine

oxidase, providing evidence that amphetamines also interact with this enzyme (Gianutsos et al., 1983). The similarities and differences in the mechanism of action of these drugs could lead to hypotheses about what genes may influence the response to both drugs as well as which genes may influence the response to only one of the drugs. For example, since both drugs interact with the dopamine, norepinephrine, and 5-HT systems these could be the source of common mechanism. In contrast, since amphetamines interact with the vesicular monoamine transporter and monoamine oxidase, but cocaine does not, these may only influence the response to amphetamines.

Ethanol Stimulation

The results for the ethanol stimulation trait are discussed in more detail, because the data presented in this dissertation focus predominately on this trait.

Gene Mapping

In this dissertation I provide evidence of a QTL for ethanol stimulation on mouse chromosome 9, where a gene accounts for 20% of the phenotypic variation in this response. D2 mice were significantly more stimulated by 2 g/kg ethanol than chromosome 9 D2.B6 congenic mice (Chapter 3). This QTL was not detected on the B6 background.

There are a number of reasons why I may have been unable to detect this QTL on the B6 background, and I was able to address some of these (see discussion of Chapter 3). I was not able to determine if the chromosome 9 QTL locus epistatically interacts with another locus. It is possible that D2 alleles are needed at another chromosomal locus to be able to observe increased locomotor stimulation with the chromosome 9 QTL. There is at least one example of epistatic interactions with a gene on chromosome 9 that affects

the acute locomotor response to ethanol. Mice lacking the dopamine D2 receptor (*Drd2*) were more stimulated by ethanol than wildtype mice when the mutation was on a B6 background, but not when the mutation was on a mixed 129S2 X 129S6 background (Palmer et al., 2003).

The QTL for ethanol-induced locomotor stimulation was more finely mapped using a congenic F_2 population derived from a cross of the chromosome 9 D2.B6 congenic mice to D2 mice. The QTL region was narrowed from a 62 to 46 cM region. Although I did not achieve as great of mapping resolution with this technique as I did using the ISCS for the methamphetamine stimulation QTL, some finer map resolution was achieved. This is the first time anyone has tried to use a congenic F_2 population for this purpose. In the future, testing more mice or using a population with more recombinants, by starting from a later intercrossed population, may provide greater resolution of the QTL region.

Behavioral pharmacology

I present data in this dissertation that acetylcholine receptor antagonists attenuate ethanol-induced locomotor activation. Similar to work in outbred NMRI mice (Blomqvist et al., 1992; Larsson et al., 2002), neuronal nicotinic acetylcholine receptors are involved in ethanol-induced stimulation in inbred D2 and selectively bred FAST mice (Chapter 2). Since these receptors are involved in ethanol activation in all three of these genetic models, together these data provide strong evidence for the involvement of nicotinic acetylcholine receptors in the locomotor activating effects of ethanol.

Mecamylamine decreased ethanol-induced stimulation in D2 and FAST mice (Chapter 2). Because mecamylamine is a nonspecific nicotinic acetylcholine receptor

antagonist, no information was provided about which type of nicotinic receptors are involved in this response. However, work with other pharmacological agents provided some insight into which types of acetylcholine receptors are not involved in this response. Hexamethonium had no effect on ethanol-induced stimulation. Since this drug does not cross the blood brain barrier these data provide evidence that neuronal nicotinic acetylcholine receptors in the central nervous system are involved in this response. Dihydro-β-erythroidine and methyllycaconitine had no effect on ethanol-induced stimulation in FAST or NMRI mice (Chapter 2; Larsson et al., 2002). These data rule out the involvement of α4β2 and α7 acetylcholine receptors. Other nicotinic acetylcholine receptors are blocked by mecamylamine that may affect this behavior. These include α3- and α6-containing receptors. There are no pharmacologic data in this dissertation that can address the specific involvement of acetylcholine receptors containing these subunits.

Prior research in the NMRI outbred mice has provided evidence that α3β2 or β3-containing nicotinic acetylcholine receptors may be involved in the acute response to ethanol, but that α6-containing acetylcholine receptors may not be involved. α-conotoxin MII blocks α3β2 nicotinic acetylcholine receptors as well as those containing a β3 or α6 subunit. When injected directly into the ventral tegmental area, α-conotoxin MII blocked ethanol-induced stimulation (Larsson et al., 2004). In contrast, the conotoxin PIA-analogue, which specifically blocks α6-containing nicotinic acetylcholine receptors, had no effect on ethanol-induced stimulation (Jerlhag et al., 2006). The β3 subunit of the nicotinic acetylcholine receptor forms functional receptors only when combined with existing α/β pairs (Groot-Kormelink et al., 1998; Broadbent et al., 2006), and is not involved in agonist binding (Boorman et al., 2003). No data exist regarding the influence

of this receptor subunit on drug responsiveness, but mice lacking this subunit had greater basal locomotor activity when compared to wildtype mice (Cui et al., 2003). Together these data suggest that α3β2 or β3-containing nicotinic acetylcholine receptors are involved in this effect of ethanol.

