QUANTITATIVE TRAIT LOCI MAPPING ANALYSIS FOR BASELINE NOCICEPTIVE SENSITIVITY AND MORPHINE-INDUCED ANTINOCICEPTION ON THE WRITHING ASSAY

by

Heather S. Hain

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

Heather S. Hain

has been approved

[redacted]
Professor in charge of thesis

[redacted]
Member

[redacted]
Member

[redacted]
Member

[redacted]
Associate Dean for Graduate Studies

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HSH

'God blesses everyone who has wisdom and common sense. Wisdom is worth more than silver; it makes you much richer than gold. Wisdom is more valuable than precious jewels; nothing you want compares with her. In her right hand Wisdom holds a long life, and in her left hand are wealth and honor. Wisdom makes life pleasant and leads us safely along. Wisdom is a life-giving tree, the source of hapiness for all who hold on to her.'

Proverbs 3:13-18

ABSTRACT

Genes have been shown to influence responses to various noxious stimuli and the inhibition of those responses. Correlation studies indicate that different genes may affect responses in distinct assays depending on the stimulus used to induce nociception. The genes themselves, however, are just beginning to be identified. The present studies describe a search using quantitative trait loci (QTL) mapping to locate chromosomal regions possibly influencing baseline nociceptive sensitivity and morphine-induced antinociception in the acetic acid-induced writhing assay. Results were compared to findings obtained with the hot-plate assay.

BXD recombinant inbred (RI) strains and an F2 intercross (B6D2F2) using the BXD progenitors, the C57BL/6 and DBA/2 inbred strains, were utilized in the QTL mapping analyses. Mice were tested for antinociceptive response under the influence of morphine and for baseline nociceptive sensitivity. Both traits were heritable in the BXD RI strain analysis, although the heritability of morphine-induced antinociception was considerably lower.

Analysis of variance revealed a strain x sex interaction in baseline nociceptive sensitivity on the writhing assay. These data suggest some genes may influence nociceptive responses of one sex differently than the other sex. Separate QTL analyses using only female, only male, and combined sex data were performed. QTL analyses of baseline nociceptive sensitivity revealed several provisional QTLs at the $p<0.05$ level. Many of these appeared to be sex-specific QTLs. Four suggestive QTLs ($p<0.002$) were detected after combining data from the BXD RI and B6D2F2 intercross analyses. One of

these appears to be a sex-specific QTL for females.

A strain x sex interaction was not found for morphine-induced antinociception on the writhing assay. Although this trait had a low heritability, seven provisional QTLs were detected at the $p<0.05$ level. One suggestive QTL was found using combined data from the BXD RI and B6D2F2 intercross analyses. This QTL did not match QTLs confirmed in the QTL analyses of morphine-induced antinociception on the hot plate.

Correlation comparisons using the BXD RI strain means revealed a non-significant correlation between baseline nociceptive sensitivity in the writhing assay and morphine-induced antinociception in the writhing test. This is unlike results from the hot plate test in which baseline nociception and morphine-induced antinociception were significantly, positively correlated. A non-significant correlation was detected between baseline nociceptive sensitivity in the writhing assay and baseline nociceptive sensitivity on the hot-plate assay. A non-significant correlation was also observed between morphine-induced antinociception in the writhing test versus morphine-induced antinociception on the hot-plate test. These results indicate both nociceptive and antinociceptive responses on the two assays are genetically independent.

Additionally, mice selectively bred for high (HMOR) and low (LMOR) morphine-induced antinociception on the hot-plate did not exhibit the same differential responses in the writhing assay. Fourth generation HMOR and LMOR mice did not differ in morphine-induced antinociception on the writhing assay, whereas a two-fold difference in antinociceptive magnitude on the hot plate was observed in previous generations. A small but significant difference in baseline nociceptive sensitivity in the

writhing assay for HMOR versus LMOR mice was revealed. This is in contrast to results using the hot plate in which no difference was found between HMOR and LMOR mice.

These results suggest that both nociceptive and antinociceptive responses in the writhing assay are influenced by different genes than these responses on the hot plate in varying ways. Further studies are needed to confirm the suggestive QTLs found in these analyses.

