GENETIC ANALYSIS OF METHAMPHETAMINE-INDUCED CHEWING STEREOTYPY IN MAPPING POPULATIONS DERIVED FROM THE C57BL/6 AND DBA/2 INBRED MOUSE STRAINS

by

Alison Lynn Atkins

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. Thesis of

Alison Lynn Atkins

has been approved

[redacted]
Professor in charge of thesis

[redacted]
Member

[redacted]
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Member

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Member

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Abstract

The goal of this project was to characterize genetic factors that contribute to the degree of expression of methamphetamine (MA)-induced stereotypic behaviors, specifically stereotypic chewing in mice derived from the C57BL/6 (B6) and DBA/2 (D2) inbred strains. Following an acute high dose of methamphetamine, both humans and rodents exhibit stereotypic behaviors, which consist of purposeless, repetitive behaviors that occur at the exclusion of other behaviors. Genetics play a significant role in degree of MA-induced stereotypy (Grisel, Belknap, O'Toole, Helms, Wenger, & Crabbe, 1997; Bergeson, Hain, Jarvis, O'Toole, Helms, & Belknap, 1999; Atkins, Helms, O'Toole, & Belknap, 2001). This is a quantitative trait, which is one that is influenced by multiple genes with a relatively small contribution per gene. A quantitative trait locus (QTL) is a site on a chromosome containing alleles that influence a quantitative trait. A QTL analysis was carried out in three mouse populations derived from B6 and D2 inbred mouse strains, BXD recombinant inbred, B6D2F_2, and selected line mice. The most promising QTL regions from a combined analysis of these three populations were located on proximal chromosome 4, mid chromosome 5, and distal chromosome 9. A significant QTL interaction was identified between QTLs on chromosomes 8 and 9. In addition to identifying QTLs for this trait, this set of experiments was designed to finely map these QTLs, to examine how they correspond to correlated behaviors, and to begin to identify candidate genes that influence chewing stereotypy. A new congenic mouse strain for the region with the most highly significant QTL, distal chromosome 9, was created to confirm the QTL effect when compared to the background strain. However, the effects of the distal chromosome 9 QTL were not statistically significant in this population.

Microarray data from whole brain samples of B6, D2, and B6D2F_2 mice were analyzed for differences in baseline gene expression in the QTL regions of the most highly significant QTL interaction. Several candidate genes emerged from the distal end of chromosome 9 and the proximal end of chromosome 8 that were related to oxidative stress and neurotoxicity. This suggests that the mechanism for MA-induced stereotypy may be related to the mechanism for MA-induced neurotoxicity. Further investigation into this mechanism could be useful in treatment and prevention of neurotoxic effects in MA users as well as possible insight into treatments for other conditions that produce stereotypic behaviors and other conditions that are caused by oxidative brain damage.

Introduction

Genetics of MA-induced chewing stereotypy. The goal of this project was to characterize genetic factors that contribute to the degree of expression of methamphetamine (MA)-induced stereotypic behaviors, specifically stereotypic chewing in mice derived from the C57BL/6 (B6) and DBA/2 (D2) inbred strains. Following an acute high dose of methamphetamine, both humans and rodents exhibit stereotypic behaviors, which consist of purposeless, repetitive behaviors that occur at the exclusion of other behaviors. Genetics play a significant role in degree of MA-induced stereotypy (Grisel, Belknap, O'Toole, Helms, Wenger, & Crabbe, 1997; Bergeson, Hain, Jarvis, O'Toole, Helms, & Belknap, 1999; Atkins, Helms, O'Toole, & Belknap, 2001). We hypothesized that the mice that show a high amount of stereotypy in response to MA may experience this drug as aversive, and those which show less stereotypy may experience it as rewarding. We hypothesized that common genes influence both MA-induced stereotypy and MA's rewarding properties.

Grisel et al. (1997) measured number of stereotypic chewing episodes in B6, D2, and 25 strains of BXD recombinant inbred mice for one minute at 33 minutes after intraperitoneal (i.p.) injection with saline or with 4, 8, or 16 mg/kg MA. They found that very few of the mice tested exhibited stereotypy following saline or the 4 mg/kg dose of MA; nearly all of the mice tested exhibited stereotypy following the 16 mg/kg dose of MA, and degree of MA-induced stereotypy differed significantly between strains following the 8 mg/kg dose of MA (Grisel et al., 1997). At the 8 mg/kg dose, genotype accounted for 33% of the total observed variance in stereotypic chewing, and the Spearman-Brown-corrected split-half reliability for this measure was 0.85 (Grisel et al.,

1997). There were no significant differences between strains in brain concentrations of MA following the 8 mg/kg dose, so differences in stereotypy are not likely to be due to pharmacokinetic differences in the distribution metabolism of MA (Grisel et al., 1997). Provisional QTLs of $p < .001$ were identified for stereotypic chewing on chromosomes 4 and X, and provisional QTLs of $p < .01$ were identified for stereotypic chewing on chromosomes 2, 4, 9, and 17 (Grisel et al., 1997).

Bergeson et al. (1999) followed up this study by measuring number of stereotypic chewing episodes for one minute at 33 minutes after 8 mg/kg MA in 400 B6D2F_2 mice. B6D2F_2 mice were derived from the C57BL/6 (B6) and DBA/2 (D2) inbred mouse strains. Mice of these strains are interbred to produce an F_1 generation, in which each mouse is genetically equivalent such that they have a heterozygous genotype (B6D2) at each differential locus. These F_1 mice are then bred with each other to produce a genetically segregating F_2 population, in which each individual is genetically unique due to segregation and variation in recombinations.

They also measured locomotor activation and body temperature, and focused their analysis on selectively genotyping 16% of the total mice tested with the highest and lowest body temperature changes (Bergeson et al., 1999), the trait of interest. Within the regions that were genotyped within this subset of mice, results were combined with those from the previous study in BXD recombinant inbred mice (Grisel et al., 1997). The most promising QTL for stereotypic chewing that emerged from this analysis was located on chromosome 17, and several other provisional QTLs were identified on chromosomes 2, 4, 5, 8, 9, and 14 (Bergeson et al., 1999).

Atkins et al. (2001) selectively bred mice of a B6D2F_2 genetic background for four generations for high (HMA) and low (LMA) numbers of stereotyped chewing episodes measured for one minute at 33 minutes after MA injection. The MA dose used for selection was 10 mg/kg in the first two generations (S1 and S2). It was changed to 7 mg/kg for the high line and 15 mg/kg for the low line in the third generation (S3) to avoid ceiling and floor effects, respectively. Selective breeding was carried out to four generations by selecting the highest scoring HMA mice and the lowest scoring LMA mice of each generation to serve as breeders of the next generation of the HMA and LMA lines, respectively.

These mice were tested on an extensive battery of stereotypic behaviors in the S3 generation measured at 33 minutes post-injection (Atkins et al., 2001). Following 7 mg/kg MA, HMA mice primarily exhibited stereotypic chewing behavior, and LMA mice primarily exhibited stereotypic circling behavior. This consisted of circling the cage in a repetitive pattern. By the fourth selected generation (S4), HMA mice showed a several-fold greater frequency of stereotypic chewing than LMA mice at most doses. Though LMA mice did exhibit some chewing stereotypy, overall this behavior was nearly eliminated in this mouse line. This is supported by dose-response data showing very low amounts of chewing stereotypy even at the very high dose of 20 mg/kg MA (Atkins et al., 2001). This suggests that alleles contributing to high expression of chewing stereotypy were largely eliminated from the population in the low line. Short-term selected mouse lines can be a useful tool for QTL mapping in cases such as this in which lines diverge rapidly over only a few generations (Belknap, Richards, O'Toole, Helms, & Phillips, 1997). The HMA and LMA mice were created to study behaviors

correlated with MA-induced stereotypy, as was done in Atkins et al. (2001), and also to provide a third population to add to the BXD recombinant inbred strains and the B6D2F_2 population for QTL analysis of MA-induced chewing stereotypy. The QTL analysis was carried out as part of the present set of experiments.

Properties of MA. MA has a high potential for abuse, and chronic use of this drug can result in long-term neurotoxic damage (Albertson, Derlet, & Van Hoozen, 1999; Chang et al., 2002). MA abuse has been linked to violent behavior, psychotic symptoms, and cognitive impairment, all of which can persist long after use has stopped (Sato, Chen, Akiyama, & Otsuki, 1983; Murray, 1998; Albertson et al., 1999; Kish et al., 1999; Chang et al., 2002; Cretzmeyer, Sarrazin, Huber, Block, & Hall, 2003; Urbina & Jones, 2004). Neurotoxicity, psychotic symptoms, and cognitive impairment are enhanced in combination with human immunodeficiency virus (HIV) infection, which occurs at higher rates among MA users than in the general population (Rippeth et al., 2004; Urbina & Jones, 2004). On a societal level, use of MA in a population is associated with increased crime including drug-induced assault, homicide, traffic accidents, organized crime related to drug trafficking, theft, and child endangerment (Cretzmeyer et al., 2003). Also, the chemicals used to manufacture MA present significant environmental and safety risks (Cretzmeyer et al., 2003).

MA acts as an indirect dopamine agonist by binding to dopamine transporter and reversing the transport process. It is released into the cytoplasm, which allows intracellular dopamine to bind to transporter. Dopamine is then transported in the opposite direction, resulting in increased dopamine release (Bannon, Granneman, & Kapatos, 1995; Fleckenstein, Metzger, Wilkins, Gibb, & Hanson, 1997; Witkin et al.,

1999; Burrows, Nixdorf, & Yamamoto, 2000). MA also acts as an indirect agonist of norepinephrine and serotonin by binding to their transporters (Fleckenstein, Gibb, & Hanson, 2000; Yu et al., 2004). MA inhibits monoamine oxidase, which normally metabolizes dopamine (Fleckenstein et al., 2000). MA inhibits vesicular monoamine transporter-2 activity, preventing intracellular dopamine from being taken up into vesicles (Fleckenstein et al., 2000; Ugarte, Rau, Riddle, Hanson, & Fleckenstein, 2003). This effect is mediated by dopamine D2 receptor activation and is positively correlated with hyperthermia, and can be counteracted by decreasing ambient temperature (Ugarte et al., 2003). Peripheral effects of MA include high blood pressure, heart arrhythmias, chest pain, shortness of breath, nausea, vomiting, diarrhea, and increased body temperature (Malay, 2001; Turnipseed, Richards, Kirk, Diercks, & Amsterdam, 2003).

Description of MA-induced stereotypy. With repeated use or following an acute high dose, MA produces stereotypy in both humans and rodents. Stereotypy consists of purposeless, repetitive behaviors that occur at the exclusion of other behaviors. In humans, MA-induced stereotypy can include repetitive scratching, washing, nail biting, visual fixation on real or imagined objects, tapping or rotating a small object, or assembling and disassembling mechanical equipment (Murray, 1998; Ridley & Baker, 1982). In rodents, stereotypy often consists of repetitive head and forelimb movements and oral behaviors, including chewing (Grisel et al., 1997; Kita et al., 2000). The degree to which this occurs has a significant genetic component (Grisel et al., 1997; Bergeson et al., 1999; Atkins et al., 2001).

The mechanism for MA-induced stereotypy likely involves the dopamine system. Acute increases in striatal dopamine levels following psychostimulant administration

positively correlate with stereotypic behavior, particularly in ventrolateral striatum (Kelley, Lang, & Gauthier, 1988; Broderick, 1993; Canales & Graybiel, 2000). However, this correlation is not consistent and additional brain areas and neurotransmitters are likely to be involved. Kuczenski (1986) found that dopamine levels in rat striatum only correlated with amphetamine dose up to 3 mg/kg, and there was no significant correlation at higher doses. Sharp et al. (1987) found that stereotypic behaviors in rats given amphetamine is correlated overall with dopamine levels in the striatum, but the changes in dopamine levels in the striatum precede the behavioral changes. This group also found that a certain type of stereotypy, repetitive sniffing, was better correlated with dopamine levels in nucleus accumbens than in striatum (Sharp et al., 1987). Kuczenski and Segal (1999) found that both D1 and D2 dopamine receptors contribute to the expression of stereotypy, but not always in a predictable manner in terms of dose and time course. Itzhak, Martin, Ali, and Norenberg (1997) found that depletion of striatal dopamine transporter does not prevent stereotypic behavior in mice following methamphetamine, and concluded that other neurotransmitters must also be involved. Kawai et al. (1997) found that cholecystokinin (CCK)-like immunoreactivity in the medial prefrontal cortex is positively correlated with MA-induced stereotypy in rats.

Extracellular serotonin is increased in the striatum following amphetamine, and stereotypy may result from an interaction between levels of dopamine and serotonin release (Kuczenski & Segal, 1989). Extracellular acetylcholine levels are also increased in striatum following MA (Kuczenski & Segal, 2001). Mice deficient in norepinephrine show increased MA-induced stereotypy (Weinshenker, Miller, Blizinsky, Laughlin, &

Palmiter, 2002). Mice lacking histamine H_3 receptors show reduced MA-induced stereotypy (Toyota et al., 2002). Pretreatment with CCK enhances amphetamine-induced stereotypy in rats (Tieppo, Felicio, & Nasello, 2001). Environmental factors also play a role in expression of stereotypy. For example, exposure to loud music following MA produces more stereotypy in mice than white noise or silence (Morton, Hickey, & Dean, 2001).

There may be an inverse relationship between psychostimulant reward and stereotypy. We hypothesized that the mice that show a high amount of stereotypy in response to MA may experience this drug as aversive, and those which show less stereotypy may experience it as rewarding. MA produces a conditioned place preference in mice at low doses and a conditioned place aversion at high doses, suggesting that this drug is experienced as rewarding at lower doses and aversive at higher doses (Cunningham & Noble, 1992). There is a positive correlation between degree of psychostimulant-induced conditioned place preference and degree of locomotor activation, both within subjects and between inbred strains (Kitahama & Valatx, 1979; Belzung & Barreau, 2000; Shimosato & Ohkuma, 2000). B6 mice show significantly more locomotor activation to a low dose of amphetamine than D2 mice, and D2 mice show significantly more stereotypy than B6 mice following 8 mg/kg MA (Castellano, Filibeck & Oliverio, 1976; Grisel et al., 1997). B6 mice also show a significantly higher conditioned place preference to low doses of amphetamine than D2 mice (Orsini, Buchini, Piazza, Puglisi-Allegra, & Cabib, 2004).

A series of experiments was planned to characterize genetic factors that contribute to degree of expression of MA-induced stereotypic behaviors, specifically, stereotypic

chewing in mice derived from the B6 and D2 inbred strains. The goal of this series of experiments was to make progress toward identification of genes that influence MA-induced chewing stereotypy. In addition to identifying QTLs for this trait, the experiments were designed to map one of these QTLs finely and to begin to identify candidate genes that influence chewing stereotypy. The first step of the project was to narrow down regions of the genome that contain genes that influence the trait. The second step of the project was to finely map QTLs identified by the first step with one or more congenic mouse strains. The third step was to identify potential candidate genes by using microarray gene expression analysis in combination with the QTL results.

Experiment 1, time course for MA-induced stereotypy. We originally planned to measure gene expression differences in a mouse strain congenic for the most highly significant QTL region for MA-induced chewing stereotypy and its background strain. We measure stereotypic behavior at 33 minutes post-injection, and this is not likely to be long enough to get appreciable new gene expression. Because of this, we wanted to test whether strain differences in stereotypic behavior last longer than 33 minutes. The 33-minute post-injection time point for measuring stereotypy was determined based on pilot data collected with B6 and D2 inbred mice (Belknap, unpublished). In general, stereotypy emerges at or before 30 minutes post-injection and continues for at least an hour. However, a systematic study of time course of amphetamine- or MA-induced stereotypy behavior in mice has not yet been reported. For these reasons, a time course for MA-induced home cage stereotypic behaviors in mice was constructed.

Psychostimulant-induced stereotypy has been studied more extensively in rats. For example, in rats given a high dose of amphetamine, stereotypic head and forepaw

movements peaked from 60-120 minutes post-injection, and remained high throughout the entire 180-minute testing period (Sharp et al., 1987). With a lower dose, stereotypy peaked at 60-80 minutes post-injection and reached near zero by 160 minutes (Sharp et al., 1987). With the lowest dose tested, stereotypy peaked at 60 minutes post-injection and reached near zero by 120 minutes. This demonstrates that time of onset of peak stereotypic behavior is similar with any dose that produces stereotypy, but duration of stereotypy increases dose-dependently.

Rats display peak stereotypy at 20-110 minutes following MA administration (Segal & Kuczenski, 1999). Leith and Kuczenski (1982) found a relative absence of locomotor activity, which they observed to indicate stereotypy, from approximately 50 to 100 minutes following amphetamine in rats. In another study, stereotypy was highest from 30-60 minutes post-injection in rats given a low dose of amphetamine, and from 20-40 minutes with a medium dose, and from 40-120 minutes with a high dose (Mueller, Kunko, Whiteside, & Haskett, 1989).

Experiment 2, MA-induced stereotypy in the absence of cage bedding. Environmental factors play a role in degree of stereotypic behavior. For example, certain types of noise in the environment can enhance the degree of stereotypy exhibited (Morton et al., 2001). In animal models of spontaneous stereotypy, changing the environment can influence the occurrence and type of stereotypic behavior (Wurbel & Stauffacher, 1996; Powell, Newman, Pendergast, & Lewis, 1999). For deer mice (*Peromyscus*), a species that show a high degree of spontaneous stereotypic behavior, an enriched environment reduces stereotypy (Powell et al., 1999). The enriched cage included items such as a running wheel, habit trails, small enclosures, nesting material, and sunflower seeds, and

items were changed and rearranged weekly. The primary stereotypic behaviors for this species are repetitive circling, jumping, and backward somersaulting. Mice in standard cages developed significantly more jumping stereotypy than mice in enriched cages, and mice in enriched cages developed significantly more circling than mice in standard cages (Powell et al., 1999). This shows that environmental factors influence type of stereotypy expressed. The authors also suggested that spontaneous stereotypy is associated with environmental restriction, and stereotypic behaviors develop from the normal behaviors appropriate to an environmental context. Wurbel and Stauffacher (1996) observed that ICR mice, an outbred strain, spontaneously develop stereotypic gnawing of the wire cage lid. They prevented the development of this behavior by reducing the distance between the bars such that the mice were unable to push their noses through them, and they did not observe the development of another stereotypic behavior in its place. Instead, they observed an increase in non-stereotypic locomotor activity.

In our studies, mice were tested in the home cage, which contains cob-type cage bedding. This is the only object available for them to chew, and the stereotypy they exhibit consists of repetitive chewing of pieces of this cage bedding. We conducted a study of MA-induced stereotypy in the absence of cage bedding to examine whether strain differences in MA-induced stereotypy persist in the absence of availability of a chewable item. We conducted this study in B6 and D2 mice rather than in HMA and LMA mice because the selected line mice were no longer in existence when this study was conducted. Behavior was only measured at the 33-minute post-injection time point and was recorded descriptively as primary behavior exhibited in this one-minute period rather than quantified.

We hypothesized that the expected genetic difference, that D2 mice exhibit more stereotypy than B6 mice, would persist in the absence of cage bedding by substitution of another oral stereotypic behavior. We expected the results of this study to provide further evidence of the genetic determination of this behavior. This would eliminate the possibility that the behavior is only expressed when relevant environmental factors are present. B6 and D2 mice were tested without cage bedding following MA administration to examine stereotypic behaviors that emerge when chewing the cage bedding is not possible.