The specific role of α3-containing acetylcholine receptors in ethanol-induced stimulation remains unknown. The α3 subunit combines with the β2 or β4 subunit to form functional receptors. No antagonists exist that can cross the blood brain barrier and selectively block α3β2 receptors. 18-methoxycoronaridine appears to be selective for α3β4 receptors (Glick et al., 2002), but is not commercially available nor is it currently available from the investigator who synthesized it. To my knowledge, no one has tried to block ethanol-induced locomotor stimulation with 18-methoxycoronaridine.

At high doses, some anticholinergic drugs have been shown to have effects on N-methyl-D-aspartate (NMDA) receptors. In CD-1 mice, 12 mg/kg mecamylamine decreased NMDA-induced lethality (McDonough and Shih, 1995), providing evidence that high doses of this drug have antagonistic effects at these receptors. Ethanol-induced locomotor stimulation was attenuated with 1 mg/kg mecamylamine in D2 mice and 1 – 3 mg/kg in FAST mice (Chapter 2). Since low doses of mecamylamine were able to attenuate ethanol-induced stimulation, I speculate that the effects observed were dependent upon mecamylamine's effects at nicotinic acetylcholine receptors and not at NMDA receptors.

Acetylcholine receptors have been implicated in other behavioral responses of ethanol. The involvement of these receptors in ethanol drinking remains unclear. While some studies have reported that mecamylamine decreased ethanol intake (Blomqvist et

al., 1996; Le et al., 2000), others have reported an increase in ethanol intake (Ericson et al., 2000) or no change in ethanol consumption (Dyr et al., 1999; Smith et al., 1999). The α3β4-specific antagonist, 18-methoxycoronaridine, decreased ethanol consumption in a two-bottle free choice paradigm (Rezvani et al., 1997). Furthermore, a restriction length polymorphism in the α4 nicotinic acetylcholine receptor gene has been associated with ethanol consumption and ethanol effects on Y-maze crossings (Tritto et al., 2001). Nicotinic acetylcholine receptors have also been implicated in ethanol withdrawal severity. Mice lacking the β2 subunit of the nicotinic acetylcholine receptor had reduced ethanol withdrawal compared to wildtype mice (Butt et al., 2004). An A/T polymorphism in the α4 subunit gene has also been associated with ethanol withdrawal severity (Butt et al., 2004).

In contrast to psychostimulants (discussed above) which interact with the dopamine, norepinephrine, and 5-HT systems, ethanol has actions on many other neurotransmitter systems. $GABA_A$, glycine, $5\text{-}HT_3$, inotropic glutamate, amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kinate, and nicotinic acetylcholine receptors are all sensitive to alcohol. Alcohol potentiates the actions of the $GABA_A$, glycine, and $5\text{-}HT_3$ receptors (Celentano et al., 1988; Lovinger, 1991; Nakahiro et al., 1991; Lovinger and Zhou, 1994; Mascia et al., 1996). In contrast, ethanol inhibits the function of inotropic glutamate and AMPA/kinate receptors (Lovinger et al., 1989, 1990; Lovinger, 1993).

Interestingly, there is a high rate of alcohol and nicotine co-abuse (Istvan and Matarazzo, 1984), and alcohols have also been shown to interact with the nicotinic acetylcholine receptors. Studies examining alcohol's effect on acetylcholine receptors

have shown a variety of results. Alcohols have been shown to potentiate ion current (Dilger and Brett, 1991; Liu et al., 1994), produce a left shift in the agonist concentration curve (i.e., make the agonist appear more potent; Forman et al., 1995), stabilize the open state (Wu et al., 1994), and act as a channel blocker (Murrell and Haydon, 1991; Wood et al., 1995). Different receptor subtypes may account for the different actions observed. For example, ethanol potentiated agonist-induced current in α2β4, α4β4, α2β2, and α4β2 receptors expressed in *Xenopus oocytes*, but α3β2 and α3β4 receptors were insensitive to this effect of ethanol. Furthermore, α7 receptors were inhibited by ethanol (Cardoso et al., 1999). Since alcohol interacts with more neurotransmitter receptors, including nicotinic acetylcholine receptors, than psychostimulants, this provides one explanation why these receptors may be involved in ethanol-induced stimulation, but not cocaine- or methamphetamine-induced locomotor activation.