INTRODUCTION

"Pain" is defined as the perception of an aversive or unpleasant sensation originating from the body (Jessel and Kelly, 1991), and pain experience is often separated into two components, motivational-affective and sensory-discriminative. "Nociception" is defined as the neural reactions to noxious or potentially harmful stimuli imposed on the body. Thus, the subjective experience of pain relief is termed analgesia, whereas antinociception describes the inhibition of nociception. Since it is difficult to ascertain the subjective experience of animals, nociception and antinociception are terms correctly used to describe responses to noxious stimuli in animal studies.

Many characteristics of the noxious stimuli may affect nociceptive and antinociceptive responses. The type, location, and intensity of a stimulus influence the pathway or mechanisms to process, encode, and respond to information about that stimulus (Millan, 1999). One of the more common types of stimuli used to measure nociceptive responses in both human and animal studies is a noxious thermal stimulus directed at cutaneous tissue. However, pain arises from stimuli applied to other tissues such as muscles, joints, and viscera, and pain can be due to more types of stimuli than just thermal stimulation. Examining nociception induced by a variety of stimuli in different tissue types, therefore, would be valuable in understanding nociceptive processes. Equally beneficial is ascertaining processes to control or inhibit nociception.

Another important consideration in interpreting nociceptive and antinociceptive processes is the organism's internal milieu or state when noxious stimuli inflicts the body. Neural systems in nociception exhibit a high degree of plasticity, so that stimuli

delivered to damaged or inflamed tissue invoke responses differently from responses evoked from healthy tissue (Levine, 1988). Hormonal levels and interactions may affect how nociception is processed in females versus males.

Additionally, the genotype of subjects may affect nociceptive and antinociceptive processes. Several studies have examined the effects of genotype on nociceptive and antinociceptive responses (Mogil et al., 1996b). Studies suggest that variations in genes may lead to differences in the response to nociceptive stimuli, but only a few have pursued possible genes that could influence nociception and antinociception (Belknap et al., 1995; Mogil and Belknap, 1996; Mogil et al., 1997; Quock et al., 1996). To adequately understand the effect of genotype on nociception and antinociception, more experiments are necessary.

The purpose of this thesis, therefore, is to explore genetic factors in the study of pain. Two broad questions are addressed. The first question is: are there genetic influences on nociceptive and antinociceptive responses in the acetic acid-induced writhing assay? The second question is: do these genetic influences differ from those for nociceptive and antinociceptive responses on other pain assays? These questions are examined using quantitative trait loci mapping analyses, correlation analyses, and selection line comparisons between nociceptive and antinociceptive responses in the writhing assay and nociceptive and antinociceptive responses on the hot plate (see Hypotheses).

The following introduction first examines aspects of acetic acid-induced writhing that may support how it is different from or similar to hot plate-induced foot withdrawal.

This is followed by a brief section regarding sex differences in pain/nociception and analgesia/antinociception. An overview of the genetics of nociception and antinociception closes the introduction.

Visceral versus Somatic Nociception

Acetic acid-induced writhing has visceral as well as somatic components. Visceral nociception differs markedly from somatic nociception in several ways. Certain types of stimuli that produce pain when delivered to somatic tissue do not produce pain sensations in humans when applied to viscera (MacKenzie, 1909, 1893; Morley, 1931). In somatic tissue, specific pain receptors or nociceptors have been found that relay sensory information about noxious stimuli. There is some disagreement whether specific nociceptors exist in the visceral nerves or whether the intensity of the noxious stimulus excites nonspecific sensory receptors to evoke a nociceptive response (see Fig. 1; Cervero and Jänig, 1992). It may be that nociception from different viscera is mediated by one or the other or both types of receptors (Häbler, et al., 1990; Sengupta et al., 1989, 1990).