Experiment 3, quantitative trait loci (QTL) analysis of MA-induced chewing stereotypy. Multiple genes influence a quantitative trait with a relatively small contribution per gene. A quantitative trait locus (QTL) is a site on a chromosome containing alleles that influence a quantitative trait. A QTL is initially detected as a chromosomal region where marker genotype is correlated with score on the quantitative trait, meaning that one or more genes in this region influence the trait. For successful QTL mapping, the trait of interest should have high heritability, high reliability, and be controlled by QTLs of relatively large effect (Belknap, Dubay, Crabbe, & Buck, 1996). QTLs are initially mapped to relatively large chromosomal segments, with resolution of 10-30 centimorgans (cM) (Crabbe, Phillips, Buck, Cunningham, & Belknap, 1999; Nadeau & Frankel, 2000). A significant association between a score on a behavior of interest and genotype at a genetic marker indicates the presence of a QTL in the same chromosomal region as the marker. Because the mouse genome is similar to the human genome at many levels, this method can provide insight into possible genetic

contributions to human behaviors, if the mouse behavior models the corresponding human behavior (Boguski, 2002).

The BXD recombinant inbred mouse strains are often used for the initial genome screen for QTLs. Inbreeding separate lines from an F_2 cross between two inbred strains, B6 and D2 mice in this case, creates recombinant inbred mouse strains. The pattern of B6 and D2 alleles in each recombinant inbred strain is unique due to different recombinations of alleles that occurred in each F_2 mouse, and subsequent generations. Mice within a given recombinant inbred strain are genetically identical to each other. Because these are inbred strains, behavioral data are cumulative.

QTL results from analysis of BXD recombinant inbred strains usually require confirmation from other genetic models (Crabbe, Belknap, & Buck, 1994). To accomplish this, only areas containing provisional QTLs identified in BXD recombinant inbred mice are examined in additional populations of the same genetic background, such as F_2, backcross, short-term selected, or congenic populations. For the F_2 step, a large number of mice are tested behaviorally, and only those scoring in the highest and lowest ends of the distribution are genotyped. This saves time and money without the loss of a significant amount of statistical power. Results from these multiple steps can then be statistically combined using Fisher's method (Belknap et al., 1996).

Several provisional QTLs were previously identified in BXD recombinant inbred strains for mean number of chewing episodes following 8 mg/kg MA. These were on mid chromosome 2, proximal chromosome 4, distal chromosome 5, distal chromosome 9, mid chromosome 17, and mid chromosome X (Grisel et al., 1997). Subsequent QTL analysis focused on these regions. Bergeson, et al. (1999) carried out a preliminary

screen of some of these provisional QTLs in B6D2F$_2$ mice. The most significant provisional QTL found for chewing stereotypy was located on proximal chromosome 17. However, not all areas of the genome implicated by the BXD recombinant inbred study as being provisional QTLs for chewing stereotypy were analyzed, since Bergeson et al. (1999) were primarily focused on MA-induced locomotor activation and changes in body temperature rather than on stereotypy. DNA samples from these animals were available for future genotyping.

Experiment 4, generation of a congenic mouse strain for the most promising QTL region. Once QTLs have been mapped to relatively large areas of a chromosome, the next step is to attain higher map resolution. This eliminates many false-positive candidate genes, helps to determine whether multiple traits that map to the same area are influenced by the same QTLs, isolates QTLs distinct from each other, and further narrows down the range of potential candidate genes (Crabbe et al., 1999; Fehr, Shirley, Belknap, Crabbe, & Buck, 2002). One way to attain higher map resolution is to generate congenic strains, which involves transfer of a chromosomal segment from one inbred strain onto the genetic background of another inbred strain by repeated backcrossing. After repeated backcrosses, mice are identical in all areas of the genome other than the QTL region. Genotyping recombinant mice in smaller intervals throughout the QTL region and testing them behaviorally narrows down the QTL region to a smaller interval (Darvasi, 1998).

The conventional method for generation of congenic strains involves selection for the allele of interest in the QTL region in each generation (Silver, 1995). Instead of following the conventional method, congenic strain development and higher resolution

mapping can be combined to attain both within six backcross generations rather than ten to twelve. This is accomplished by carrying out the "speed" congenic method of simultaneously selecting for donor alleles flanking the QTL and against donor strain alleles throughout the rest of the genome (Wakeland, Morel, Achey, Yui, & Longmate, 1997). The sixth backcross generation offspring are equivalent to a tenth backcross generation produced by conventional methods in terms of the proportion of the genome comprised of donor strain regions outside of the QTL region (Wakeland et al., 1997).

Chromosome nine congenic mice on a B6 background with a heterozygous B6D2 congenic segment from 9-55 cM were available for testing. We behaviorally tested these mice in order to determine whether it would be necessary to create a new congenic mouse strain to capture the QTL effect.

Experiment 5, MA preference drinking. B6 mice drink significantly more 40 mg% cocaine solution than D2 mice when given a choice between cocaine and water, and D2 mice show significantly more stereotypy than B6 following 15 mg/kg cocaine (Morse et al., 1993). Neither B6 nor D2 mice showed a significant preference for 10 ug/mL amphetamine over water in a two-bottle choice test (Meliska, Bartke, McGlacken, & Jensen, 1995). However, though B6 mice did not show any significant preference or aversion, males did consume significantly more of the highest concentration tested than D2 males, and B6 females consumed more of all solutions tested except for the lowest concentration than D2 females (Meliska et al., 1995).

Degree of MA-induced stereotypy could also provide information about preference for other drugs of abuse, such that high stereotypy would predict less drug reward. For example, Sardinian non-alcohol-preferring rats showed significantly more

stereotypy following 1 mg/kg amphetamine than Sardinian alcohol-preferring rats (D'Aquila et al., 2002). Voluntary consumption of 6% ethanol and amphetamine-induced locomotor activation were significantly positively correlated in rats, with high ethanol preferring rats showing greater locomotor activation in response to amphetamine (Fahlke et al., 1995). We hypothesized that mice which show a high amount of stereotypy in response to MA would experience this drug as aversive and therefore not show a preference for MA, and mice that show less stereotypy would experience MA as rewarding and therefore show a preference for MA.

Experiment 6, microarray analysis of baseline gene expression. QTL analysis identifies genes related to a phenotype positionally, whereas expression mapping identifies functional genetic differences (Carter, Del Rio, Greenhall, Latronica, Lockhart, & Barlow, 2001). Combining QTL and microarray analysis by treating expression levels as quantitative traits can identify pathways involved in the trait of interest (Schadt et al., 2003; Hitzemann et al., 2004). RNA arrays measure expression levels of thousands of genes at a time, which is useful in studying how sets of genes interact to influence traits that are determined by complex interactions between multiple genes (Carter et al., 2001; Hoffman et al., 2003). Long-lasting adaptive changes in the brain such as addiction are likely to involve changes in gene expression in the brain (Hoffman et al., 2003). Microarray analysis can identify which genes within the QTL region are expressed in a brain area of interest and which show different levels of expression between mice of different genotypes (Belknap, Hitzemann, Crabbe, Phillips, Buck, & Williams, 2001; Luo & Geschwind, 2001).

For this project, MA-induced stereotyped chewing was measured acutely at 33 minutes post-injection, which is not enough time to get new gene regulation resulting from exposure to the drug. Because of this, measurement of baseline gene expression comparing the congenic mice with their background strain was planned, on the presumption that the important effects of gene expression on stereotypy exist prior to drug exposure.

Methods

General methods. Mice were housed 2-4 animals per plastic shoebox cage (internal dimensions 28.5 x 17.5 x 12 cm) with Bed-o-Cob™ bedding, filtered cage lids, and with Purina 5001 Labdiet™ food and water freely available. Animals were kept on a 12-hour light/dark cycle with lights on at 6:00 a.m. and off at 6:00 p.m. Ambient temperature of the colony room and the two testing rooms ranged from 19°-22° C. Approximately equal numbers of male and female mice of each line were used for each study. All procedures were approved by the VA Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health guidelines for animal care and use.

Unless otherwise noted, behavioral testing in all studies was conducted in the home cage to minimize handling the animals in order to minimize potential stress effects. Experimentally naïve mice were injected intraperitoneally with 10 mg/kg (+)-MA sulfate (Sigma, St. Louis, MO). Following injection, animals were returned to the home cage. The home cages of the animals being tested on a given trial were lined up on a table, with lids on when the animals were not being observed and removed just before each observation. The 10 mg/kg dose was chosen because it brought out the full range of

responses in B6D2F$_2$ mice during pilot testing before the selected lines were produced, meaning that individual mice ranged from no response to mainly locomotor activation to mainly stereotypic behaviors. Stereotypic behaviors infrequently occur at baseline, but the rate of such behaviors did not differ significantly from zero, so we did not include saline groups in these studies (Atkins et al., 2001). Following injection, animals were returned to the home cage.

Experiment 1, MA behavior time course. This study was planned to determine whether genetic differences in MA-induced stereotypy behavior last longer than 33 minutes, which is the time at which it was measured in the other studies in this set of experiments. We hypothesized that the differences observed at 33 minutes post-injection would persist beyond this time point. HMA and LMA mice of the fourth selected generation (S4; n=12 per line) were tested. Mice were videotaped for one minute every five minutes for a total of sixty minutes. Videotapes of behavior were later scored for number of chewing episodes, time spent chewing, number of circling episodes, and time spent circling in each one-minute block. Data analysis consisted of separate repeated measures ANOVA for each variable, with score at each time point as the dependent measure, time point as the repeated measure, and genotype as a factor. For number of chewing episodes and time spent chewing, scores at the 5-minute time point were not included in the data analysis because all scores were zero.

Experiment 2, MA-induced stereotypy in the absence of cage bedding. In the studies described in this set of experiments, mice were tested in the home cage, which contains cob-type cage bedding. This was the only object available for them to chew, and the stereotypy they exhibited consisted of repetitive chewing of pieces of this cage

bedding. We conducted this study of MA-induced stereotypy in the absence of cage bedding to examine whether strain differences in MA-induced stereotypy persist in the absence of availability of a chewable item. We hypothesized that strains that scored differently on stereotypic chewing would also score differently on some other stereotypic behavior that can occur in an environment with out cage bedding, providing further evidence of the genetic determination of this type of behavior. Because this study was conducted after HMA and LMA mice were no longer available for testing, B6 (n=20) and D2 mice (n=21) were tested. Following injection, animals were placed in a new cage of the same type as the home cage, either containing the usual cob-type cage bedding or containing nothing. Primary behavior, defined as behavior that the animal exhibited for the majority of the one-minute observation period was recorded at 33 minutes post-injection. This was the time point at which stereotypy was measured in the studies that preceded the present set of experiments. The data were not statistically analyzed; the goal of this study was to obtain a description of behaviors exhibited by B6 and D2 mice following 10 mg/kg methamphetamine in the absence of cage bedding.

Experiment 3, QTL analysis of MA-induced chewing stereotypy. To complete the QTL analysis for MA-induced chewing stereotypy, B6D2F_2 mice were genotyped in all areas identified as containing provisional QTLs in BXD recombinant inbred mice. The BXD recombinant inbred behavioral testing was previously completed (Grisel et al., 1997). Updated marker data for these strains were available for analysis (Williams, Gu, Qi, & Lu, 2001). All of the B6D2F_2 behavioral testing and some of the genotyping had been previously completed (Bergeson et al., 1999). We expanded this work by selecting 152 animals with the highest and lowest stereotypic chewing scores and genotyping these

animals in all areas implicated by the BXD recombinant inbred or the B6D2F_2 QTL analyses. In some cases, the genotyping was partially done, and in other cases, none of it had been done. DNA samples from HMA and LMA mice of the third (S3) and fourth (S4) selected generations were then genotyped in regions that were identified in both the BXD recombinant inbred and B6D2F_2 mice as containing provisional QTLs in order to confirm these results.

The markers used for QTL analysis were simple sequence length polymorphisms, or microsatellites, which are naturally occurring variations in the number of repetitive base pair sequences produced by mispairing during recombination or replication within the tandem repeat sequence (Silver, 1995; Belknap et al., 1996). Specifically, we used microsatellite markers of the MIT series (Dietrich et al., 1992). They were genotyped by amplification using oligodeoxynucleotide primer pairs specific to each marker by polymerase chain reaction (PCR). This was followed by resolution of PCR products representing alleles on agarose gels. PCR makes it possible to quickly amplify specific sequences, even when present in very low concentrations, in order to distinguish polymorphisms between strains. In some cases, the marker used in previous studies was no longer available. In most of these cases, a marker in the exact location of the previous marker was substituted. In just a few cases, we could not find a substitute marker in the exact location of the previous marker. In these cases, a marker within 5 cM of the previous marker was chosen, and results were combined despite the fact that the location was not exactly the same.

To check for additional QTLs that may have been missed in the BXD screen due to relatively small influence on the trait, pooled DNA samples were created for selected

line mice, such that there were four separate samples containing DNA from HMA females, HMA males, LMA females, and LMA males. These pooled samples were genotyped at markers throughout the genome, with at least one marker per 20 cM. This was done in order to carry out an inexpensive and non-labor intensive screen of the entire genome rather than individually genotyping each sample to screen for additional QTLs. Of the 220 markers initially screened, 80 were selected as good candidates for analysis by DNA pooling. To be a good candidate, the marker had to produce bands that were bright, distinct, of similar density, and with a large separation between the B6 and D2 alleles. The gel densities of standards including B6, D2, B6D2F_1, a 3:1 ratio of B6:D2, and a 3:1 ratio of D2:B6 were measured to create a standard curve, and the gel densities of the HMA and LMA pooled samples were then compared to the standard curve after background subtraction. The gel density is a measure of allele frequency, which can then be used to estimate the percentages of B6 and D2 alleles in the pooled samples. This method was used as a quick screen for possible additional markers that differed between the selected lines that may have been missed in the initial BXD recombinant inbred screen. Promising markers were defined as those for which there was a 50% difference ($p < .01$) in B6 alleles between HMA and LMA mice after background subtraction. The promising markers were then genotyped on individual mice.

A QTL was detected when mice with one allele, or genotype, at a marker locus scored differently on number of chewing episodes in one minute measured at 33 minutes after MA injection than those with the other allele, or genotype. A significant difference indicates that a gene locus at or near the marker, the QTL, contributes to the observed trait differences. After the genotyping was completed, results from the separate analyses

of BXD recombinant inbred, B6D2F_2, and selected line mice were statistically combined using a Mathcad worksheet created by John Belknap to combine p values from multiple experiments testing the same hypothesis using R.A. Fisher's method (Sokal and Rohlf, 1995). Using this method, the sum of -2ln(p) over t experiments is a chi square distributed variable with degrees of freedom equal to 2t. The p values from each individual analysis were entered and the worksheet calculated a chi-square value that was associated with a combined P value from all three populations. This method is more conservative than simply adding the LOD scores from multiple experiments. The worksheet was set up such that individual p values from up to five different populations testing the same hypothesis could be entered. In most cases, we had data from three populations for this initial analysis. In cases for which we only had data from two populations, the worksheet was set up to accommodate these analyses also.

The QTL analysis was carried out with Map Manager QTX software (Meer, Cudmore, & Manly, 2002). This software uses behavioral and genotyping data to generate a likelihood ratio statistic that a QTL exists, testing for the association of the behavioral score with marker loci. This statistic is a chi-square value. Interactions between QTLs in the F_2 population were also analyzed with Map Manager QTX. To do this, the software uses a model that measures the main effects of each locus and interactions between pairs of loci, including additive and dominance effects and the four possible types of interaction (additive by additive, additive by dominance, dominance by additive and dominance by dominance). These four types are reported as a single total interaction effect.

Provisional QTL interactions were then followed up using a MathCad spreadsheet designed by John Belknap to implement the Cheverud and Routman (1995) method of analyzing an F_2 for epistasis between pairs of loci with two possible alleles, B6 and D2 in this case. Using the cell means for each of nine possible genotypes and total and within-groups sums of squares from a two-way ANOVA, two QTL are tested for additive by additive, additive by dominance, dominance by additive and dominance by dominance components of epistasis.

QTL interactions were also analyzed using Pseudomarker software, which identifies interactions with a combination of a Bayesian method and an independent sample Monte Carlo algorithm that sequentially samples from possible QTL locations and genetic models that describe the relationship between genotype and phenotype (Sen & Churchill, 2001). A Bayesian method allows the use of prior information to evaluate relative probabilities of different hypotheses. A Monte Carlo algorithm uses randomly generated or sampled data and computer simulation to approximate a solution. In the Pseudomarker program, this is based on a virtual dense set of completely genotyped markers, called pseudomarkers, for which genotypes can be inferred from linkage information in actual marker data, with different possible versions that are consistent with the data. The phenotype is regressed on each pseudomarker for each possible set, and the LOD scores are averaged in a way that gives more weight to genotypes that explain more of the phenotypic variation. QTL location is estimated based on a 2.5 cM pseudomarker grid, which approximates interval mapping at this resolution.

To analyze QTL interactions, LOD scores are computed for each pair of pseudomarkers. Then main effects and interactions are combined to produce a single

model that describes both main QTL effect and QTL interactions. This full model is compared to a null model for which there are no QTL effects on the phenotype. The LOD scores have four degrees of freedom for the interactions and eight degrees of freedom for the full model. This differs from the two degrees of freedom in conventional QTL analyses for an F_2. This method allows simultaneous assessment of the combined influence of multiple loci on the trait of interest, which can provide insight into multi-locus pathways that influence the trait (Phillips & Belknap, 2002). This program has been used effectively in previous genome scans for QTL epistasis (Sen & Churchill, 2001; Shimomura et al., 2001; Sugiyama et al., 2001).

All provisional QTL interactions identified by any type of analysis were followed up with two-way ANOVAs, with genotype at pairs of markers as factors and chewing stereotypy score as the dependent measure. A significant interaction suggests that both loci influence the same pathway or pathways influencing stereotypy.

Experiment 4, generation of distal chromosome 9 congenic strain. The first step was to test the available congenic mouse strain with a B6D2 heterozygous segment from 9-55 cM on chromosome 9 on a B6 background and compare these mice to their B6 background strain to determine whether it was necessary to create a new congenic strain to capture the QTL region. To do this, we measured number of stereotypic chewing and circling episodes and time spent chewing and circling at 33 minutes post-injection following 10 mg/kg MA i.p. in these mice.

Based on the fact that we did not find a significant difference between the available congenics and their background strain on MA-induced chewing stereotypy, the decision was made to develop a new congenic strain with a more distal congenic segment

to confirm QTL at 71 cM on distal chromosome nine. Starting with males of a B6D2F_1 population mated with females of the B6 background strain, a backcross was produced. These mice were genotyped to identify males with a heterozygous (B6D2) genotype at 15-71 cM on chromosome nine and a low frequency of D2 alleles throughout the rest of the genome. The male with no crossovers in this region with the smallest proportion of D2 alleles throughout the rest of the genome was selected to mate with several B6 females to generate the next backcross generation. A male was selected rather than a female because larger numbers of progeny can be bred per individual male breeder mated to multiple females.

With each successive generation, genotyping was only carried out for markers in regions known to have the B6D2 genotype from the prior generation, such that fewer markers were used in each successive generation. The non-recombinant heterozygotes with the smallest proportion of D2 alleles from each generation were selected as breeders. Males were selected in order to maximize number of offspring whenever possible, but female congenic mice were also chosen as breeders when necessary. Starting with the third backcross generation, all mice were behaviorally tested to determine whether genotype in the QTL region was associated with behavioral scores. Starting with the sixth backcross generation, separate congenic lines were maintained from the recombinant mice in addition to the main congenic line derived from the non-recombinant mice. The pattern of which recombinant lines show the QTL effect and which do not provides increased mapping resolution (Fehr et al., 2002).

Because Map Manager QTX software was not equipped to handle this dataset, data were analyzed using R/qtl, a program for mapping QTLs in R software. This

program was designed to analyze QTLs in backcross populations, such as congenic strains, and intercross populations, such as an F_2 (Broman, Wu, Sen, & Churchill, 2003). It takes missing and partially missing genotyping data into account, and identifies potential genotyping errors.