Gene expression

Chrna3 expression was examined in the FAST and SLOW mice to determine if differential expression of this gene may account for the behavioral differences between these lines of mice. *Chrna3* expression differences were not observed between these lines of mice. Gene expression differences in *Chrna3* may not be responsible for differential sensitivity to ethanol stimulation. Alternatively, I may not have been able to detect mRNA expression differences between these two selected lines of mice for a variety of reasons. First, QTL for ethanol-induced stimulation have not been mapped in the FAST and SLOW mice. Therefore, it is not known if a gene on chromosome 9 influences this trait in these mice. If *Chrna3* expression is influenced by only cis-

regulatory regions, and if the FAST and SLOW mice are genetically identical in this region of chromosome 9, I may not have expected to see differences in gene expression. Both cis- and trans-regulatory regions influence the expression of *Chrna3* (www.genenetwork.org), therefore even if these mice were genetically similar in this region of chromosome 9, trans-activation of expression could still exist. Second, the comparison between FAST and SLOW mice may be difficult to interpret because these lines also differ in the sedative effects of ethanol. FAST mice are extremely sensitive to the stimulant effects of ethanol, whereas SLOW mice are insensitive to the stimulant effects of ethanol and are additionally more sensitive to the sedative effects (Phillips et al., 2002b). In fact, SLOW mice are sedated (as defined by a decrease below saline activity) during the first six min after an acute injection of ethanol as well as when assessed by the loss of righting reflex (Phillips et al., 2002b). The genes that influence sensitivity to the stimulant effects of ethanol may be different than those that influence locomotor sedation. Therefore, a comparison that may be more easily interpreted may be between two strains of animals that both display locomotor stimulation, but to different degrees and that do not differ in sensitivity to the sedative effects of ethanol. Third, the α3 subunit is not widely expressed throughout the brain. Gene expression assays in brain regions known to have high expression of this subunit may yield different results. Therefore, the fact that *Chrna3* expression differences were not detected between whole brain samples from the FAST and SLOW mice does not eliminate the possibility that receptors containing this subunit are involved in ethanol stimulation.

In contrast to FAST and SLOW mice, *Chrna3* expression differences were detected between the chromosome 9 D2.B6 congenic and D2 control mice using both

whole brain and regional samples. These data support that *Chrna3* is a candidate gene for the chromosome 9 ethanol stimulation QTL. D2 mice that are highly stimulated by ethanol have less *Chrna3* expression in the whole brain, ventral midbrain, and striatum compared to chromosome 9 D2.B6 congenic mice that are less stimulated by ethanol (Chapter 3). In contrast, expression of *Chrna5* and *Chrnb4* are similar between these two strains. Both the D2 and D2.B6 congenic mice are stimulated during the first five min after an acute inject of ethanol (Figure 13) and currently no other data exist regarding the sensitivity to the sedative effects of ethanol in these mice.

While we were able to detect *Chrna3* expression in the FAST, SLOW, D2, and D2.B6 congenic strains, it should be noted that the Allen Brain Atlas (http://www.brainatlas.org/aba/) indicates very low levels of *Chrna3* expression in the mouse. Furthermore, most of the original work localizing the expression of this gene was done in rats. Further work should be done to confirm the presences of this gene in the mouse brain and examine cellular localization in this species. Confirming the observed differences in *Chrna3* expression in the D2 and D2.B6 congenic strains with another method, such as in situ hybridization, may also strengthen these findings.

As discussed in the appendix of Chapter 3, the assays currently used to examine acetylcholine receptor protein levels, including those for the α3 subunit, may not be adequate. Therefore, although it appears that the difference in *Chrna3* expression between the chromosome 9 D2.B6 congenic and D2 control mice did not correspond with an increase in α3 protein in the D2.B6 congenic strain (Chapter 3 appendix) these results could be due to an inadequate assay. I may have been unable to detect differences in α3 protein levels because the cytisine-resistant epibatidine binding assay used in the current

study is not solely measuring acetylcholine receptors with this subunit (see discussion of Chapter 3 appendix). Prior studies examining nicotinic acetylcholine receptor subunits have also reported a discordance between mRNA and protein levels (Huang and Winzer-Serhan, 2006).