Visceral pain has been described as having poor localization and a diffuse nature compared to somatic pain. One possible explanation is that somatic and visceral afferents converge peripherally, at the spinal cord, and supraspinally, and their signals are integrated or misinterpreted leading to a diffuse sensation (Ruch, 1961). Another theory is that activation of visceral afferents causes a general excitability which facilitates or excites somatic neurons in the vicinity of the stimulated area or at the spinal cord level, hence leading to a nociceptive sensation from those somatic areas as well

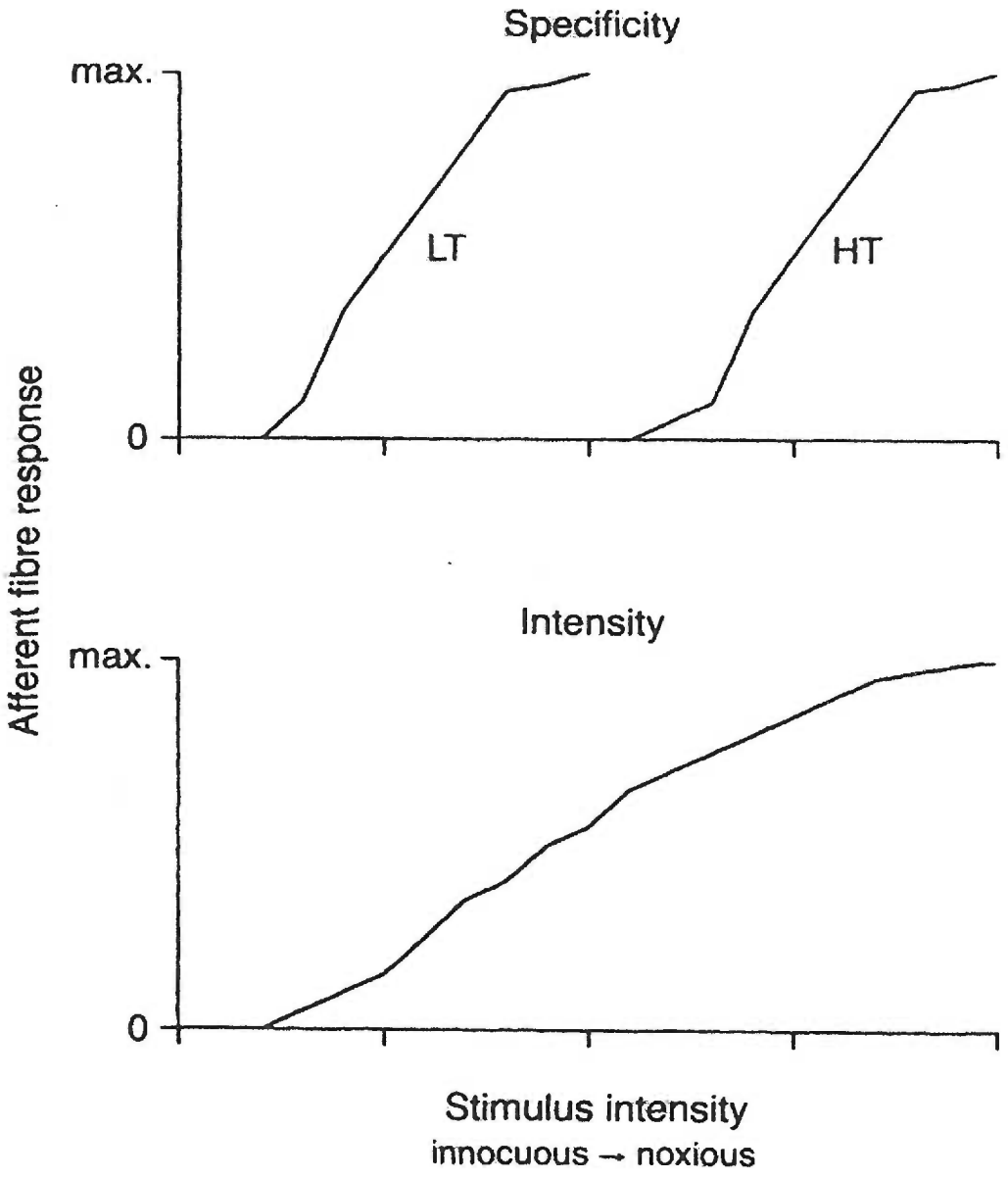

Figure 1. Two classical theories for encoding noxious stimuli by peripheral afferents. *Specificity:* two different classes of primary afferents exist; low threshold (LT) fibers and high threshold (HT) are responsible for encoding innocuous and noxious stimuli, respectively. *Intensity:* a single homogenous population of afferents are capable of encoding stimulus intensities from innocuous to noxious.
taken from Cervero and Jänig, 1992