Experiment 5, MA preference drinking. Mice heterozygous for a congenic segment from 15-71 cM on chromosome 9 and their background strain (B6) littermates of the third to fifth backcross generations were tested on MA preference drinking. All mice had previously been tested on MA-induced stereotypy as part of the QTL study. We hypothesized that mice with high MA-induced stereotypy would not show a preference for MA, and mice with low MA-induced stereotypy would show a preference for MA. On the day before preference testing began, mice were given two bottles of water in order to habituate them to the procedure. On days 2-4, one of the bottles contained 5 mg/L MA, and concentration was increased to 10 mg/L on days 5-7, and to 20 mg/L on days 8-10, with one bottle containing water and one bottle containing MA. In each case, drug was available 24 hours per day. These concentrations were previously determined to differ significantly between 24 BXD recombinant inbred mouse strains plus the progenitor B6 and D2 strains (Belknap, unpublished). Bottle position was switched on days 2, 5, and 8. To reduce the risk of excessive body weight loss due to drug-induced anorexia, higher-calorie Purina Labdiet Mouse Diet 9F™ was available to the animals throughout the study.

Both bottles were weighed every day throughout the study. Total water intake was calculated for all 10 days of the study, as was total fluid intake (water plus MA). MA preference was calculated as percent of total liquid consumed by weight that came

from the MA bottle. Preference for each concentration was averaged over the second and third days of that concentration. This was done to allow mice the opportunity to learn which bottle contained drug and which contained water on the first day of each new concentration. Amount of MA consumed per day (mg/kg/day) was calculated based on body weight (which was taken on the first day of the study and on the first day of each new concentration; days 1, 4, 7, and 10 of the study), average volume consumed on the second and third days of that concentration, and concentration. One-way ANOVAs for preference for MA and for amount of MA consumed per day as a function of genotype at the chromosome 9 congenic segment were carried out for each concentration separately. Data were also analyzed by Pearson correlation between number of chewing episodes and preference score and between number of chewing episodes and amount of MA consumed per day for each concentration separately.

Experiment 6, microarray analysis of baseline gene expression. Because the QTL analysis in the distal chromosome 9 congenic strain did not detect a QTL on distal chromosome 9, the microarray comparison of these congenic mice compared to their background strain was not pursued for the purposes of this project. However, there was a suggestive QTL for MA-induced chewing stereotypy at 71 cM on chromosome 9 in the $B6D2F_2$, and B6 and D2 mice have been shown to differ significantly in MA-induced chewing stereotypy, with D2 mice showing more stereotypy than B6 mice (Grisel et al., 1997). A database of baseline gene expression differences in 6 B6 and 6 D2 mice and a second database of baseline gene expression differences in 56 $B6D2F_2$ mice in whole brain measured by Affymetrix microarray using the 430A chip were available for

analysis (Hitzemann et al., unpublished; WebQTL.org). These data were analyzed for differences in baseline gene expression in the QTL region.

The 430A chip contains probes for 14,484 full-length genes, 4371 expressed sequence tags, and 3371 additional probes. Each probe is a labeled, single-stranded DNA or RNA molecule of specific base sequence that is used to detect the complementary base sequence by hybridization. A probe set represents a gene, and each one consists of 11 probe pairs of 25 nucleotides in length (25-mers), each uniquely derived from the same DNA sequence. Each probe set includes a perfect match probe and a mismatch probe with a single base mismatch in the middle of the oligonucleotide sequence. In some types of background correction, expression of the mismatch probe is subtracted from expression of the perfect match probe to correct for non-specific binding.

There are three stages for probe level analysis of microarray data; background subtraction, normalization, and summarization. Background subtraction removes background noise, which includes both the chip surface itself and non-specific binding, from signal intensities using information from only one chip. Normalization reduces variation between chips due to experimental factors unrelated to transcript expression levels using information from multiple chips. Summarization reduces the 11 probe intensities on each array to a single intensity value for each probe set.

It is necessary to background correct and to normalize microarray data in order to make comparisons between arrays, because overall brightness of the scanned image varies (Åstrand, 2003). There are several different methods for doing this. Microarray Suite (MAS) 5.0 is the Affymetrix background correction and normalization method. The chip is broken into a grid of sixteen rectangular regions, and for each region the

lowest two percent of probe intensities are used to compute a background value for that grid. Each probe is then adjusted based upon a weighted average of the backgrounds for each of the regions. The weights are based on the distances between the location of the probe and the centroids of sixteen different regions. Each perfect match probe, which matches a gene, is paired with a mismatch probe that is identical to the perfect match probe except that one base is changed in the middle of the probe. The expression measure is a robust average of the log of perfect match probe expression value minus mismatch probe expression value. The MAS method uses constant normalization, which scales all of the arrays so that they have the same mean value (Åstrand, 2003). The MAS background correction subtracts an ideal mismatch from the perfect match. The MAS summarization method is a robust average using one-step Tukey biweight on a $\log_2$ scale to reduce the influence of outliers, or near outliers, on the results.

The second method examined, robust multi-array analysis (RMA) method, is a stochastic model-based procedure (Irizarry et al., 2003; Wu & Irizarry, 2004). Using this method, perfect match probe intensities are corrected by using a global model for the distribution of probe intensities. The observed perfect match probes are modeled as the sum of a normal noise component and an exponential signal component. RMA adjusts only perfect match values and ignores mismatch probes. The RMA summarization method is median polish, for which a multi-chip linear model is fit to data from each probe set, and the expression values are in $\log_2$ scale.

The third method employed, dChip, uses invariant set normalization, which uses a baseline array to normalize arrays by selecting invariant sets of genes or probes and using them to fit a non-linear relationship between arrays (Li & Wong, 2001a). This method

uses no background correction, only taking perfect match probes into account. It uses a summarization method fitting a multi-chip model to each probe set as described by Li and Wong (2001a, 2001b, 2003).

The fourth method, position dependent nearest neighbor (PDNN), estimates gene-specific and non-specific binding using a different weight factor for each nucleotide position for each probe on the chip. This is similar to the dChip method, except that it can be applied to single arrays, whereas dChip requires a series of replicates. This method has been shown to produce lower variances than MAS 5.0 or dChip, is an effective means of detecting outliers, and does not require the use of mismatch probes (Zhang, Miles, & Aldape, 2003).

The B6 vs. D2 expression data were analyzed using the BioConductor Affymetrix package in R (www.bioconductor.org, version 1.9). Using this package, the raw data (CEL files) were analyzed with MAS 5.0, RMA, dChip, and PDNN, and t-tests were run comparing expression levels of each transcript between the two strains. Statistical power, the probability that the t-test will correctly identify a significant difference between groups of a given magnitude, was also calculated for each transcript for all four datasets. This calculation takes into account the sample size, the effect size, and the alpha level. The equation is as follows:

$t_{beta} = [(n \times \text{effect size})/(1\text{-effect size})]^{1/2} - t_{alpha}$.

The effect size is the proportion of the trait variance accounted for by the two-group difference in scores on the dependent variable. Effect size is calculated based on the sample size and the t value from a one-sample t test comparing groups on the dependent variable. The equation is as follows:

Effect size= $t^2/(t^2+n)$.

The alpha level is the significance level (p) set by the experimenter. The t_{alpha} term is the t value associated with this p value. Alpha is the probability of a type I error, that is, of rejecting the null hypothesis when it is true. Beta is the probability of a type II error, that is, of accepting the null hypothesis when it is false. Power is one minus beta.

A graph plotting each of the four power estimates against rank order was then created, and the comparison with the most power to detect gene expression differences between the two strains was chosen for all subsequent analyses. The q value, which is an estimate of false discovery rate, was then calculated for each comparison. This value is indicative of the proportion of declared statistically significant results in the dataset that are expected to be false positives (Storey, 2003). This method corrects for non-normality and non-independence among the tests. It is particularly important to use such a method when dealing with a large dataset with many comparisons such as in a microarray study.

The PDNN analysis method was then used on the $B6D2F_2$ expression data. Genes whose expression on chromosome 9 significantly differed between the B6 and D2 strains at $q< .01$ were extracted. These were analyzed in the $B6D2F_2$ using R/qtl to determine which genes had expression QTLs in the chromosome 9 region. This means that expression levels of each transcript in this region were treated as quantitative traits for QTL analyses. Transcripts with expression QTLs of LOD=2 or greater were selected. Dominance was then assessed to select those for which direction of dominance matched that for the chewing stereotypy QTL, which was D2 partial to complete dominant. The calculation for dominance takes into account the average values (number of chewing episodes for the behavioral measure, expression levels for the transcripts) for each of the

three possible genotypes and the overall standard deviation. The equation for dominance effects is as follows:

$$d = B6D2 - \frac{[(B6B6 + D2D2)/2]}{\text{Standard deviation}}$$

If there is complete dominance, the heterozygote value will be the same as one of the homozygote values. If there is partial dominance, the heterozygote value will be significantly closer to one of the homozygote values than to the other. If there are only additive effects, the heterozygote value will be exactly between the homozygote values. The equation for additive effects is as follows:

$a = [(D2D2 - B6B6)/2]/\text{standard deviation}$.

The ratio of d/a can range from negative one, indicating complete B6 dominance, to zero, indicating no dominance, to one, indicating complete D2 dominance.

Results

Experiment 1, MA behavior time course. There were significant differences in number of chewing episodes by genotype [$F(1, 22)=8.2$, $p= .009$), time point [$F(10, 220)=5.1$, $p< .001$], and genotype x time point [$F(10, 220)=2.0$, $p= .04$] (Figure 1). HMA mice had more chewing episodes than LMA mice, and the difference was significant between 25 and 40 minutes post-injection [25 minutes $F(1, 22)=17.1$, $p>.001$; 30 minutes $F(1, 22)=13.9$, $p= .001$; 35 minutes $F(1, 22)=6.6$, $p= .02$; 40 minutes $F(1, 22)=5.1$, $p= .04$]. For LMA mice, number of chewing episodes increased slightly at 15 minutes post-injection and continued to increase slightly throughout the testing period. For HMA mice, number of chewing episodes increased sharply at 25 minutes post-injection and began to decrease slightly at 45 minutes post-injection.

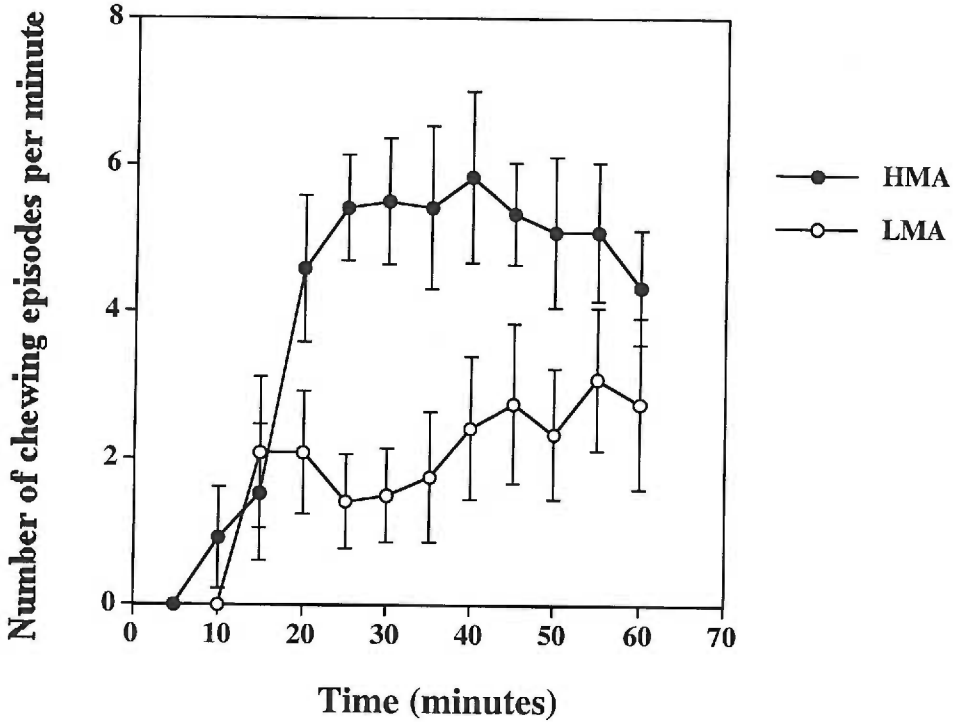

Figure 1. Mean (± s.e.m.) number of chewing episodes per minute after 10 mg/kg i.p. methamphetamine in HMA and LMA mice of the fourth selected generation.

For time spent chewing, there were significant effects of genotype [F (1, 22)=32.0, p< .001], time point [F (10, 220)=20.4, p< .001], and genotype x time point [F (10, 220)=11.8, p< .001] (Figure 2). HMA mice spent more time chewing than LMA mice, and this difference was significant between 20 and 60 minutes after injection [20 minutes F (1, 22)=15.2, p= .001; 25 minutes F (1, 22)=26.7, p< .001; 30 minutes F (1, 22)=27.6, p< .001; 35 minutes F (1, 22)=23.4, p< .001; 40 minutes F (1, 22)=46.7, p< .001; 45 minutes F (1, 22)=30.0, p< .001; 50 minutes F (1, 22)=42.0, p< .001; 55 minutes F (1, 22)=29.7, p< .001; 60 minutes F (1, 22)=22.2, p< .001]. HMA mice had a significant increase in time spent chewing per minute at 20 minutes post-injection and

this continued to increase until HMA mice reached near-maximal scores at the end of the test period. Time spent chewing increased slightly for LMA mice from 50 to 60 minutes post-injection, but remained near zero throughout the entire testing period.

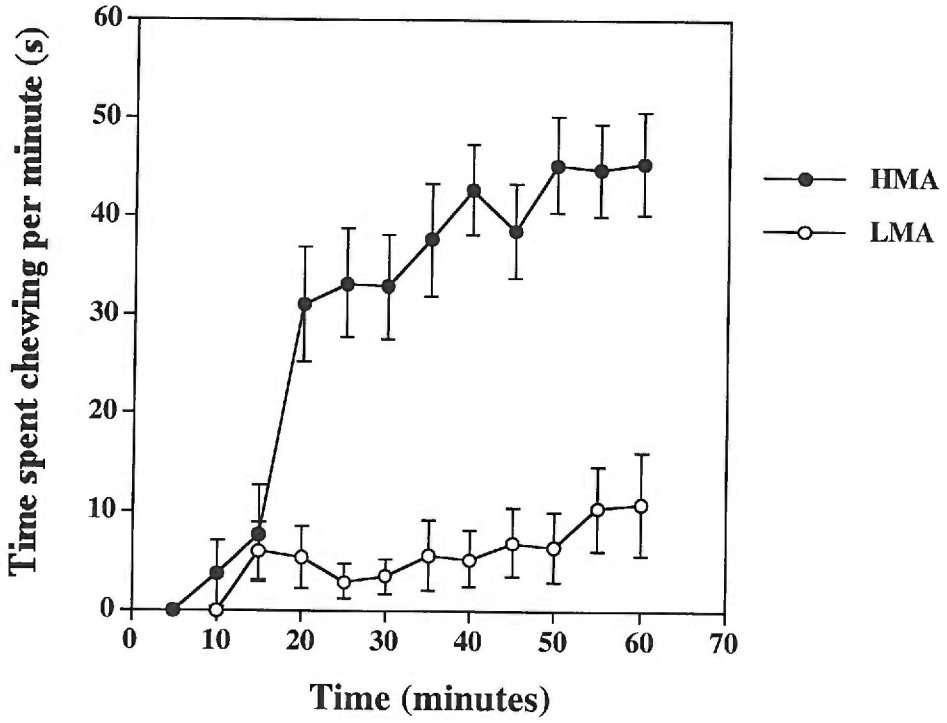

Figure 2. Mean (± s.e.m.) time spent chewing per minute after 10 mg/kg i.p. methamphetamine in HMA and LMA mice of the fourth selected generation.

For number of circling episodes, there were significant effects of genotype [F (1, 22)=16.1, p= .001], time point [F (11, 242)=4.6, p< .001], and genotype x time point [F (11, 242)=3.5, p< .001] (Figure 3). LMA mice had more circling episodes than HMA mice. These differences reached statistical significance at 15 minutes post-injection, after which point LMA mice reached near-maximum scores and HMA mice scored near zero [15 minutes F (1, 22)=5.9, p= .02; 20 minutes F (1, 22)=15.2, p= .001; 25 minutes F (1,

22)=10.9, p= .003; 30 minutes F (1, 22)=22.5, p< .001; 35 minutes F (1, 22)=21.4, p< .001; 40 minutes F (1, 22)=17.1, p< .001; 45 minutes F (1, 22)=7.5, p= .01; 50 minutes F (1, 22)=9.5, p= .006; 55 minutes F (1, 22)=11.9, p= .002; 60 minutes F (1, 22)=16.5, p= .001]. For LMA mice, number of circling episodes remained at about three per minute throughout the entire testing period. For HMA mice, number of circling episodes started out as comparable to LMA mice, but dropped sharply to near zero by 15 minutes post-injection, and remained near zero for most of the remaining test period.

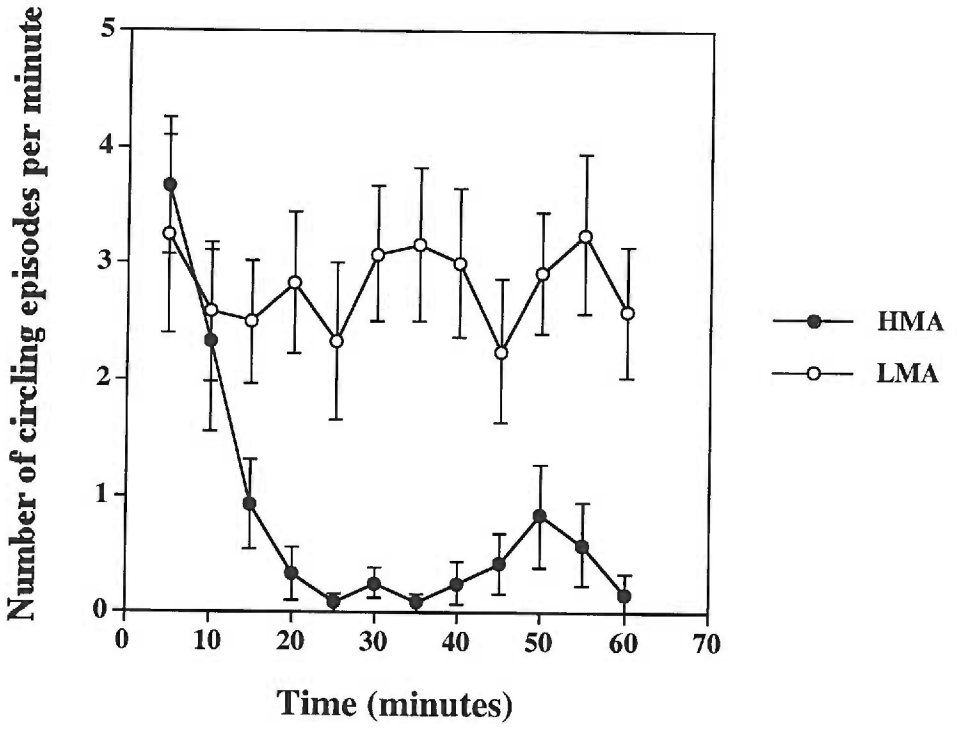

Figure 3. Mean (± s.e.m.) number of circling episodes per minute after 10 mg/kg i.p. methamphetamine in HMA and LMA mice of the fourth selected generation.

For time spent circling, there was a significant effect of genotype [F (1, 22)=12.4, p< .005] (Figure 4). LMA mice spent more time circling than HMA mice over the entire 60 minutes, but this did not vary significantly by time point. The pattern was similar to

that for number of circling episodes, but the high amount of variation between individual mice resulted in a non-significant line x time point interaction.