However, it is possible that a difference in mRNA and protein expression may exist. There could be differences in mRNA, but not protein, levels of this gene. This disparity could arise for at least two reasons. There could be differential mRNA degradation between the two strains of mice or there could be translational regulation such that the same number of receptors is produced in both strains.

Stimulation as an Endophenotype

In this dissertation the acute locomotor response to ethanol, cocaine, and methamphetamine was used as an endophenotype for ethanol and drug abuse. As discussed in the general introduction there is considerable human literature to suggest that there is a positive relationship between the acute stimulant response to these drugs and the likelihood of further abuse of these substances. The data regarding this relationship in rodents is less clear.

There is a considerable amount of evidence in the animal literature to suggest that there is a positive relationship between drug stimulation and intake. For example, the FAST and SLOW mice selectively bred for their initial sensitivity to the locomotor stimulant effects of ethanol differ in ethanol intake, such that the FAST mice consumed more ethanol than SLOW mice (Risinger et al., 1994). Additional evidence of a positive relationship comes from lines of rats selectively bred for ethanol consumption or preference. The P, HAD, sP, and UChB selected lines of rats (all selected for high

ethanol consumption or preference) were more stimulated by an acute injection of ethanol than the corresponding line selected for low ethanol intake (NP, LAD, sNP, and UChA, respectively; Waller et al., 1986; Krimmer and Schechter, 1992; Quintanilla, 1999; Agabio et al., 2001; Rodd et al., 2004). These data provide evidence of a positive genetic correlation between ethanol stimulation and consumption.

Although there are a number of studies in rodents that show a positive relationship between the acute locomotor response to ethanol and ethanol consumption, other selected lines have not shown this relationship. For example, mice that were selectively bred for high (HAP) or low (LAP) ethanol consumption are equally sensitive to the stimulant effects of ethanol (Grahame et al., 2000). Furthermore, a negative genetic correlation ($r = -0.48$ to $r = -0.68$) between ethanol stimulation and consumption was reported when the BXD RI strain means for both of these traits were examined (Phillips et al., 1995). To my knowledge only one study has examined the correlation between psychostimulant activation and consumption. The HMACT and LMACT lines selectively bred for their initial response to methamphetamine stimulation showed a negative genetic correlation with methamphetamine drinking (Kamens et al., 2005), such that the line that was more stimulated by methamphetamine consumed less drug. It was speculated in this paper that extreme locomotor stimulation may be aversive. It is possible that another example of this may be the D2 inbred strain. These mice are extremely stimulated by ethanol (Dudek et al., 1991; Crabbe et al., 1994), but consume little ethanol in a 2-bottle choice paradigm (Wahlsten et al., 2006). Together these data suggest that the acute locomotor response to ethanol and psychostimulants may predict further drug intake, but that this relationship is likely complicated by multiple factors.

Furthermore, it does appear that understanding the biological basis of this response may lead to a great understanding of drug abuse.

Genetic Correlations and Pleiotropy

Genetic correlations have been observed between the acute locomotor responses to amphetamines, cocaine, and ethanol (Marley et al., 1998; Bergstrom et al., 2003; Kamens et al., 2005, 2006). These data provide evidence that a common gene(s), and thus neural mechanism, may influence the response to these drugs. The data presented in this dissertation do not support the hypothesis that neuronal nicotinic acetylcholine receptors (and the genes that encode these receptors) have a common role in ethanol-, cocaine-, and methamphetamine-induced locomotor stimulation, because nicotinic drugs did not effect psychostimulant-induced activation.

Although the genes encoding the nicotinic acetylcholine receptors on chromosome 9 do not appear to pleiotropically influence the acute response to ethanol, cocaine, and methamphetamine, it is possible that a different gene on mouse chromosome 9 could influence the response to all of these drugs. Alternatively, it is possible that a gene on another chromosome pleiotropically influences these behaviors. At least two genes have been shown to influence the acute locomotor response to ethanol and psychostimulants. The gene encoding the dopamine D4 receptor maps to chromosome 7 and influences the locomotor response to these drugs. Mice lacking this receptor were supersensitive to the locomotor stimulant effects of cocaine, ethanol, and methamphetamine (Rubinstein et al., 1997). A QTL for the acute locomotor response to all of these drugs has also been mapped on this chromosome (Cunningham, 1995; Miner and Marley, 1995; Phillips et al., 1996; Grisel et al., 1997; Phillips et al., 1998; Downing