(MacKenzie, 1909; Sinclair et al., 1948). In addition, encoding of visceral input to the thalamus is not topographically organized as is somatic input (McMahon, 1994). The receptive fields of visceral afferents responding to nociception appear to be larger, weakly-defined, and more complex when compared to somatic receptive fields (Morrison, 1977).

Evidence suggests variations in number, innervation density, and fiber type may lead to differences in the processing of nociceptive stimuli from somatic versus visceral tissues. Fewer afferents appear to innervate the viscera as compared to somatic tissue. For example, it is estimated in the thoraco-lumbar region of the spinal cord, where input from visceral afferents from the upper abdominal viscera and somatic afferents from the abdomen and back are received, fewer than 10% of afferents are relegated to innervating visceral structures while the remainder transmit somatic information (Cervero et al., 1984). Likewise, only 5-15% of dorsal root ganglia cell bodies at the spinal segments receiving visceral afferent input are constituted of visceral afferents (Cervero and Tattersall, 1987). Of these afferents, the proportion of the three classifications or fiber types (Aβ, Aδ, and C) innervating somatic versus visceral tissue also appears to vary. Most notably, few or no large, fast conducting, myelinated Aβ fibers innervate the visceral structures. In somatic nerves, Aβ fibers account for approximately 20-25% of the population. The majority of fibers found innervating the viscera are small myelinated Aδ and unmyelinated slow conducting C fibers (Cervero, 1988; McMahon, 1994, 1997; Millan, 1999).

Some neuropeptides have been shown to be differentially localized in primary

afferent fibers of visceral versus somatic nerves. For instance, substance P and vasoactive intestinal peptide (VIP) appear to be more prominent in visceral afferents as opposed to somatic afferents whereas somatostatin is found in virtually no visceral afferents (McMahon, 1997; Lawson et al., 1997; De Groat, 1986). Since substance P and VIP are pronociceptive and somatostatin inhibits nociception, the varying localization between tissues types may affect nociceptive and antinociceptive processes.

Inflammation

Visceral pain is often accompanied or sustained by inflammatory responses. Several animal models of visceral pain include inflammatory components. Injection of acetic acid to induce writhing, for example, causes an inflammatory reaction in the peritoneal cavity (Northover, 1963; Gyires and Knoll, 1975; Deraedt et al., 1980). Under conditions of inflammation, mechanisms of nociceptive processing may be altered as compared to processing in normal tissue. Noxious stimuli that cause interactions of immunological cells such as macrophages and mast cells lead to the production and release of mediators which result in an inflammatory state in tissue (Levine, 1988). Inflammation causes an change in nociceptive processing with a sensitization of peripheral and central nervous systems' circuits (Häbler, 1988; McMahon, 1988; Ness and Gebhart, 1990). This excitability and sensitization is the result of a cascade of neurotransmitters, neuropeptides, and other inflammatory mediators such as intracellular signals, including adenylyl cyclase and calcium (see Fig. 2).

Nociception caused by inflammatory agents can be due to direct stimulation of receptors on primary afferents or indirectly through the release of other mediators that