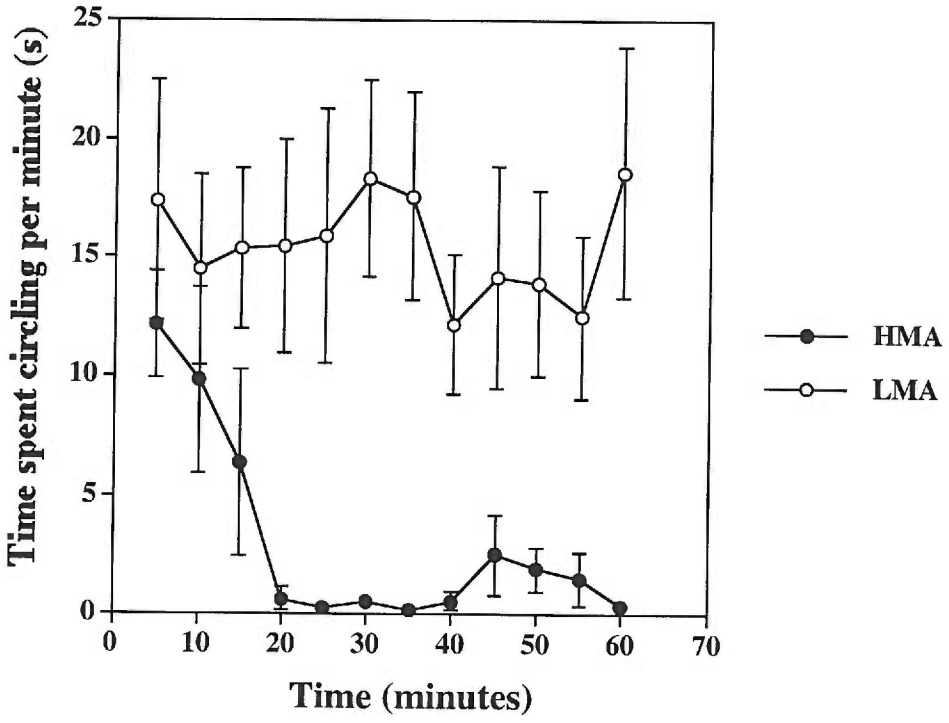

Figure 4. Mean (± s.e.m.) time spent circling per minute after 10 mg/kg i.p. methamphetamine in HMA and LMA mice of the fourth selected generation.

Experiment 2, MA-induced behaviors in the absence of cage bedding. For mice with cage bedding available, chewing was the primary behavior for both B6 and D2 mice, with all ten D2 mice chewing and six B6 mice exhibiting chewing as the primary behavior (Figures 5 and 7). Primary behaviors for the rest of the B6 mice with cage bedding included two that were sniffing, one that was nose poking in the hole in the cage available for drinking water, and one that was licking the cage wall (Figure 5). For mice without cage bedding, the most common behavior was licking the cage floor, with five B6 mice and six D2 mice exhibiting this as the primary behavior (Figures 6 and 8). Of

the remaining B6 mice, two were sniffing as the primary behavior and three were licking the cage wall (Figure 11). Of the remaining D2 mice, three were sniffing as the primary behavior and two were attempting to bite the cage wall (Figure 8).

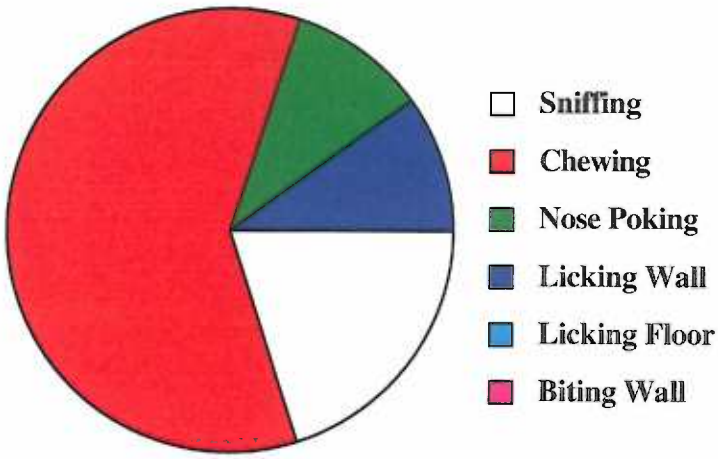

Figure 5. Primary behaviors exhibited by B6 mice (n=10) with cob cage bedding 33 minutes after 10 mg/kg MA.

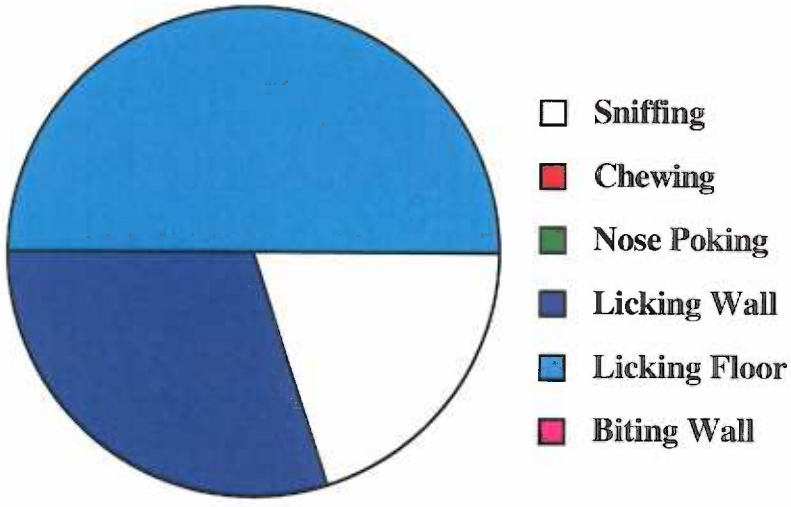

Figure 6. Primary behaviors exhibited by B6 mice (n=10) without cob cage bedding 33 minutes after 10 mg/kg MA.

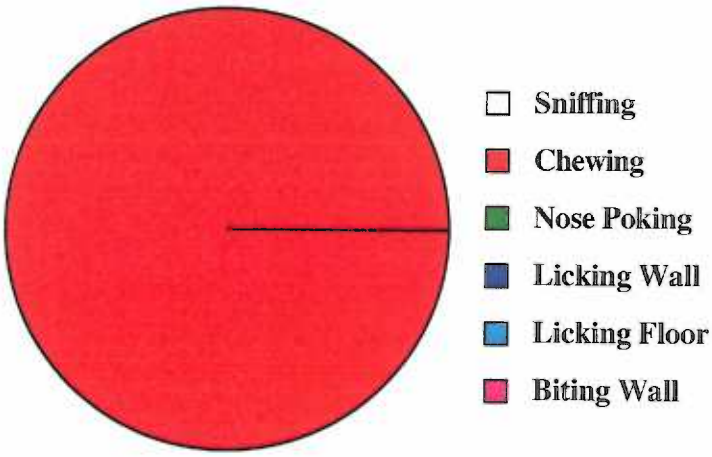

Figure 7. Primary behaviors exhibited by D2 mice (n=10) with cob cage bedding 33 minutes after 10 mg/kg MA.

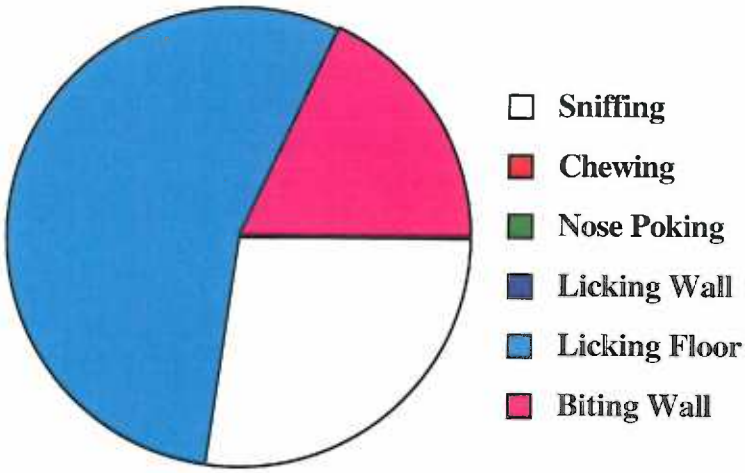

Figure 8. Primary behaviors exhibited by D2 mice (n=10) without cob cage bedding 33 minutes after 10 mg/kg MA.

Experiment 3, QTL analysis of MA-induced chewing stereotypy. Data from the S3 and S4 mice were combined, since both showed significant effect of genotype on number of chewing episodes [S3, $F(1, 45)=200.2$, $p< .001$; S4, $F(1, 121)=14.8$, $p< .001$]. From the combined analysis of BXD recombinant inbred, B6D2F_2, and selected line mice, the most promising regions for QTLs were proximal chromosome 4, mid chromosome 5, and distal chromosome 9 (Table 1). At the chromosome 4 QTL, HMA mice had higher chewing scores than LMA mice across genotypes, and the effect was greatest for animals with B6B6 and heterozygous genotypes (Figure 9). The D2D2 genotype at the nearest marker was associated with higher circling in both HMA and LMA mice, and LMA mice with B6B6 and heterozygous genotypes had higher circling scores than HMA mice with these genotypes (Figure 9). At the chromosome 5 QTL, HMA mice had higher chewing scores than LMA mice across genotypes, and LMA mice did not exhibit much chewing regardless of genotype (Figure 10). LMA mice of B6B6 and D2D2 genotypes had higher circling scores than HMA mice with these genotypes, and heterozygous mice of both lines had similar circling scores (Figure 10). At the chromosome 9 QTL, HMA mice had higher chewing scores than LMA mice across genotypes, and the difference between lines was greatest for mice with B6B6 and heterozygous genotypes (Figure 11). LMA mice had higher circling scores than HMA mice of B6B6 and D2D2 genotypes (Figure 11).

Chromosome	Map Location (cM)	Combined LOD	Combined p Value
4	21.9-22.5	2.25	.006
5	44-45	1.97	.01
9	71-74	3.09	.0008

Table 1. QTLs of $p\leq .01$ in the combined BXD recombinant inbred, B6D2F_2 and selected line populations.

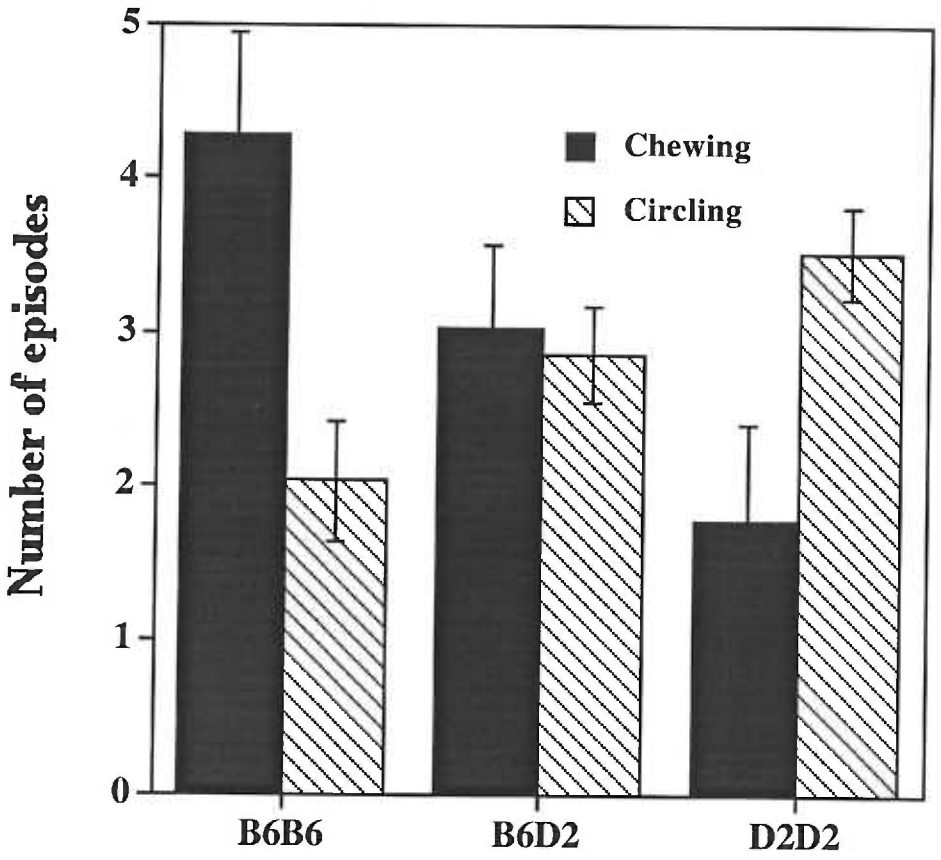

Figure 9. Mean (± s.e.m.) number of chewing and circling episodes at 33 minutes after 10 mg/kg i.p. MA by genotype at the marker D4Mit111 in selected line mice of the S3 and S4.

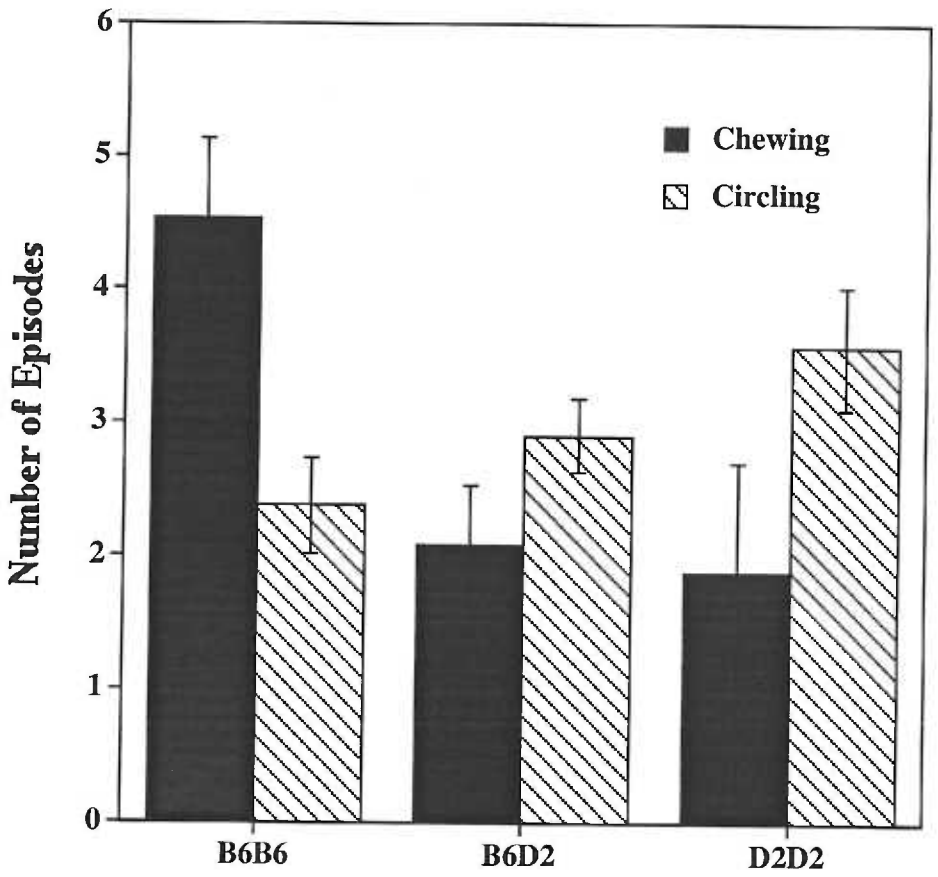

Figure 10. Mean (± s.e.m.) number of chewing and circling episodes at 33 minutes after i.p. MA by genotype at the marker D5Mit197 in selected line mice of the S3 and S4.

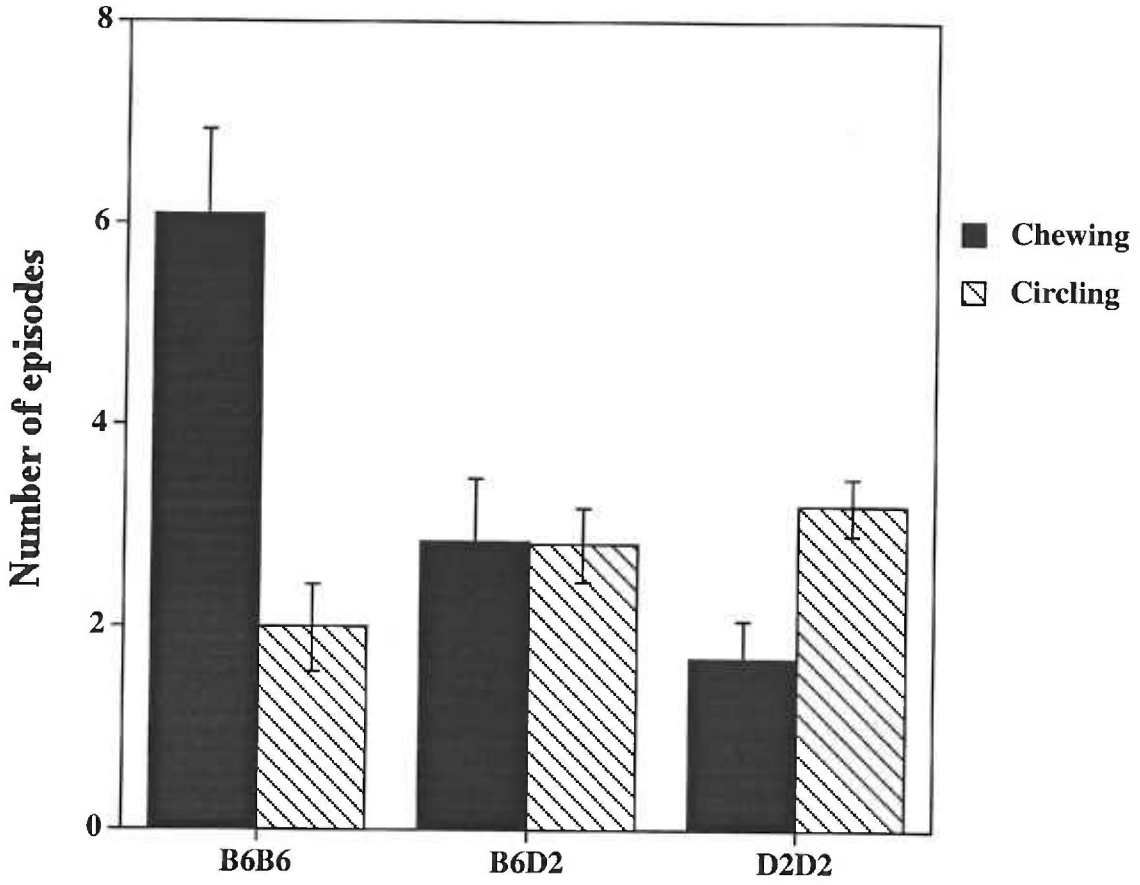

Figure 11. Mean (± s.e.m.) number of chewing and circling episodes at 33 minutes after i.p. MA by genotype at the marker D9Mit18 in selected line mice of the S3 and S4.

Provisional QTLs in BXD mice on distal chromosome 2, proximal chromosome 14, and mid chromosome 17 were not pursued further because results for B6D2F_2 mice were not supportive; the trend (n.s.) was in the opposite direction (Table 2).

Chromosome, Map Location (cM)	BXD Allele Associated with High Chewing	F_2 Allele Associated with High Chewing	Selected Lines Allele Associated with High Chewing	Combined LOD	Combined p Value
2, 45	B6	B6	D2	1.75	.02
2, 69-71	D2	B6	Not measured	1.10	.08
5, 54	B6	B6	D2	1.61	.02
8, 10-12	B6	B6	D2	1.37	.04
8, 14-15	B6	B6	D2	2.79	.002
14, 16.8	D2	B6	Not measured	1.73	.02
17, 15-16	B6	D2	D2	1.49	.03
17, 24.5	B6	D2	Not measured	1.59	.03
17, 32.3-34.3	B6	D2	B6	1.38	.04
17, 47.4-55.7	B6	D2	B6	1.80	.02
X, 38.3-43.2	D2	B6	D2	3.04	.0009

Table 2. Provisional QTLs that were not pursued further because the direction of the QTL was not consistent in all three mapping populations. Some markers were not analyzed in the selected line population because the direction of the QTL did not match in the BXD recombinant inbred and B6D2F_2 populations.