et al., 2006; Palmer et al., 2006). The vesicular monoamine transporter 2 that maps on chromosome 19 has also been implicated in the behavioral response to ethanol, cocaine and amphetamine, although a QTL has been mapped in this region for only cocaine and ethanol sensitivity (Phillips et al., 1998; Gill et al., 2000). Mice having only one functional copy of the vesicular monoamine transporter 2 gene were more sensitive than wildtype mice to the locomotor stimulant properties of amphetamine, cocaine, and ethanol (Wang et al., 1997). These genes, or other yet to be identified, may pleiotropically influence the locomotor response to ethanol and psychostimulants and thus account for the observed genetic correlations among these traits.

Progressing from Candidate Gene to Quantitative Trait Gene (QTG)

The evidence that *Chrna3* is a candidate gene for ethanol-induced locomotor activation comes mostly from the gene expression differences observed between the chromosome 9 D2.B6 congenic and D2 strains. Additionally, behavioral pharmacology studies have implicated the involvement of neuronal nicotinic acetylcholine receptors in this behavior, although there is no direct pharmacologic evidence that α3-containing receptors are involved. Variants in the sequence of a gene can have two different effects. Polymorphisms in the non-coding region of the gene can influence gene expression, while variants in the coding region can have functional consequences. Both of these types of polymorphisms may affect complex traits (Flint et al., 2005), but it has been suggested that genes underlying complex traits are more likely due to polymorphisms that result in gene expression differences (Glazier et al., 2002; Korstanje and Paigen, 2002).

In this dissertation, data is presented that *Chrna3*, but not *Chrna5* or *Chrnb4*, is differentially expressed between the chromosome 9 D2.B6 congenic and D2 control

strains (Chapter 3). Given the idea that genes influencing quantitative traits are likely due to differences in gene expression (Korstanje and Paigen, 2002), from these data *Chrna3* would be the most promising candidate gene of those in the acetylcholine receptor cluster on chromosome 9. In contrast, if the QTL arises because of a polymorphism in the coding sequence of the gene, *Chrnb4* appears to be a more promising candidate. There are no coding single nucleotide polymorphisms (SNP) in the *Chrna3* coding sequence between the B6 and D2 inbred strains. Interestingly, there are coding sequence SNP in the other two genes in the chromosome 9 cluster. There is a silent SNP in exon 5 of *Chrna5*. In contrast, there are 4 SNP in the coding region of *Chrnb4*. One SNP in exon 2 is silent, but there are other SNP in exons 3, 4, and 6, of which the one in exon 6 is known to be a missense SNP (www.genenetwork.org). It is possible that these SNP have functional effects that influence locomotor stimulation. I know of no data regarding if these SNP cause functional changes on the subunit. Furthermore, there are many other genes in the QTL region that could also have SNP that influence gene expression or protein function.

Drd2 should also be regarded as candidate, because it maps in the region captured by the ethanol stimulation QTL. Mice lacking one or two functional copies of the *Drd2* gene were more stimulated than wildtype mice by 2 g/kg ethanol when baseline locomotor differences were taken into account (Cunningham et al., 2000; Palmer et al., 2003), but this effect is dependent on the genetic background of the mouse (see Palmer et al., 2003).

Chrna3 and *Drd2* are both candidate genes for ethanol-induced stimulation, but a body of convergent evidence is needed to determine if one of these genes (or another yet

to be identified) is the quantitative trait gene (QTG). As discussed in the literature recently, there is no exact formula for proclaiming that the QTG has been identified (Belknap et al., 2001; Glazier et al., 2002; Phillips et al., 2002a; Abiola et al., 2003), rather evidence from multiple methods provides this proof. These methods can include genetically engineered mice, gene expression, protein expression, and functional studies. Additional work will be needed to determine the QTG that underlies the acute locomotor response to ethanol on mouse chromosome 9.

Conclusions and Future Directions

In this dissertation I provided evidence of a QTL for the acute locomotor responses to the psychostimulants cocaine and methamphetamine on mouse chromosome 9. The QTL for methamphetamine stimulation was more finely mapped using ISCS to a 24 Mb region on the distal portion of this chromosome. Examination of gene expression differences in genotypes relevant to this phenotype identified four genes in the QTL region that should be regarded as candidate genes, although others could exist.