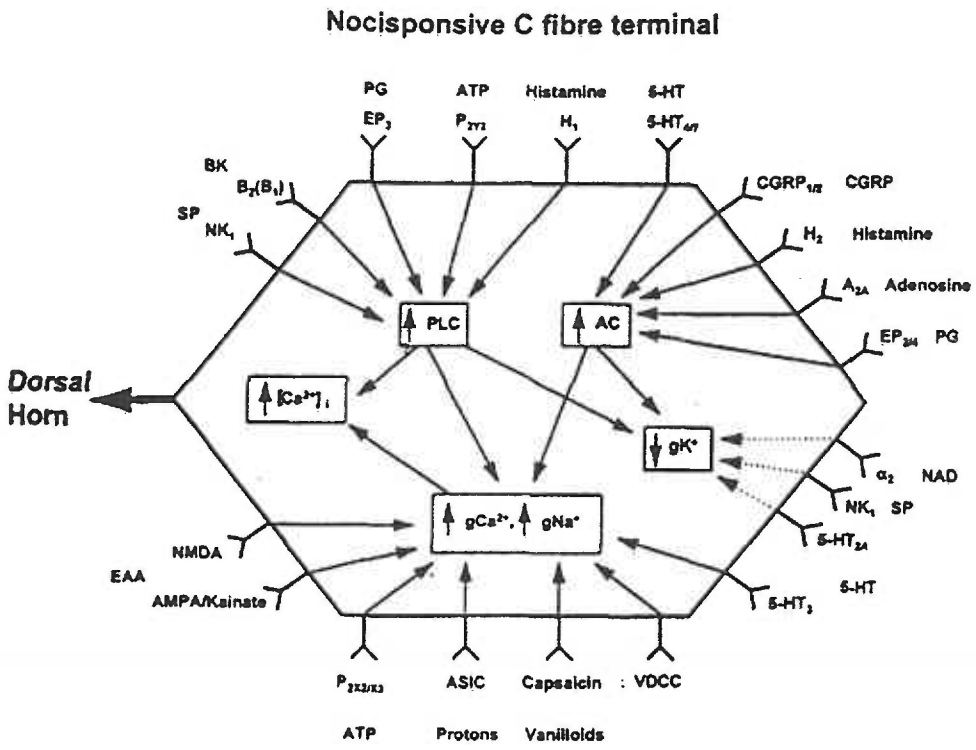

Figure 2. Possible mediators of nociception, their receptors, and intracellular signals at a polymodal C fiber.
taken from Millan 1999

stimulate nerve endings. For example, nociceptive responses to acetic acid injected into the peritoneal cavity of rodents is mediated indirectly through the release of prostaglandins (Deraedt et al, 1976, 1980). Prostaglandins directly stimulate primary afferent fibers or enhance the release of other pronociceptive agents via secondary messengers such as cyclic AMP and ion conductance changes. In addition, inflammatory actions appear to either activate a separate population of afferents that are 'silent' under normal tissue conditions or sensitize afferents that are normally high threshold to respond at lower thresholds (Häbler et al., 1990; Cervero, 1994; McMahon, 1997).

Thermal versus Chemical Stimuli

Nociceptive responses in the writhing assay are the result of neural reactions to a chemical stimulus, whereas responses in the hot plate are reactions to a thermal stimulus. Noxious thermal stimuli excite thermosensitive, mechanothermosensitive, and polymodal afferents; chemical stimuli appear to excite mostly polymodal afferents (Davis et al., 1993; Lang et al., 1990; Jessel and Kelly, 1991; Bonica, 1990). The polymodal C-fibers are excited by mechanical, thermal, and chemical stimuli (Bessou and Perl, 1969; Kumazawa et al., 1987). Some authors contend the intensity of noxious stimuli is important to consider when examining differences between nociceptive assays, as different intensities may evoke different nociceptors (Parsons and Headley, 1989). For example, if thermal stimulation is applied so that it elicits responses mediated by C-fibers rather than Aδ-fibers, morphine is more potent as an antinociceptive agent (Yeomans et al., 1996; Lu et al., 1997). The dose of morphine needed to produce a 50% antinociceptive response (AD_{50}) in mice is approximately 10-fold lower in the writhing

assay as compared to the hot-plate test, suggesting the intensity of the former is lower (see Mogil et al., 1996a). The variation in fiber types excited by thermal versus chemical stimuli may partly explain differences in nociceptive processing between the hot-plate and writhing assays.