In a number of cases, when there appeared to be a provisional QTL in two of the three mapping populations analyzed, the direction of the QTL was the opposite for the third population (Table 2). For example, if the B6 genotype at a particular marker was associated with high chewing in the BXD recombinant inbred and B6D2F_2 populations, but the D2 genotype at the same marker was associated with high chewing in the selected line population, this would suggest that a true QTL does not exist in this region. The same genotype would be associated with high scores on the trait in all populations tested if a true QTL exists in the region. None of the provisional QTLs for which this was the case was pursued. Further analysis focused on those provisional QTLs that were in the same direction for all three mapping populations.

Circling stereotypy was identified as an alternative stereotypic behavior induced by MA in HMA and LMA mice (Atkins et al., 2001). For the majority of the markers analyzed, the direction of the provisional QTL for circling stereotypy was in the opposite direction to that for chewing stereotypy, as expected (Table 3). These analyses were only conducted in the selected lines because this was the only population in which circling stereotypy was measured.

Chromosome	Map Location (cM)	Selected Lines LOD, Chewing	Selected Lines p Value, Chewing	Selected Lines LOD, Circling	Selected Lines p Value, Circling	Allele Associated with High Chewing and Low Circling
2	45	.46	.35	.44	.37	D2
4	14.5	.45	.35	.49	.33	B6
4	21.9	.63	.24	.27	.57	B6
5	5	1.10	.08	1.09	.08	B6
5	24	1.45	.04	1.67	.02	B6
5	36	1.39	.04	1.41	.04	B6
5	54	.43	.37	.4	.40	D2
5	65	.45	.35	.47	.34	B6
5	84	1.13	.07	1.28	.05	D2
8	10	.48	.33	.44	.36	D2
8	15	.52	.30	.47	.34	D2
8	33	.57	.27	.58	.26	D2
8	59	.67	.21	.69	.20	B6
8	73	.53	.30	.57	.27	B6
9	15	1.17	.07	1.45	.04	D2
9	31	.80	.16	.70	.20	D2
9	50	.42	.38	.43	.37	B6
9	55	.46	.34	.41	.39	B6
9	61	.60	.25	.64	.23	B6
9	71	1.65	.02	1.74	.02	B6
17	16	.98	.10	1.16	.07	D2
17	34.3	.50	.32	.46	.35	B6
17	55.7	.89	.13	1.34	.05	B6
X	43.2	.37	.42	.48	.33	D2

Table 3. QTLs for which the direction of the QTL for chewing stereotypy was opposite to the direction of the QTL for circling stereotypy in the selected mouse line population.

From DNA pooling in the selected lines, three additional provisional QTLs were identified on mid chromosome 6, mid chromosome 15, and mid chromosome 16. Individual genotyping was then carried out for these markers in the B6D2F_2 and selected line populations. For the chromosome 6 QTL, results were in the opposite direction in the two populations (46.3 cM, combined LOD= .21, p= .62). The same was true for the chromosome 16 QTL (38 cM, combined LOD=1.79, p= .02). The QTL on chromosome 15 was consistent in the direction of effect, but was not as promising as the others found in the three-population combined analysis (29.2 cM, combined LOD=1.47, p= .03).

Provisional QTL interactions in the B6D2F_2 followed up using a MathCad spreadsheet designed by John Belknap based on Cheverud and Routman's method included QTLs on chromosomes 4 and 5, chromosomes 5 and 9, and chromosomes 8 and 9 (Table 4). Similarly, Provisional QTL interactions were identified by two-way ANOVA between QTLs on chromosomes 4 and 5, chromosomes 5 and 9, and chromosomes 8 and 9 (Table 5).

QTL 1 (Chr., Map Location)	**QTL 2 (Chr., Map Location**	**F Epistasis**	**Additive x Additive p Value**	**Additive x Dominance p Value**	**Dominance x Additive p Value**	**Dominance x Dominance p Value**
4, 14.5	5, 39	4.81	.83	.002	.03	.06
5, 39	9, 61	5.17	.78	.31	.006	.0008
8, 14	9, 61	6.44	.10	.005	.03	.002
8, 22.5	9, 61	5.99	.07	.001	.03	.03

Table 4. QTL interactions of $p \leq .01$ in the B6D2F_2 identified using the method of Cheverud and Routman (1995) for analyzing an F_2 for epistasis between pairs of loci.

QTL 1 (Chromosome, Map Location in cM)	QTL 2 (Chromosome, Map Location in cM)	F Value	p Value
4, 14.5	5, 36	3.7	.007
4, 14.5	5, 39	4.0	.004
5, 36	9, 61	3.2	.01
5, 39	9, 61	3.6	.007
8, 14	9, 61	4.9	.001
8, 22.5	9, 61	4.8	.001

Table 5. QTL interactions of $p \leq .01$ in $B6D2F_2$ mice as identified using two-way ANOVA.

In BXD recombinant inbred strains, provisional QTLs were identified using the Pseudomarker program on chromosomes 1, 2, 4, 5, 14, and 17 (Table 6, Figure 12). A number of significant QTL interactions were also identified, and these are summarized below (Table 7, figure 13). This figure represents a full-genome search using all pairs of markers analyzed for interactions. The lower triangle shows the full model, which includes the main effects of both markers plus their interaction, and the upper triangle shows just the interactions. Regions associated with behavioral score at a p value of less than .05 are colored progressively toward the red end of the color spectrum as a function of increasing LOD scores.

Chromosome	Map Location	LOD	p Value
1	10	1.44	.01
2	30	1.34	.01
4	15	1.74	.005
5	50	2.15	.002
14	20	1.74	.005
17	25	1.67	.006

Table 6. Provisional QTLs of $p \leq .01$ identified by the Pseudomarker program in BXD recombinant inbred strains.

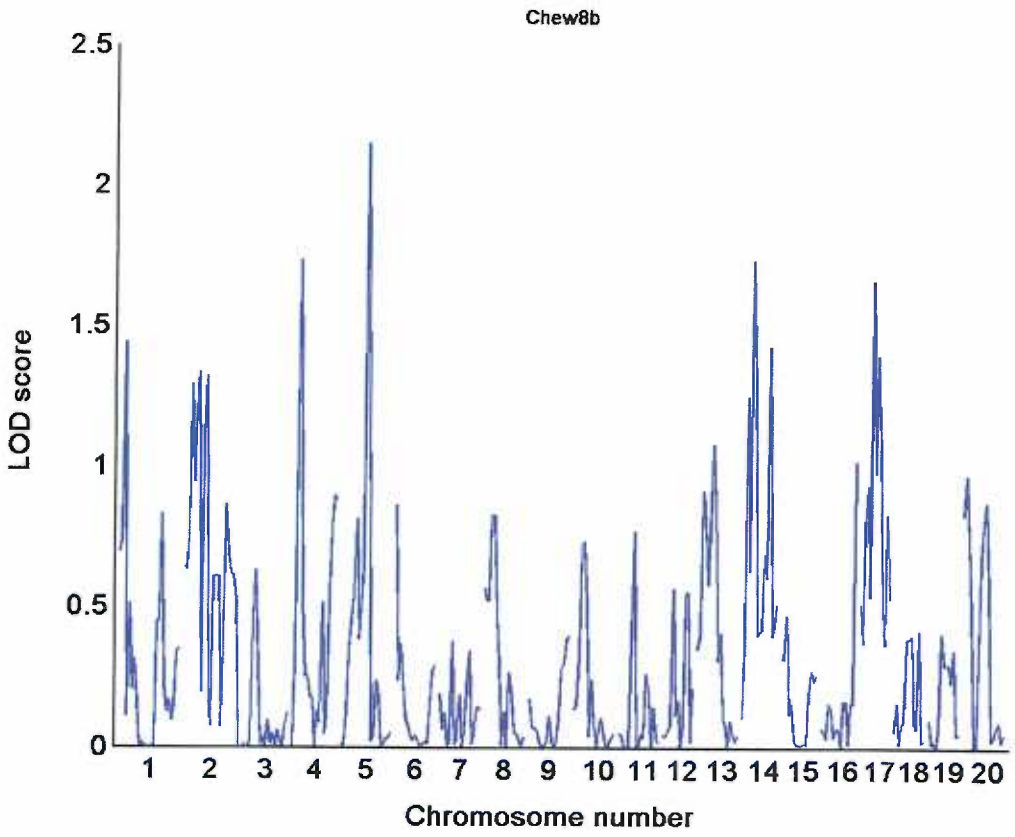

Figure 12. Graphical representation of provisional QTLs identified in the Pseudomarker program in BXD recombinant inbred strain mice. The y-axis represents LOD score and the x-axis represents location. For example, the provisional QTL with the highest QTL score is one at 50 cM on chromosome 5, with a LOD score of 2.15.

QTL 1 (Chromosome, Map Location in cM, LOD)	**QTL 2 (Chromosome, Map Location in cM, LOD)**	**Interaction LOD**	**Full Model LOD**
1, 25, .27	X, 0, .90	7.32	8.42
2, 105, .96	5, 55, 1.69	4.26	6.48
5, 55, 1.62	11, 60, .55	5.63	7.43
5, 50, 2.19	14, 10, 1.29	3.65	7.09
5, 50, 2.61	17, 50, .83	4.05	7.04
5, 50, 2.28	X, 5, 1.10	3.94	7.19
14, 50, .61	X, 5, .97	5.69	7.27
17, 45, .42	X, 5, .90	6.52	7.91

Table 7. QTL interactions of $p \leq .0001$ when $p \leq .001$ for the full model in BXD recombinant inbred mouse strains were identified using the Pseudomarker program.

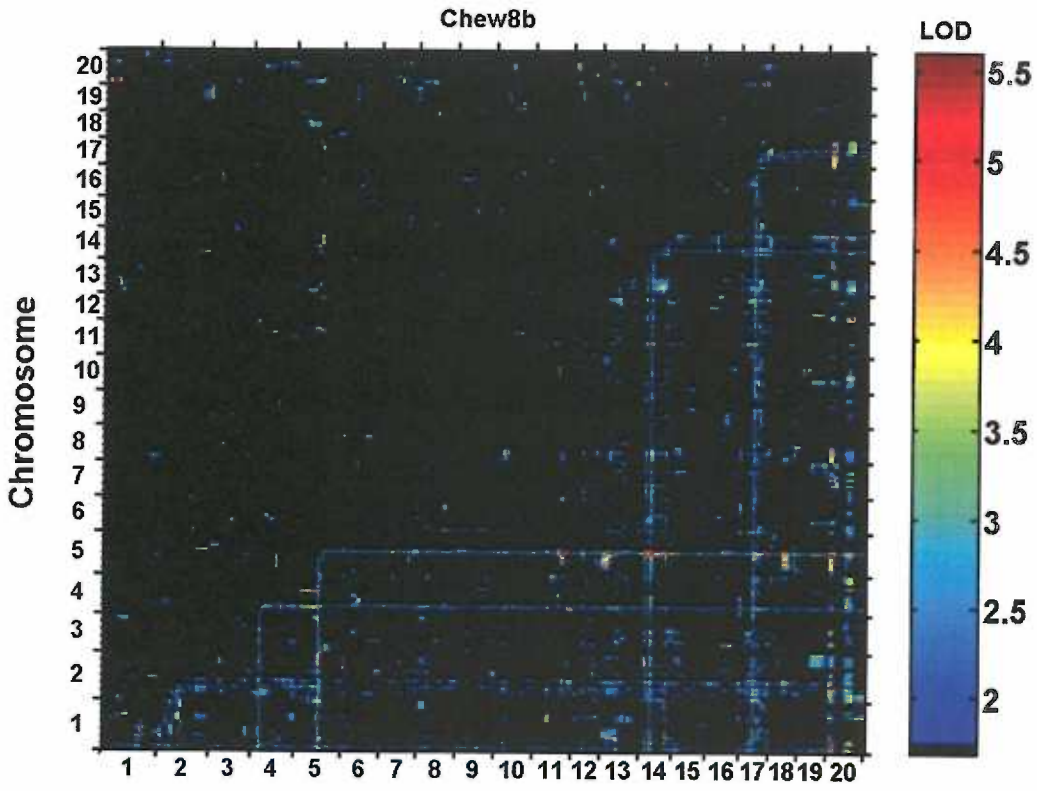

Figure 13. Interactions between pairs of QTLs for chewing stereotypy QTL in BXD recombinant inbred mice analyzed by the Pseudomarker program at a 2.5 cM resolution, meaning that there is one pseudomarker located every 2.5 cM throughout the genome, with genotype information inferred from actual marker data and linkage information. Dividing the square into two triangles from the lower left corner to the upper right corner, the lower triangle shows the full model, which includes the main effects of both markers and their interaction. The upper triangle shows the interactions alone. All black areas are $p > .05$ and the significance level of colored areas is based on the scale to the right of the figure.

Two provisional QTLs were identified in the B6D2F_2 population using the Pseudomarker program, on chromosomes 5 (18 cM, LOD=2.27, p= .005) and 9 (35 cM,

LOD=2.51, p= .003). A QTL interaction was identified between QTLs on chromosomes 8 and 9 (chromosome 8, 22.5 cM, individual LOD= .61 x chromosome 9, 62.5 cM, individual LOD=1.46, interaction LOD=4.13, full model LOD=6.14, full model p= .0008) (Figure 14).

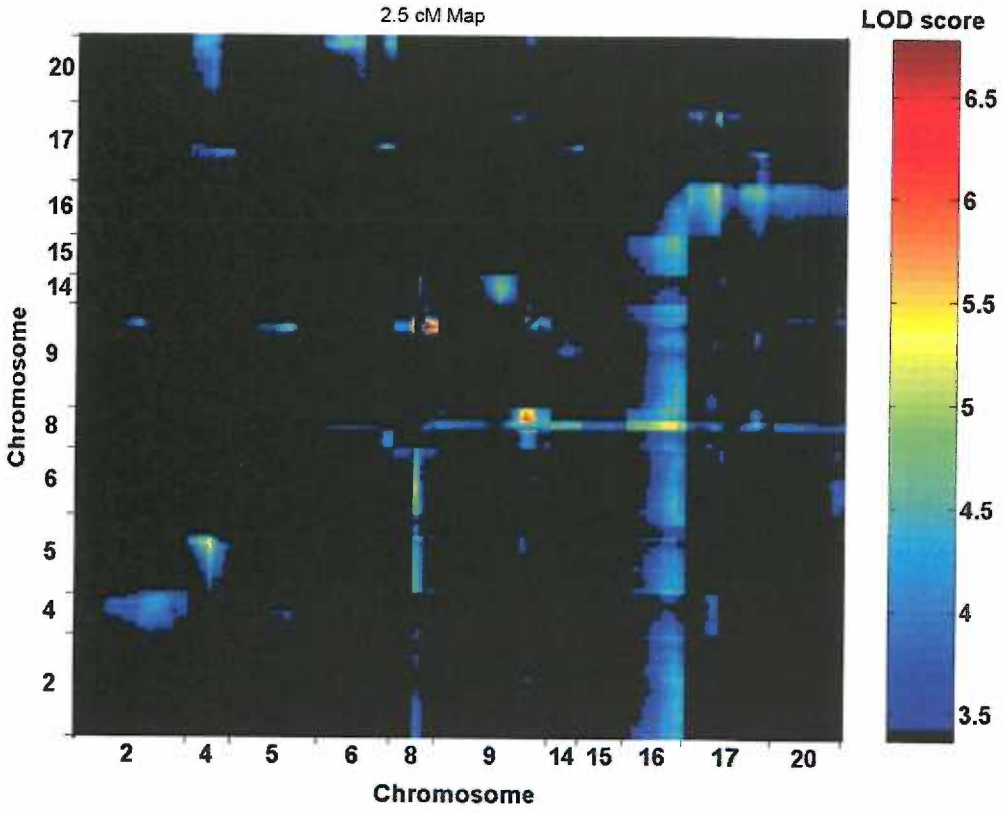

Figure 14. Interactions between pairs of QTLs for chewing stereotypy QTL in B6D2F_2 mice analyzed by the Pseudomarker program at a 2.5 cM resolution, meaning that there is one pseudomarker located every 2.5 cM throughout the genome, with genotype information inferred from actual marker data and linkage information. Dividing the square into two triangles from the lower left corner to the upper right corner, the lower triangle shows the full model, which includes the main effects of both markers and their interaction. The upper triangle shows the interactions alone. All black areas are $p > .05$ and the significance level of colored areas is based on the scale to the right of the figure.

Experiment 4, QTL results in the congenic strains. There was no significant difference between mice heterozygous for a segment from 9-55 cM on chromosome 9 and the B6 background strain for chewing stereotypy [$F(1, 40) = .09$, $p = .77$] (Figures 15

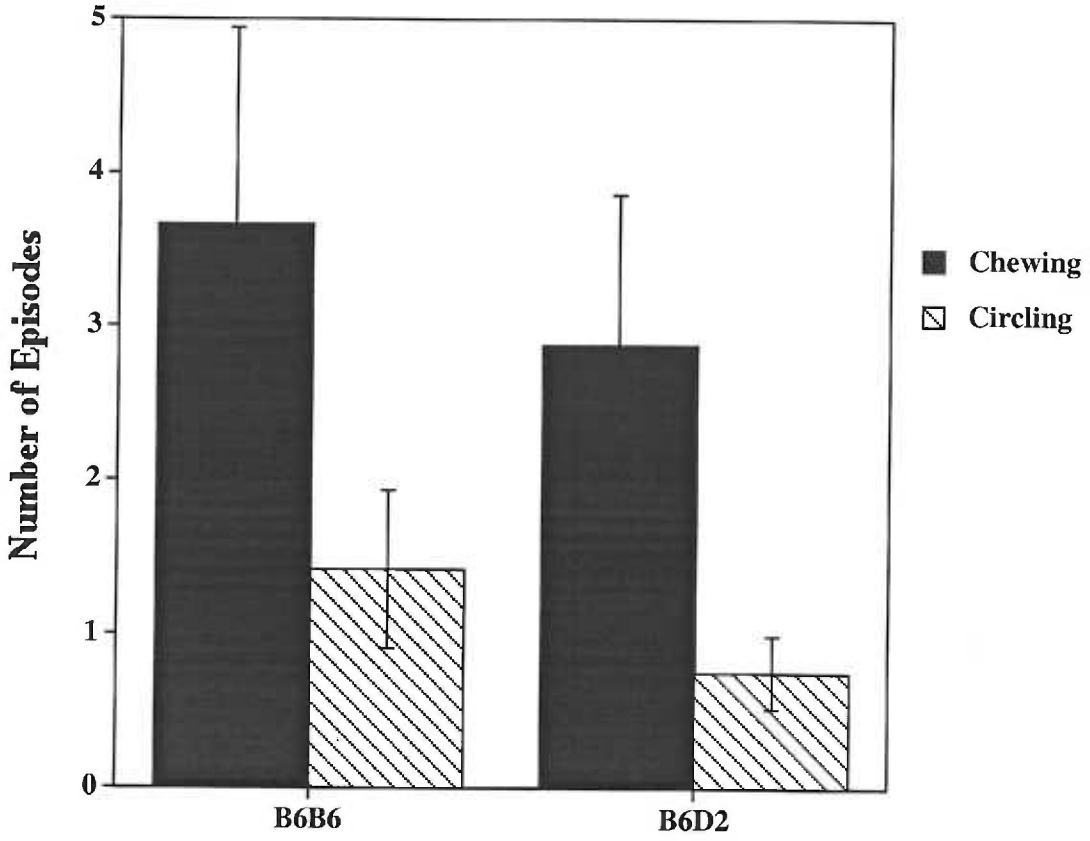

Figure 15. Mean (± s.e.m.) number of chewing and circling episodes per minute at 33 minutes after 10 mg/kg i.p. MA in B6 mice (n=12) and mice with a B6D2 segment from 9-55 cM (n=30) on chromosome 9 on a B6 background.