New ISCS are currently being created to more finely map the QTL for methamphetamine stimulation. Once the location of the QTL is narrowed multiple approaches will be taken to identify the QTG. To determine which genes are the most promising candidates, genes will be identified that are expressed in the brain, have functional SNP, and/or are differentially expressed. With a combination of these approaches one or more candidate genes should be identified that can be further examined using other approaches, such as pharmacology or genetically engineered mice.

Although there was some evidence of a QTL for cocaine stimulation on mouse chromosome 9, this evidence was weaker than that for methamphetamine stimulation. To

follow up on these data female mice of the chromosome 9 ISCS panel could be tested to finely map this QTL with the goal of identifying candidate genes.

In this dissertation the presence of a QTL on mouse chromosome 9 for ethanol-induced locomotor activation was confirmed. Through pharmacological studies and gene expression assays I determined that nicotinic acetylcholine receptors are involved in this response. Furthermore, some data is provided that suggests that *Chrna3* is a candidate gene for this QTL, at least in D2 mice.

While the nicotinic acetylcholine receptor appears to be involved in the acute locomotor response to ethanol, further work is needed to determine which type of acetylcholine receptors are involved in this response. To determine if α3-containing nicotinic acetylcholine receptors are involved in this response it would be ideal to test if 18-methoxycoronaridine, or another α3 specific antagonist, blocks ethanol-induced locomotor stimulation. Unfortunately, this drug is not commercially available. Moreover, when the investigator who uses 18-methoxycoronaridine was asked for some of the drug, he said that an insufficient quantity exists to share. No other antagonists specific for α3-containing acetylcholine receptors that can cross the blood brain barrier exist.

Conotoxins are peptides that derived from the cone snail's venom. These are useful for studying the role of nicotinic acetylcholine receptors, because there are many varieties that specifically block different acetylcholine receptor subtypes (Nicke et al., 2004). Unfortunately, conotoxins do not cross the blood brain barrier and thus have to be administered intra-cerebrally. A number of conotoxins that block α3-containing acetylcholine receptors currently exist including: α-conotoxin Vc1.1, α-conotoxin OmIA,

and α-conotoxin AuIB (specific for both types of α3-containing receptor, α3β2, or α3β4 receptors, respectively; Clark et al., 2006; Talley et al., 2006). Administration of these conotoxins can be used to more specifically address the involvement of α3-containing nicotinic acetylcholine receptors in ethanol stimulation. Prior studies have shown that acetylcholine receptor blockers administered directly into the ventral tegmental area decreased ethanol-induced stimulation in NMRI mice (Larsson et al., 2004). If α3 acetylcholine receptors are involved in this behavioral response to ethanol, administration of these conotoxins into this region in D2 mice may decrease locomotor stimulation.

A number of other techniques could be used to examine the involvement of α3-containing nicotinic receptors. α3 homozygous knockout mice do not survive past 8 weeks of age (Xu et al., 1999), therefore these animals are not useful for testing the acute response to ethanol. However, heterozygous mice lacking one of the two copies of this gene are viable and could be tested. In fact, these mice have been useful for testing the involvement of α3-containing acetylcholine receptors in nicotine behaviors (Salas et al., 2004). There are at least two ways gene expression can be decreased *in vivo*. Antisense oligonucleotides and small interfering RNA (siRNA) decrease mRNA, and thus protein levels, of a specific gene of interest (Cejka et al., 2006; Corey, 2007). To date no studies have used siRNA to decrease expression of the α3 subunit of the nicotinic acetylcholine receptor, but antisense oligonucleotides have been used to examine the function of this receptor (Adams et al., 2004). Since the chromosome 9 D2.B6 congenic mouse had more *Chrna3* expression, but was less stimulated by ethanol than the D2 strain, α3 antisense oligonucleotides (or siRNA) may increase the acute locomotor response to ethanol in this congenic strain.

Finally, further work could be done to more finely map the QTL for ethanol-induced locomotor stimulation by creating ISCS from the chromosome 9 D2.B6 congenic strain. Alternatively, advanced intercross lines may also provide greater map resolution of this QTL (Palmer and Phillips, 2002). Either of these approaches could narrow the QTL region, thus narrowing the number of potential candidate genes for this response.

In general, significant progress has been made in reducing the chromosome 9 ethanol and methamphetamine stimulation QTL regions. Some of the data presented in this dissertation suggest that the QTG for these two drugs on chromosome 9 may be different. However, much work is yet to be done to definitively identify the QTGs underlying these drug sensitivity traits.

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