Morphine Antinociception

Several endogenous opioid peptides have been detected that, among other functions, act as modulators to inhibit nociceptive processing (Hughes et al., 1975; Loh et al., 1976; Goldstein et al., 1979). Morphine mimics the actions of endogenous opioids, particularly those which bind μ-opioid receptors. Three subtypes of opioid receptors have been classified: the mu (μ), delta (δ), and kappa (κ) receptors. All three subtypes are thought to play a role in mediating antinociceptive actions even though they have different patterns of expression (Mansour et al., 1986, 1988). Through immunohistochemistry, receptor autoradiography, and light microscopy techniques, opioid receptors have been localized in much the central nervous system in many brain areas, including those thought to be associated with nociception such as the periaqueductal gray (Mansour et al., 1986, 1988; Tempel and Zukin, 1987; Baush et al., 1995). Opioid receptors have also been located on primary afferent terminals in the superficial layers of the dorsal horn in the spinal cord (Atweh and Kuhar, 1977; Besse et al., 1990; see Coggeshall and Carlton, 1997 for review). Evidence suggests that different types of nociception may utilize the receptor subtypes in varying ways. For example, morphine may exert its antinociceptive actions primarily through a summative activation of the μ- and κ-opioid receptors in inflammatory nociception, whereas the primary

antinociceptive action of morphine for inhibition of thermal nociception is through its activation of the μ-opioid receptor (Schmauss and Yaksh, 1984; Millian and Colpaert, 1991; Murray and Cowan, 1991; Ward and Takemori, 1983; but see Parsons and Headly, 1989). Opioid receptors are G-protein coupled receptors with seven transmembrane spanning domains. Activation of these receptors and their respective G-proteins results in either an inhibition of adenylyl cyclase or activation of ion channels.

Systemically administered morphine has been shown to inhibit nociceptive transmission through descending modulatory pathways from the periaqueductal grey (PAG) and the rostral ventral medulla (RVM) to the spinal cord and by inhibiting nociceptive input at the spinal cord (see Fig. 3; Basbaum and Fields, 1984; Fields et al., 1992). Supraspinally, opioids activate descending antinociceptive outflows using disinhibition (Fields, et al., 1992). For example, the stimulation of μ-opioid receptors inactivates γ-aminobutyric acid (GABA) inhibition (disinhibition) by hyperpolarizing GABAergic cells in the periaqueductal gray (Lipp, 1991; Vaughan et al., 1997). In the spinal cord, morphine binds to presynaptic and postsynaptic opioid receptors and inhibits nociception by both direct and indirect mechanisms. The direct mechanism is a presynaptic or postsynaptic inhibition of pronociceptive neurotransmitters or neuropeptides such as substance P (Go and Yaksh, 1987). The indirect mechanism consists of disinhibition or hyperpolarization of neurons involved in nociception transmission.

In addition to their actions in the central nervous system, endogenous opioids and morphine also may cause antinociception by peripheral mechanisms, particularly when