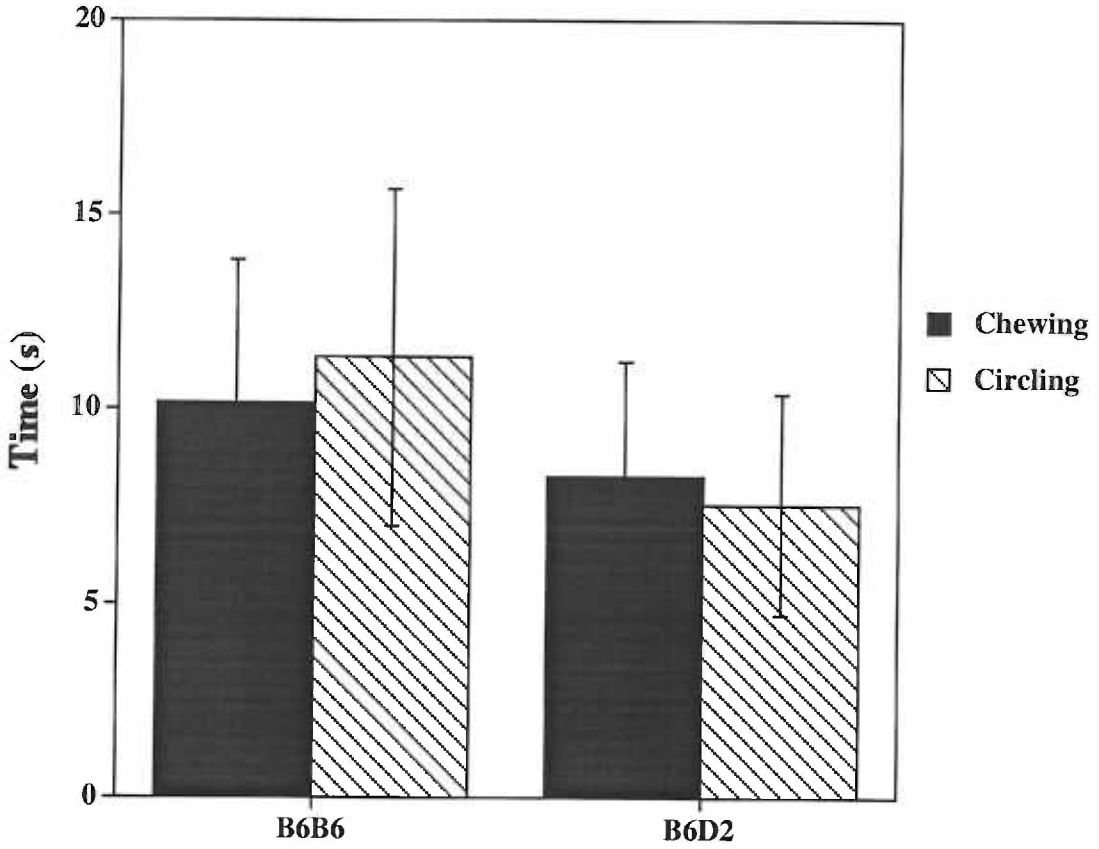

Figure 16. Mean (± s.e.m.) time spent chewing and circling per minute at 33 minutes after 10 mg/kg i.p. MA in B6 mice and mice with a B6D2 segment from 9-55 cM on chromosome 9 on a B6 background.

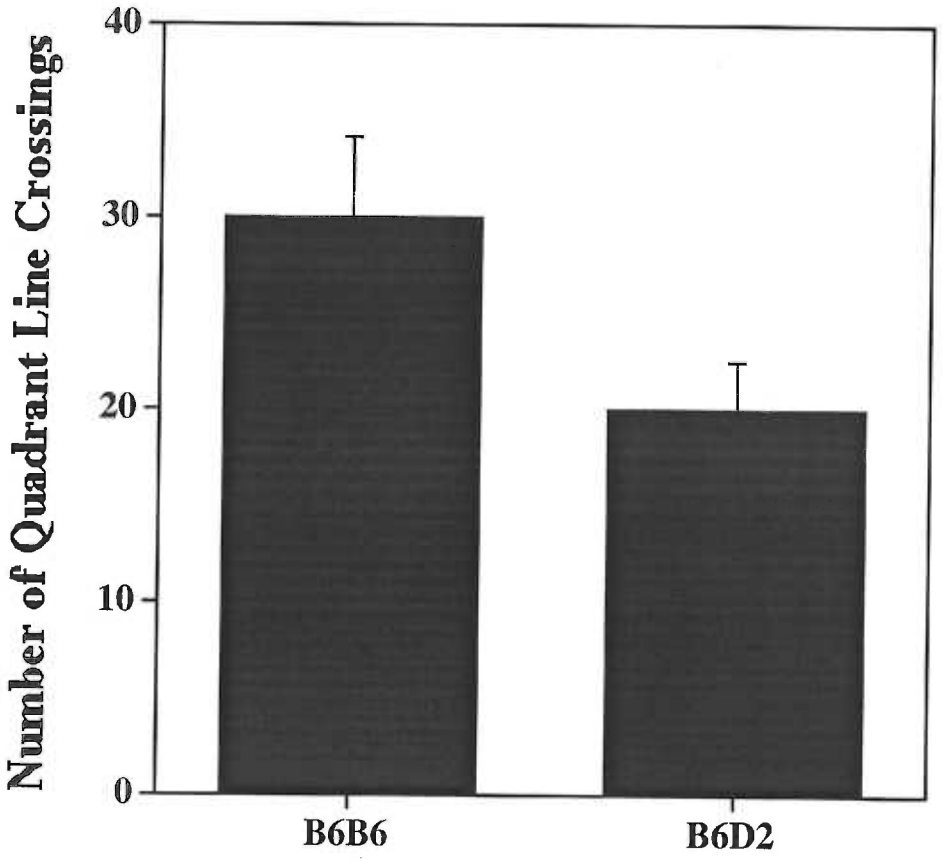

Figure 17. Mean (± s.e.m.) number of quadrant line crossings per minute at 17 minutes after 10 mg/kg MA in B6 mice and mice with a B6D2 segment from 9-55 cM on chromosome 9 on a B6 background.

The data from the third, fourth, fifth, and sixth backcross generations were combined (n=174). The association between number of stereotypic chewing episodes and genotype at the 71 cM marker on chromosome 9 was not statistically significant [F (1, 172)= .01, p= .93] (Figure 18). In this population, the distal chromosome 9 QTL was not statistically significant [LOD= .682, p= .21]. Combining results from the BXD recombinant inbred, B6D2F2, selected line, and distal chromosome 9 congenic populations, the distal chromosome 9 QTL was less significant than it had been in the

first three populations [LOD= 2.99, p= .001]. The chromosome 9 QTL peak LOD score was at 26 cM [LOD=1.46, p= .009]. The association between number of stereotypic chewing episodes and genotype at the 71 cM marker on chromosome 9 was stronger in the third backcross [F (1, 43)= 9.4, p= .004] than in the fourth [F (1, 24)= 1.4, p= .25], fifth [F (1, 48)= .61, p= .44], or sixth backcross generations [F (1, 51)= 2.5, p= .12] (Figure 19). Thus, results were most promising in the third backcross generation, and less so in later generations. Because we could not reliably detect this QTL in the congenic, we decided that the pursuit of higher resolution mapping for this QTL using the congenic strain was not warranted.

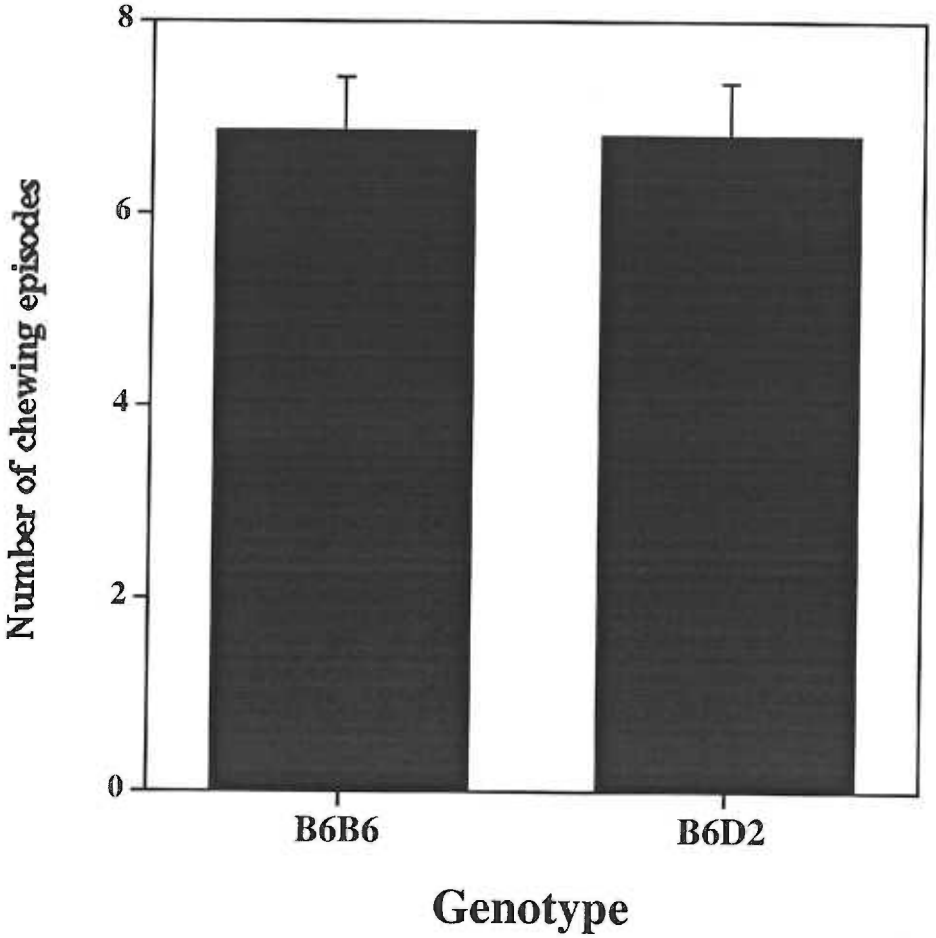

Figure 18. Mean (± s.e.m.) number of stereotypic chewing episodes in one minute at 33 minutes after 10 mg/kg MA i.p. as a function of genotype at D9Mit18.

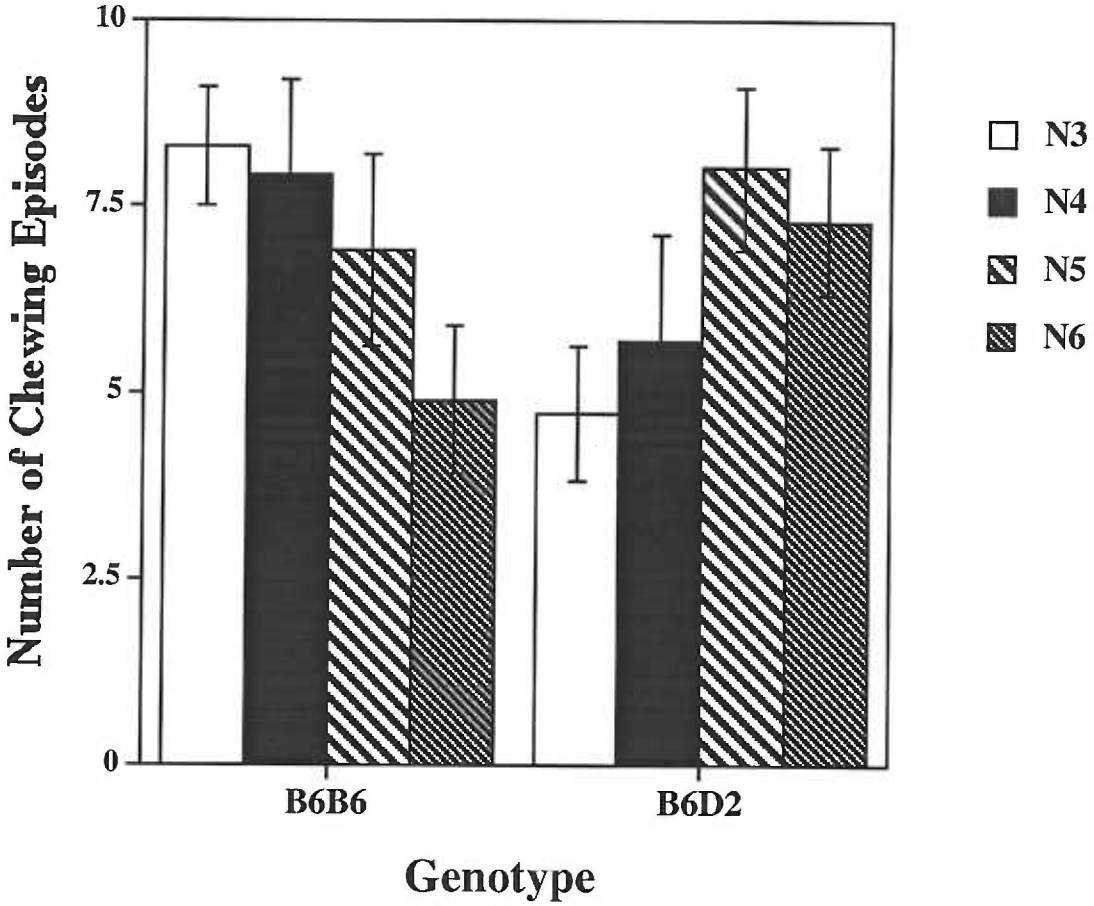

Figure 19. Mean (± s.e.m.) number of stereotypic chewing episodes in one minute at 33 minutes after 10 mg/kg MA i.p. as a function of genotype at D9Mit18 by generation in distal chromosome 9 congenic mice.

Experiment 5, MA preference drinking. Mice with the B6D2 segment from 15-71 cM on chromosome 9 did not differ significantly from the B6 background strain in total water intake over all ten days of the study [F (1, 52)= .09, p= .76] or in total fluid intake over all ten days of the study [F (1, 52)= .7, p= .38]. Mice with the B6D2 segment from 15-71 cM on chromosome 9 did not differ significantly from the B6 background strain on preference for 5 mg/L MA [F (1, 52)= .006, p= .94], 10 mg/L MA [F (1, 52)=2.7, p= .11], or 20 mg/L MA [F (1, 52)= .02, p= .88] (Figure 20). MA preference was higher in mice heterozygous for this segment, particularly for the 10 mg/L concentration, but the groups

were not significantly different (Figure 20). Number of chewing episodes was not significantly correlated with preference for 5 mg/L MA ($r = .10$, $p = .47$), 10 mg/L MA ($r = .11$, $p = .42$), or 20 mg/L MA ($r = .06$, $p = .66$) (Figure 21).

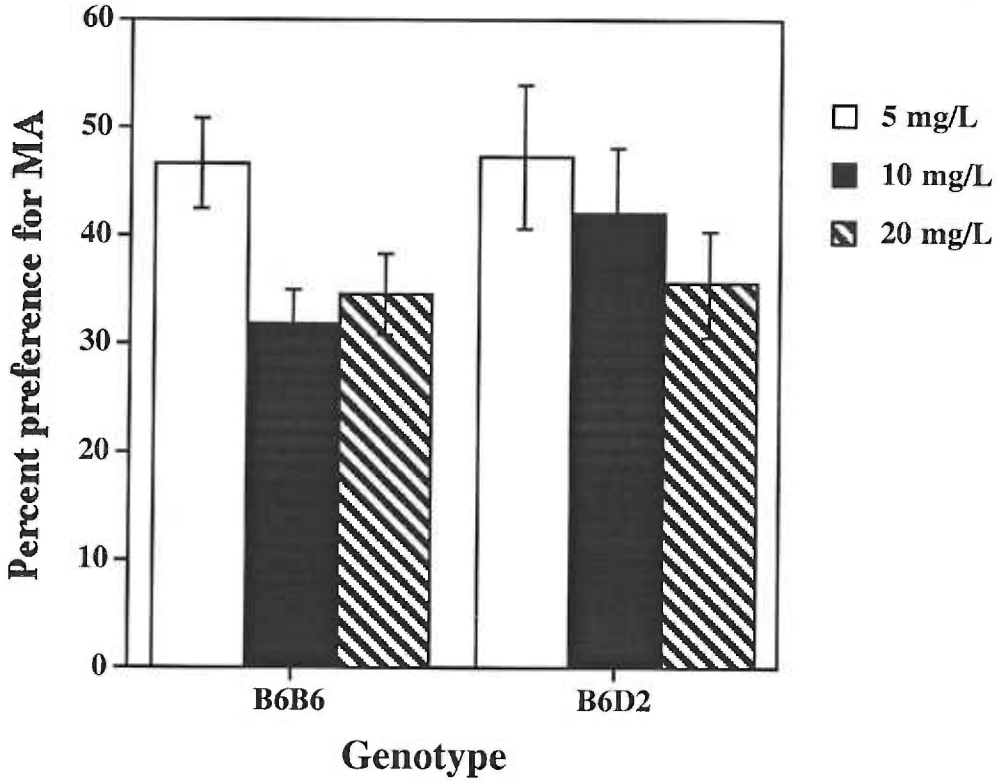

Figure 20. Mean (± S.E.M.) preference for MA vs. water in a two-bottle choice test in mice heterozygous for a congenic segment from 15-71 cM on chromosome 9 and the B6 background strain from the third to fifth backcross generations.

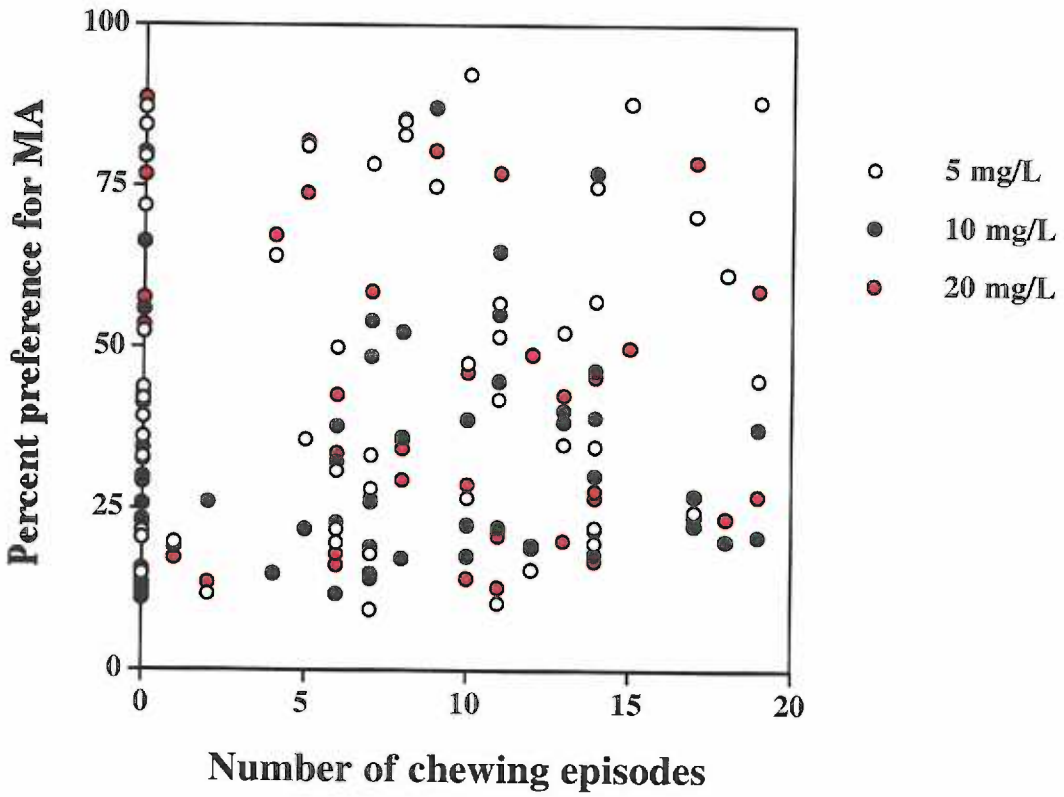

Figure 21. Individual scores for number of chewing episodes and preference for 5, 10, and 20 mg/L MA in mice heterozygous for a congenic segment from 15-71 cM on chromosome 9 and B6 background strain of the third to fifth backcross generations.

Mice with the B6D2 segment from 15-71 cM on chromosome 9 did not differ significantly from the B6 background strain on amount consumed per day of 5 mg/L MA [F (1, 52)= .80, p= .36], 10 mg/L MA [F (1, 52)=1.6, p= .21], or 20 mg/L MA [F (1, 52)= .10, p= .77] (Figure 22). Amount of MA consumed was slightly higher in B6 mice at the 5 and 20 mg/L concentrations, and slightly higher for heterozygous mice at the 10 mg/L concentration, but the groups were not significantly different. Number of chewing episodes was not significantly correlated with amount of MA consumed for 5 mg/L MA (r= .0001, p= .99), 10 mg/L MA (r= .04, p= .78), or 20 mg/L MA (r= .07, p= .60) (Figure 23).

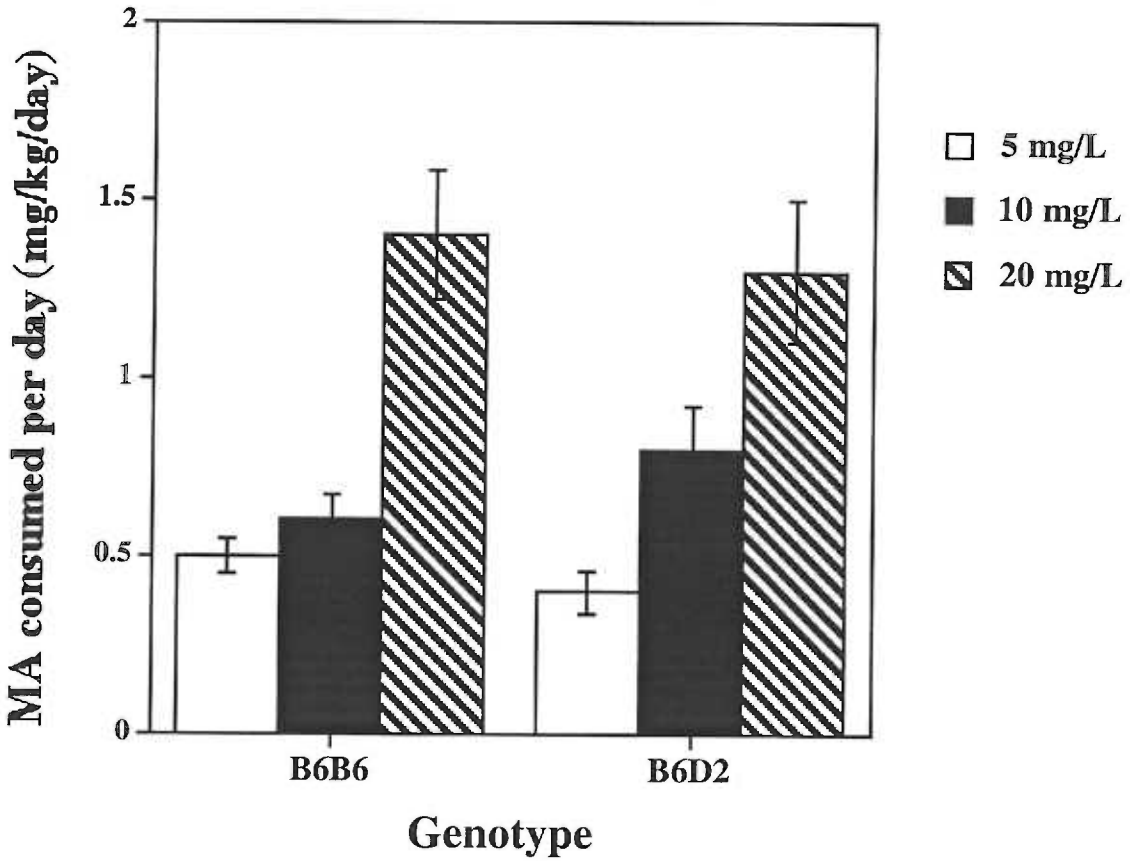

Figure 22. Mean (± s.e.m.) MA consumption per day in a two-bottle choice test in mice heterozygous for a congenic segment from 15-71 cM on chromosome 9 and the B6 background strain from the third to fifth backcross generations.

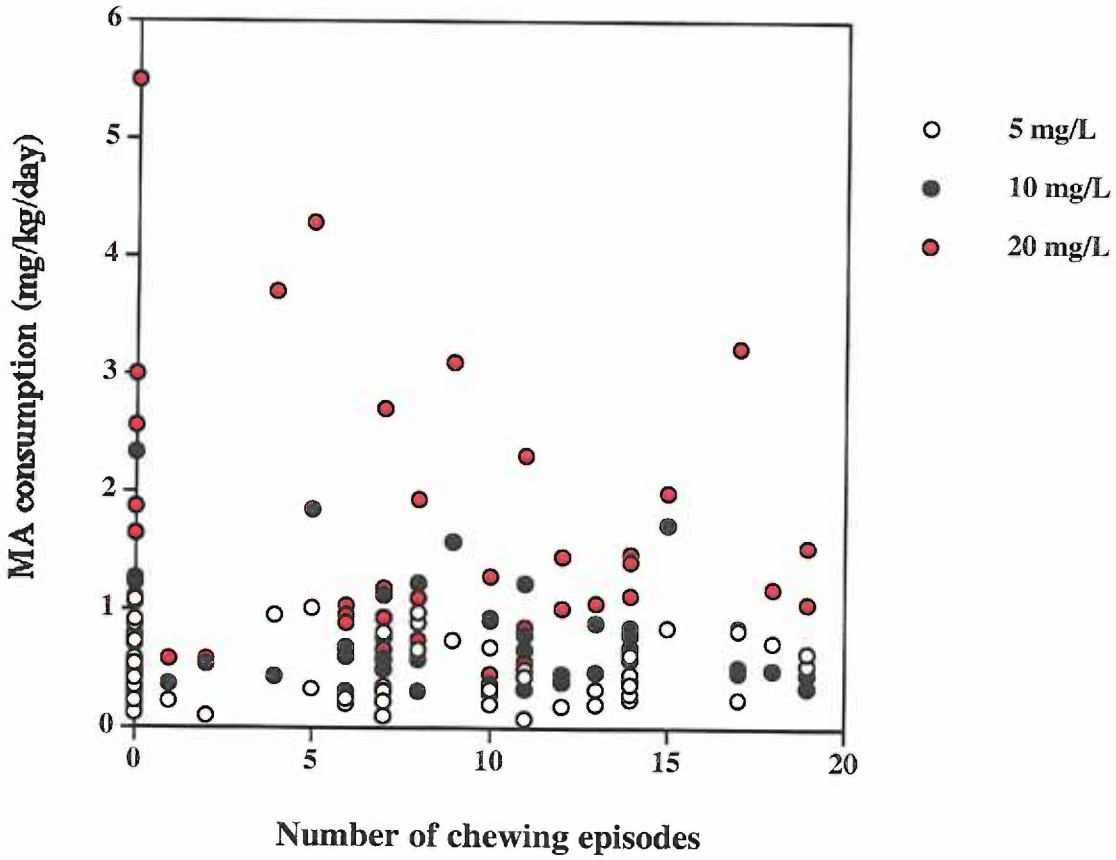

Figure 23. Individual scores for amount of MA consumed per day (mg/kg/day) of 5, 10, and 20 mg/L MA in mice heterozygous for a congenic segment from 15-71 cM on chromosome 9 and B6 background strain of the third to fifth backcross generations.

To support these results, data from separate groups of BXD recombinant inbred mice previously tested in the Belknap laboratory on MA preference drinking and MA-induced stereotypy were analyzed to examine relationships between these behaviors. In the BXD recombinant inbred strains, we found that MA-induced stereotypy following 8 mg/kg MA was not significantly correlated with preference for 20 mg/L MA (r= .06, p=.8) or 40 mg/L MA (r= -.11, p= .6).

Experiment 6, microarray analysis of baseline gene expression. The graph plotting power variables for MAS 5.0, RMA, dChip, and PDNN against rank order of

transcript t test values revealed that PDNN was the comparison with the most power to detect gene expression differences between the two strains (Figure 22). For this reason, only the PDNN data set was used for subsequent analyses.

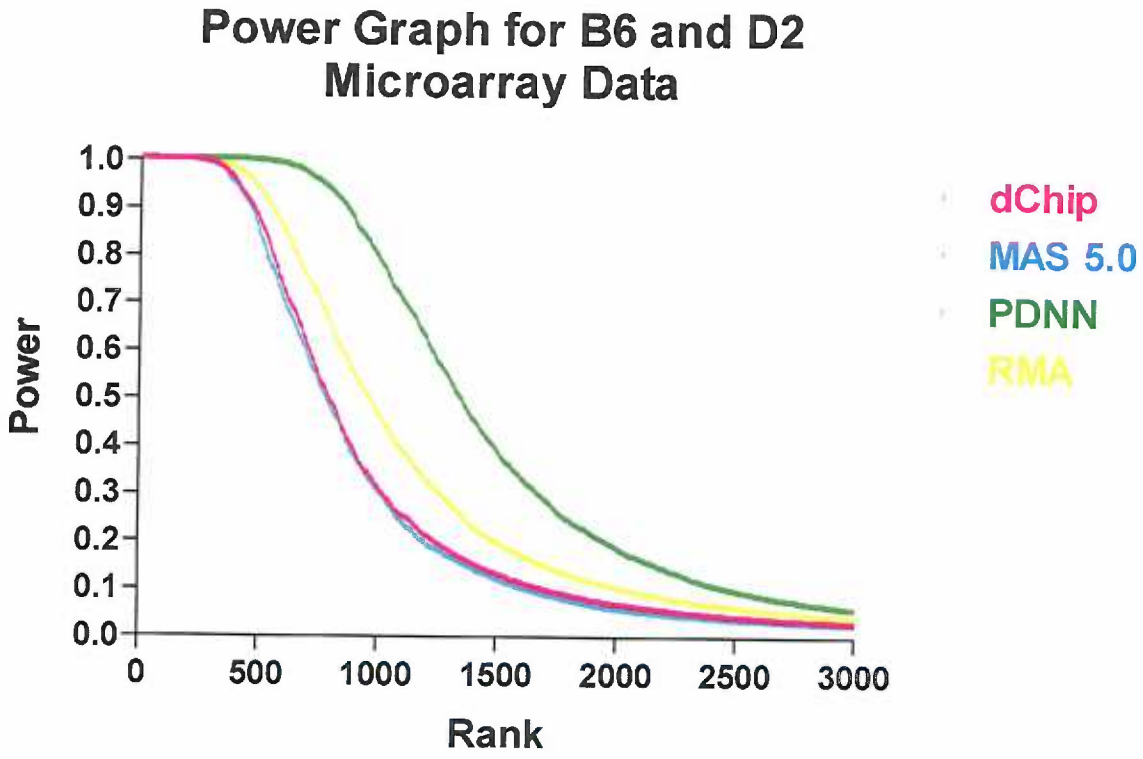

Figure 24. Power of each of the four normalization methods as a function of rank order for microarray data comparing baseline gene expression between B6 and D2 mice.

There were 52 transcripts on chromosome 9 that significantly differed between strains at $q < .01$. Of these, 32 transcripts were determined using R/qtl to have significant expression QTLs in the F_2. In the B6D2F_2, the D2 allele is dominant at the QTL for MA-induced chewing stereotypy ($p < .03$). Dominance was calculated for each of the 32 transcripts on chromosome 9 to have expression QTLs in this region, and only those with

significant D2 dominance were selected, which narrowed the list down to six transcripts (Table 8). The marker for the QTL, D9Mit18, is located at 71 cM and 129.7 megabases (Mb). Three of the transcripts, Tpbg, Zic1, and Mras, mapped to the distal end of chromosome 9, though not as distal as the QTL region. The cM location of the gene was only known in one case, for Zic1, which is located at 61 cM.

Gene Symbol	**Gene Name**	**Gene cM**	**Peak LOD (cM) for Expression QTL**
9030425E11Rik	RIKEN cDNA 9030425E11	21	15
Sorl1	Sortilin-related receptor, LDLR class A repeats-containing	24	50
Arpp19	cAMP-regulated phosphoprotein 19	Unknown	42
Tpbg	Trophoblast glycoprotein	48	46.5
Mras	Muscle and microspikes RAS	54	50
Zic1	Zinc finger protein of the cerebellum 1	61	42

Table 8. Differentially expressed transcripts on chromosome 9 with significant expression QTLs with D2 dominance in a B6D2F_2 population.

Due to the likely interaction between QTLs for MA-induced chewing stereotypy on chromosome 8 and chromosome 9, microarray data from chromosome 8 were also analyzed. There were 44 transcripts on chromosome 8 that significantly differed between strains at $q < .01$. Of these, 32 transcripts were determined using R/qtl to have significant expression QTLs. In the B6D2F_2, the putative QTL at D8Mit4 for MA-induced chewing

stereotypy is additive. Dominance was calculated for each of the 32 transcripts on chromosome 8 to have expression QTLs, and only those with no significant dominance were selected, which narrowed the list down to 15 transcripts (Table 9). The marker for the QTL, D8Mit4, is located at 14 cM and 31 Mb. Five of the transcripts, Plat, 2410018G23Rik, BC019943, Gsr, and 3110010F15Rik, mapped near the region of the behavioral QTL.

Gene Symbol	Gene Name	Gene cM	Peak LOD (cM) for Expression QTL
2410018G23Rik	RIKEN cDNA 2410018G23 gene	8	17
Plat	Plasminogen activator, tissue	9	12
BC019943	cDNA sequence BC019943	13	12
Gsr	Glutathione reductase 1	14	7
3110010F15Rik	RIKEN cDNA 3110010F15 gene	18	7
2510049I19Rik	RIKEN cDNA 2510049I19 gene	31	32
Lpl	Lipoprotein lipase	33	27
BC053440	cDNA sequence BC053440	33	22
9130011J15Rik	RIKEN cDNA 9130011J15 gene	Unknown	42
None	None	Unknown	32
None	None	Unknown	27
D8Ertd812e	DNA segment, Chr. 8, ERATO Doi 812, expressed	39	22
Man2b1	Mannosidase 2, alpha B1	37	47
Fts	Fused toes	41	52
Rab4a	RAB4A, member RAS oncogene family	67.5	54

Table 9. Differentially expressed transcripts on chromosome 8 with significant expression QTLs with additive effects in a B6D2F_2 population.

In addition, expression QTLs on chromosome 9 for the chromosome 8 transcripts were analyzed to further examine the interaction between QTLs on chromosomes 8 and

9. Five of the transcripts with additive effects had significant expression QTLs on chromosome 9 (Table 10). In all cases, the location of the gene on chromosome 8 was between 68.9 and 71.8 Mb and the peak LOD score for the expression QTL was at 8 cM on chromosome 9. The chromosome 8 genes are not as proximal as the chromosome 8 behavioral QTL, and the expression QTL on chromosome 9 is proximal rather than distal. This suggests that this interaction does not represent one between QTLs on proximal chromosome 8 and distal chromosome 9. However, it still represents an area where gene expression is significantly different between B6 and D2 mice, and could still be relevant in terms of baseline differences between the strains that underlie their differential responses to MA despite the fact that it does not help to clarify the QTL of interest in this set of experiments.

Gene Symbol	**Gene Name**	**Gene cM**	**Peak LOD (cM) for Expression QTL**
Lpl	Lipoprotein lipase	33	8
9430098E02Rik	RIKEN cDNA 9430098E02 gene	33	8
9130404D08Rik	RIKEN cDNA 9130404D08 gene	33.5	8
Plvap	Plasmalemma vesicle associated protein	33.5	8
Gtpbp3	GTP binding protein 3	33.5	8

Table 10. Differentially expressed transcripts on chromosome 8 with significant expression QTLs on chromosome 9 with additive effects in a B6D2F_2 population.

Discussion

Experiment 1, MA behavior time course. The results of the present study were consistent with those of previous studies in that onset of chewing stereotypy did not begin until about 20 minutes post-injection and remained at peak levels at 60 minutes post-injection. Though there is some variation in previous studies, they generally show that animals do not begin to show significant amounts of stereotypic behavior until at least 20 minutes post-injection, amount of stereotypy peaks around 60 minutes post-injection regardless of dose, and duration of stereotypy is dose-dependent such that duration increases with dose.

There are several important differences between the previous studies and the present study. First, the previous studies were conducted with rats rather than mice. The definition of stereotypic behavior in rodents generally includes repetitive head and forepaw movements, but specific definition varies from study to study, and also differs between species. In most of the previous studies, amphetamine was used rather than MA, and drugs were administered subcutaneously rather than intraperitoneally. Amphetamine and MA have the same mechanism of action but differ in potency, and are generally discussed interchangeably in terms of their effects. However, some studies do show differences between effects of these drugs. For example, rats given chronic amphetamine develop tolerance to working memory impairment on the T-maze and to the reduction in food consumption produced by the drug, whereas mice given chronic MA do not (Shoblock, Maisonneuve, & Glick, 2003). Lastly, animals were tested in novel environments in the previous studies, with specific type of novel environment differing between studies, rather than in the home cage, which minimizes possible confounds of

novelty- and handling-induced stress. The fact that these results matched previous results despite these differences is encouraging in terms of comparing data on psychostimulant-induced stereotypy behaviors across studies, despite differences in species, drug, procedure, and definition of stereotypic behavior.

The testing time used for selection in creating the selected mouse lines, 33 minutes post-injection, captured maximal separation between the selected lines for both chewing stereotypy and circling stereotypy, an additional behavior that correlates negatively with chewing. Because of this negative correlation, we hypothesized that at gene loci that influence stereotypic behaviors, one allele on a given gene locus would produce high chewing and low circling, and a different allele on the same gene locus would produce low chewing and high circling. This hypothesis was explored in the QTL analysis of these mice.

The stereotypic behaviors observed in these mice reached near-maximal levels by 15 to 20 minutes post-injection and remained there for the entire 60 minutes of testing. This indicates that stereotypic behavior lasts long enough in order to affect new gene expression, making it worthwhile to study differences in gene expression in MA-treated vs. saline-treated animals. However, the differences between HMA and LMA mice in number of chewing episodes per minute were only significant from 20 to 50 minutes post-injection, so this may not be an effective measure for comparing mice of different genotypes at time points later than 50 minutes. On the other hand, baseline differences were evident at 20 to 30 minutes; this is too short an interval for MA to induce much gene regulation. The measure of time spent in stereotypy per minute would be more

effective, since the lines were highly significantly different on this measure at 20 minutes and all later time points.

The time course for stereotypic behaviors in the mouse following MA administration is similar to that for stereotypy in the rat following amphetamine administration, despite the many differences between studies. In addition, stereotypy measured at 33 minutes post-injection for selection affected the entire time course rather than being specific to the 33-minute time point. This indicates that selection changed expression of the behavior in general rather than only at a specific point in the time course of the behavior.

Experiment 2, MA-induced behaviors in the absence of cage bedding. Even in a restricted environment with no objects available for mice to chew, stereotypic behaviors that occurred following MA administration were mainly oral behaviors. In the absence of cage bedding, the majority of the mice tested repetitively licked the floor or the wall of the cage. One B6 mouse that did have cage bedding available licked the cage wall as its primary behavior. This illustrates that this behavior is in the repertoire even when cage bedding is available, but chewing the cage bedding is more common than licking the cage wall when cage bedding is available. Sniffing is a behavior that is not necessarily stereotypic and often occurs in the absence of drug administration. It is possible that for the animals that primarily exhibited sniffing behavior, this dose of MA did not produce stereotypy. Environmental context influences the specific MA-induced stereotypic behaviors that occur in these mouse strains, but the general nature of the stereotypy remains similar, at least in the two simple environments tested.

Experiment 3, QTL analysis of MA-induced chewing stereotypy. Based on combined QTL analysis of three different mouse populations derived from a B6 and D2 background, the most highly promising QTL (highest LOD score) is located on the distal end of chromosome 9, with a significant QTL interaction between a QTL on proximal to mid chromosome 8 and a QTL on distal chromosome 9. Based on these results, the next step was to generate a congenic mouse strain for the QTL region on distal chromosome 9. It was necessary to add a fourth population to the QTL analysis because the most promising QTL was not statistically significant (LOD=3.09, p= .0008). The congenic strain also provides the opportunity for finer mapping of the QTL region.

Experiment 4, QTL results in the congenic strain. Rather than confirming QTL results from the first three combined populations, including the distal chromosome nine congenic strain in the QTL analysis weakened the evidence that a QTL for MA-induced chewing stereotypy exists at this location. This is likely related to the fact that the evidence for a QTL interaction between QTLs on proximal chromosome 8 and distal chromosome 9 was stronger than evidence for main effects of either QTL by itself. Selecting against heterogeneity everywhere in the genome other than chromosome 9 may have eliminated this interaction. To test this hypothesis, a congenic strain could be produced for the chromosome 8 QTL region and these animals could then be crossed with animals of the chromosome 9 congenic strain. If only the double congenic animals showed the QTL effect, this would support the idea that the interaction between these two QTLs is the relevant factor that led to emergence of a QTL on distal chromosome 9.

These mice will also be tested in the context of other drug abuse-related QTLs that are located on the region of chromosome 9 captured in this congenic strain. For

example, there is a QTL for two-bottle choice ethanol preference drinking proximal to mid chromosome 9 (Phillips, Belknap, Buck, & Crabbe, 1998; Tarantino, McClearn, Rodriguez, & Plomin, 1998; Belknap & Atkins, 2001).

Experiment 5, MA preference drinking. MA reward as measured by two-bottle choice drinking preference has no relationship to MA-induced chewing stereotypy. This suggests that MA-induced chewing stereotypy and MA reward occur by different mechanisms. Because stereotypic chewing is not closely related to the other behaviors we measured, there are not likely to be QTLs in common between them. This provides evidence against the hypothesis that stereotypy is inversely related to drug preference. A recent study revealed that mice selectively bred for high and low locomotor activation following 2 mg/kg MA show significantly different preference for 20 mg/L and 40 mg/L MA in a two-bottle choice test, with the low line showing greater preference than the high line (Kamens, Burkhart-Kasch, McKinnon, Reed, & Phillips, 2004). This supports the finding that preference for MA is not associated with high locomotor activation and low stereotypic behavior as we initially predicted. Another study showed that rats previously exposed to a neurotoxic dose of MA that produced stereotypy later demonstrated greater reward in response to a lower dose of MA than control animals that did not receive the previous neurotoxic dose (Gehrke, Harrod, Cass, & Bardo, 2003).

It could be that two-bottle choice preference drinking is not the appropriate measure of MA reward. Humans who abuse this drug usually take it intravenously, intranasally, or by smoking rather than consuming it orally (Matsumoto et al., 2002; Harris, Boxenbaum, Everhart, Sequeira, Mendelson, & Jones, 2003). Some other possible methods to measure drug reward in rodents that may yield different results

include conditioned place preference and intravenous self-administration (Gehrke et al., 2003; Harrod, Dwoskin, Green, Gehrke, & Bardo, 2003; Mizoguchi et al., 2004; Nakajima et al., 2004).

Experiment 6, microarray analysis of baseline gene expression. After a QTL has been finely mapped, the next step is to identify and evaluate candidate genes. The fraction of the mouse genome containing functional genes is approximately 5 to 10 percent (Silver, 1995). Though dopamine is the primary neurotransmitter involved in the mechanism for MA-induced stereotypy, we did not find any QTLs for chewing stereotypy near known dopamine-related genes, so genetic variability in dopamine activity in these strains may not underlie genetic differences in stereotypy. Pharmacokinetic differences probably do not account for the differences between strains, since there are no strain differences in brain concentrations of MA following administration in BXD recombinant inbred mice, and this measure was not correlated with chewing stereotypy or with any of the other behavioral measures (Grisel et al., 1997). There is only one case of identification of a gene resulting from a QTL analysis of drug abuse-related traits (Fehr et al., 2002). Combining QTL results with microarray gene expression provides stronger evidence that a candidate gene is involved in the mechanism for the behavior of interest. Using real-time quantitative PCR to measure gene expression levels in rat lines selected for high and low two-bottle choice ethanol preference, a gene influencing ethanol preference was identified within the region of a QTL for this trait (Liang et al., 2003). By integrating QTL and microarray data from mouse lines selected for high and low acute functional tolerance to the incoordinating effect of ethanol, a candidate pathway that mediates this behavior was identified

(Tabakoff, Bhave, & Hoffman, 2003). It is also possible to do this type of analysis in reverse and integrate QTL and microarray data to facilitate correctly identifying a known gene influencing a trait (Hitzemann et al., 2004).

In the microarray analysis of baseline gene expression in a B6D2F_2 population, three genes on distal end of chromosome 9 had significant expression QTLs in the F_2 in this region. The first gene, zinc finger protein of the cerebellum 1, is a zinc finger protein involved in central nervous system development, particularly in the cerebellum (Aruga et al., 1998). Knockout mice lacking this gene have a low survival rate, and those that do survive exhibit severe abnormalities in motor behavior (Aruga et al., 1998). Mice heterozygous for the knocked-out gene show reduced locomotor activity, motor coordination, and muscle tone as compared to wild-type mice (Ogura, Aruga, & Mikoshiba, 2001). The primary function of zinc finger protein of the cerebellum 1 occurs during development, and is not likely to be relevant to acute response to MA in adult animals. However, this gene is also expressed in adults with unknown function, so it is possible it contributes to response to MA in some unknown way, possibly influencing expression of stereotypic behaviors through its role in development of motor behaviors.

The second gene, trophoblast glycoprotein, is involved in embryogenesis and the homologous human gene is involved in tumor formation (King, Sheppard, Westwater, Stern, & Myers, 1999). Again, its primary function occurs during development, but it is also expressed in adults with unknown function, so may contribute to MA response in some unknown way.

This third gene, muscle and microspikes RAS, is involved in small GTPase mediated signal transduction and GDP/GTP binding (Matsumodo, Asano, & Endo,

1997). Activated M-Ras can morphologically transform mammary epithelial cells and induce tumor formation (Ward, Zhang, Somasiri, Roskelley, & Schrader, 2004). M-Ras and possibly trophoblast glycoprotein, contribute to tumor formation. The link between oxidative stress and tumor formation may be relevant. One role of endogenous and exogenous antioxidants is to inhibit tumor formation and growth (Watson, Cai, & Jones, 2000; Zhao et al., 2001; Genter, Burman, Vijayakumar, Ebert, & Aronow, 2002; Burman, Shertzer, Senft, Dalton, & Genter, 2003; Bagchi, Sen, Bagchi, & Atalay, 2004). It is possible that animals with higher levels of expression of genes that promote tumor formation are also more susceptible to oxidative damage following exposure to a stressor.

Five genes on the proximal end of chromosome 8 had significant expression QTLs in this region. The function of three of them is unknown. Tissue plasminogen activator is a serine protease that converts serum zymogen plasminogen to plasmin, which then degrades extracellular matrix components and activates proteases and growth factors during early development (Carroll, Richards, Darrow, Wells, & Strickland, 1993). In adults, this protein is involved in the initiation of activity-dependent plasticity in the brain (Hashimoto, Kajii, & Nishikawa, 1998). In rats, acute MA induces tissue plasminogen activator expression in cortical regions that project to the striatum from one to four hours after injection, with higher expression for a higher dose that produced stereotypic behavior, and this effect is dopamine-dependent (Hashimoto et al., 1998). The authors hypothesized that the expression of this protein may be involved in the mechanism for development of sensitization to the effects of MA (Hashimoto et al., 1998).

Glutathione reductase is an antioxidant enzyme that regenerates glutathione from its oxidized form, which enhances the ability of glutathione to protect neurons against oxidative stress (Vexler et al., 2003). Reduced glutathione reductase activity is associated with increased oxidative damage to neurons under oxidative stress (White et al., 1999). For example, glutathione reductase activity is reduced following a neurotoxic dose of ethanol (Heaton, Madorsky, Paiva, & Mayer, 2001). Glutathione reductase activity in the brain is most prevalent in cerebellum, striatum, cortex, hippocampus, and septum (Calabrese, Scapagnini, Ravagna, Fariello, Stella, & Abraham, 2002).

There were five transcripts on chromosome 8 with significant expression QTLs on chromosome 9. The function of three of them is unknown. Lipoprotein lipase is involved in lipid recycling and protection of neurons from oxidized lipoproteins (Blain, Paradis, Gaudreault, Champagne, Richard, & Poirier, 2004; Paradis et al., 2004). In brain, it is mainly expressed in hippocampus and expressed to a lesser extent in globus pallidus, striatum, nucleus accumbens, amygdala, cortex, and cerebellum (Lorent, Overbergh, Moechars, De Strooper, Van Leuven, & Van Den Berghe, 1995; Paradis et al., 2004). Expression of lipoprotein lipase is increased in response to brain injury (Blain et al., 2004; Paradis et al., 2004).

GTP binding protein is a mitochondrial GTPase that is involved in tRNA modification (Li & Guan, 2003). This protein may be relevant to MA-induced stereotypy in terms of playing a role in the synthesis of other proteins that affect the behavior.

The relationship between stereotypy and neurotoxicity. Several of the candidate genes that emerged by combining the QTL results with microarray gene expression were related to oxidative stress and neurotoxicity. Degree of MA-induced stereotypy is

positively correlated with degree of neurotoxicity (Wallace, Gudelsky, & Vorhees, 1999). Genetic differences in degree of MA-induced stereotypy are likely to be related to MA-induced neurotoxicity. D2 mice, which demonstrate more MA-induced stereotypy than B6 mice, have a higher sensitivity to MA-induced striatal dopaminergic neurotoxicity than B6 mice (Kita, Paku, Takahashi, Kubo, Wagner, & Nakashima, 1998). Repeated high doses of MA result in long-term reductions of striatal dopamine and serotonin (Ellison, Eisen, Huberman, & Daniel, 1978; Ricaurte, Schuster, & Seiden, 1980). A single high dose can also have these effects (Fukumura, Cappon, Pu, Broening, & Vorhees, 1998).

The mechanism for MA-induced neurotoxicity may be directly related to the mechanism for MA-induced stereotypy. Mice lacking the dopamine transporter do not show changes in dopamine levels or free radical formation following repeated administration of MA, but do show reduced serotonin levels in striatum and hippocampus, indicating that the dopamine transporter is required for MA-induced dopaminergic but not serotonergic neurotoxicity (Fumagalli, Gainetdinov, Valenzano, & Caron, 1998). MA neurotoxicity involves an increase in dopamine release resulting in increased extracellular striatal concentrations of dopamine, and NMDA receptor activity may further increase this release (Boireau, Bordier, Dubédat & Doble, 1995). However, some studies suggest that an increase in striatal glutamate is not necessary for the mechanism of MA neurotoxicity (Wallace et al., 2001). Dopamine synthesis and receptor antagonists, competitive antagonists at glutamatergic site, and non-competitive antagonists at PCP/MK-801 site of NMDA receptors all reduce degree of MA-induced neurotoxicity (Boireau et al., 1995). Haloperidol, a dopamine D2 receptor antagonist,

reduces the glutamate increase and prevents long-term dopamine depletion, and the NMDA antagonist MK-801 reduces MA-induced loss of dopamine (Wallace et al., 2001). Both haloperidol and MK-801 reduce MA-induced hyperthermia, which is related to neurotoxicity (Wallace et al., 2001).

MA induces formation of reactive oxygen species by oxidation of cytoplasmic dopamine, and oxidative damage to proteins occurs after long-term exposure, eventually resulting in cell death (Yamamoto & Zhu, 1998; Lotharius & O'Malley, 2001; Jimenez et al., 2004). Activation of microglia, indicative of neural damage, occurs following a neurotoxic dose of MA, and there is in inverse relationship between increased microglial counts and dopamine depletion in the striatum (Guilarte, Nihei, McGlothan, & Howard, 2003; LaVoie, Card, & Hastings, 2004; Thomas, Walker, Benjamins, Geddes, & Kuhn, 2004). Brain areas affected include striatum, thalamus, dorsal raphé, periaqueductal gray, hippocampus, and inferior colliculus (Guilarte et al., 2003). Gene transcription and mRNA translation are required for the mechanism for MA-induced neurotoxicity. Both mRNA synthesis inhibitor actinomycin-D and protein synthesis inhibitor cycloheximide blocked MA-induced neurotoxicity to dopaminergic neurons, and did so without changing body temperature, dopamine transporter function, or brain levels of MA (Xie et al., 2002).

MA users have significantly lower dopamine transporter availability than controls, and degree of damage to striatal dopamine transporters was related to cognitive and motor impairment (Volkow et al., 2001). Deficits in reaction time and cognitive tasks that require working memory correlate with reduced relative regional cerebral blood flow in the basal ganglia, frontal, and parietal brain regions, attributed to neurotoxic

effects on the dopaminergic system (Chang et al., 2002). Psychotic symptoms, particularly positive symptoms including conceptual disorganization, tension, mannerism and posturing, grandiosity, hostility, suspiciousness, hallucinatory behavior, uncooperativeness, unusual thought, and excitement, were negatively correlated with dopamine transporter binding potential in striatum as well as in orbitofrontal cortex and dorsolateral prefrontal cortex (Sekine et al., 2001; Sekine et al., 2003). MA users who had been abstinent from the drug for an average of three years showed significantly reduced density of striatal dopamine transporter, and this appeared to be related to damage of striatal dopamine axons and axon terminals (McCann, Wong, Yokoi, Villemagne, Dannals, & Ricaurte, 1998, Guilarte et al., 2003). Microglial activation occurs before axonal damage, which suggests that reactive microgliosis contributes to the damage rather than just being a marker for it (LaVoie et al., 2004).

Results from microarray studies support the idea that genes involved in the brain's response to oxidative stress show altered expression following MA administration. A study comparing gene expression in striatum following 40 mg/kg MA or saline in B6 mice revealed several upregulated genes in mice given MA hypothesized to be involved in MA neurotoxicity (Thomas, Francescutti-Verbeen, Liu, & Kuhn, 2004). For example, heat-shock protein 70, now called heat shock protein 1B, was upregulated at 3 hours post-injection. This protein is involved in anti-apoptosis, DNA repair, inhibition of caspase activation, and ATP binding. Microarray analysis of changes in gene expression in CD-1 mice following 40 mg/kg MA revealed upregulation of several genes involved in apoptosis, DNA repair, and response to oxidative stress at 2, 4, and 16 hours post-injection (Cadet, Jayanthi, McCoy, Vawter, & Ladenheim, 2001). Increased cytochrome

c oxidase subunit 1 expression in ventral midbrain is associated with MA-induced neurotoxicity to dopamine neurons when measured 12 and 24 hours after administration of 45 mg/kg MA (Xie et al., 2002). This gene, now called prostaglandin-endoperoxide synthase 1, is involved in oxidoreductase and peroxidase activity.

Implications for research on MA-induced stereotypy. The results of the series of experiments presented here suggest that the mechanism for MA-induced stereotypy is related to the mechanism for MA-induced neurotoxicity. Knowledge of this mechanism could be useful in treatment of addicts who have already endured neurotoxic damage after long-term use of MA or related compounds. Increased occurrence of stereotypic behaviors with repeated drug is related to the development of amphetamine psychosis (Murray, 1998; Ridley & Baker, 1982). Insight into the mechanism of MA-induced stereotypy could be useful in treating addicts who develop psychosis with repeated use, perhaps by restoring a protein that has been depleted in the brains of these individuals. In terms of prevention of such damage, there are known factors that limit the amount of oxidative damage that occurs in response to a stressor. Exercise, eating fruits and vegetables, and taking vitamins are associated with high antioxidant response to oxidative stress, and smoking, exposure to ultraviolet radiation, and psychological stress are associated with low antioxidant response to oxidative stress (Lesgards et al., 2002). Foods or vitamin supplements with antioxidant properties, such as vitamin C, vitamin E, carotenoids, and anthocyanins found in berries and red wine, provide protection against oxidative damage in the brain (Klein & Ackerman, 2003; Bagchi et al., 2004).

In addition to being drugs of abuse, MA and related substances such as amphetamine and methylphenidate have approved medical uses. These drugs are

prescribed for conditions such as attention deficit disorder, obesity, narcolepsy, and depression. Research on the mechanism for MA-induced stereotypy could be helpful in prevention of possible neurotoxic effects in these patients.

The mechanism for MA-induced stereotypy may also be the same as or related to the mechanism for stereotypic behaviors that occur in human conditions such as autism, Tourette's syndrome, and schizophrenia (Garner, Meehan, & Mench, 2003). A treatment that prevents the expression of MA-induced stereotypy may also be useful in treating one or more of these disorders of which stereotypic behaviors are a symptom.

Though not directly related to stereotypy, oxidative stress also contributes to neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease as well as to the normal aging process (Watson et al., 2000; Perry et al., 2002; Klein & Ackerman, 2003; Alexeyev, LeDoux, & Wilson, 2004). Any treatment that is developed to counteract neurotoxic effects of MA may also be useful in treating oxidative damage that occurs in neurodegenerative disorders, and could potentially be used to slow normal aging.

The next logical step from this series of studies is to test the effects of the most promising candidate genes on MA-induced stereotypic chewing. First, the roles of the differentially expressed candidate genes should be followed up by measuring changes in protein levels that follow from changes in gene expression (Hoffman et al., 2003). Once the gene products are identified and measured, if pharmacological agents that enhance or block the effects of a candidate gene are available, the effect of the gene product on the behavior could be studied this way. In the absence of the appropriate agonists and antagonists, the function of these genes could be examined by constructing knockout

mice with a null mutation for the gene of interest or transgenic mice that overexpress or underexpress the gene to examine its role in the behavior. These studies would further confirm that the candidate gene does influence the behavior, and should then be followed up by examination of the function of the gene in the context of a pathway that influences expression of the behavior.

In conclusion, MA-induced chewing stereotypy is a heritable behavior that is determined by many genes, each of which has a small effect on the phenotype. The most promising main effect QTL was located at the distal end of chromosome 9. However, a congenic mouse strain produced to capture this QTL region did not show the QTL effect. This is likely due to a background effect, which could be due to a significant interaction between QTLs on distal chromosome 9 and proximal chromosome 8 or some other interaction that did not exist in the congenic mice due to homogeneity in the region of the second QTL. Combining the QTL results with microarray gene expression results in a population derived from the same two inbred mouse strains, several candidate genes emerged related to the brain's response to oxidative damage. This suggested a common mechanism for MA-induced stereotypy and MA neurotoxicity. Further investigation into this mechanism could be useful in treatment and prevention of neurotoxic effects in MA users as well as possible insight into treatments for other conditions that produce stereotypic behaviors and other conditions that are caused by oxidative brain damage.

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