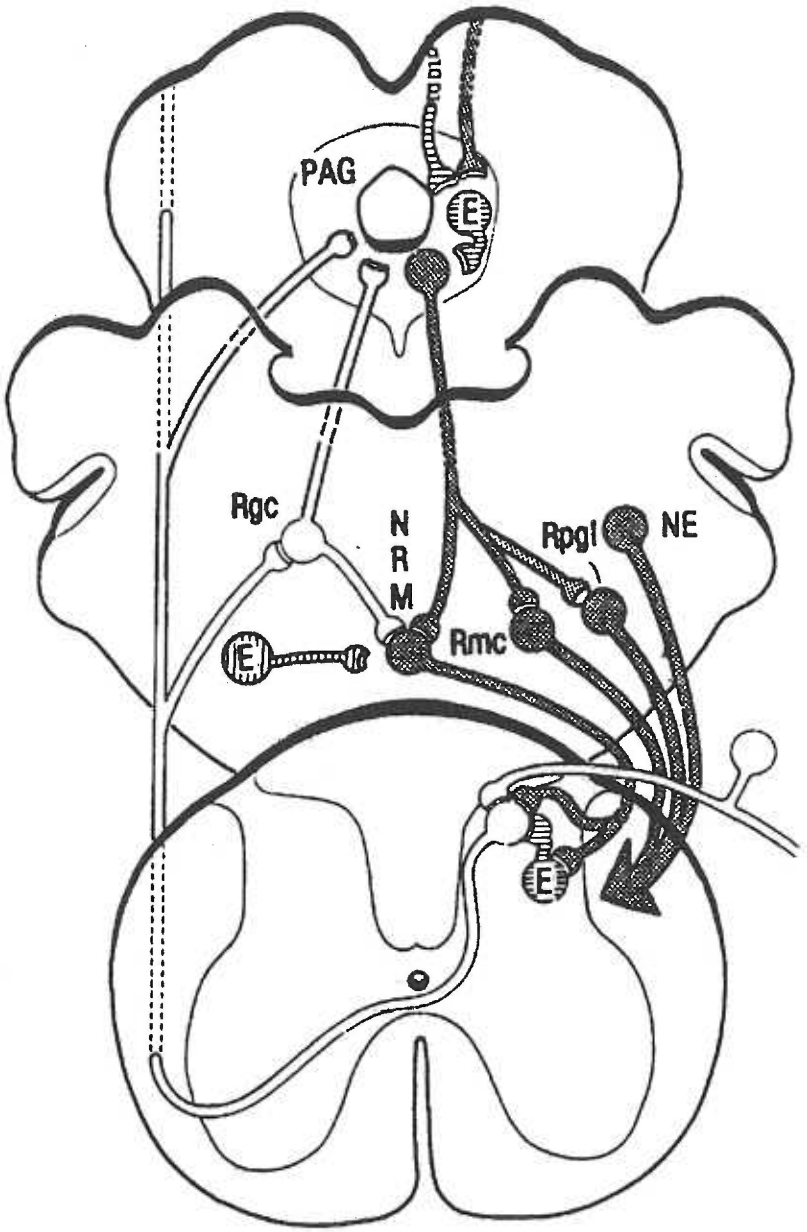

Figure 3. Major components of a descending antinociceptive opioid pathway. Stippled neurons indicate the connections between the periaqueductal gray (PAG) and subregions of the rostral ventral medulla [nucleus raphae magnus (NRM), nucleus reticularis magnicellularis (Rmc), nucleus reticularis paragigantocellularis lateralis (Rpgl)], and from the medulla to the spinal cord.
taken from Basbaum and Fields, 1984

the nociception is affected by inflammatory states. This may be due to an up-regulation of opioid receptors in peripheral primary afferents after inflammation (Hassan et al., 1993). For example, acetic acid-induced writhing has been shown to be inhibited by morphine within a short time, which may implicate binding to peripheral sites on primary afferents (Bentley et al., 1981). Opioid receptors have been found on primary afferents in normal and inflamed somatic and deep somatic tissue (Fields et al., 1980; Yaksh, 1988; Stein et al., 1990). Moreover, studies using morphine congeners with poor transport across the blood-brain-barrier (such as N-methyl-morphine) have demonstrated antinociceptive effects that can be inhibited by antagonists that also lack accessibility to central sites (Smith et al., 1982; Rios and Jacob, 1982; Follenfant et al., 1988). Opioid receptors and peptides that may be inhibiting nociception due to inflammation have been found in immunocompetent cells such as lymphocytes and mononuclear phagocytes (Stein et al., 1990; Carr et al., 1989; Makman, 1994).

Sex Differences in Nociception and Antinociception

Another aspect to consider when examining nociception and antinociception is that many studies only observe males and do not determine whether sex differences influence responses in the assays used. Recently, clinical studies have begun investigating whether differences exist between males and females on measures of pain sensitivity and analgesia. Some studies suggest no sex difference in pain sensitivity while others find that women have a lower threshold for pain sensitivity (Neri and Agazzani, 1984; Bush et al., 1993; Jensen et al., 1992; Meh and Denislic, 1994). Similarly, experiments find no difference in analgesic magnitude between males and

females or that men have higher analgesic responses to analgesic compounds (Lehmann and Tenbuhs, 1986; Gourlay et al.,1988; Burns et al., 1989). The outcomes of these studies are complicated by variables such as stimulus type or intensity, hormonal influence, anxiety or stress, age, and social interactions which may influence the pain and analgesic sensitivity or response to these variables in females and males (see Fillingim and Maixner, 1995, and Berkley, 1997 for reviews). For example, Lautenbacher and Rollman (1993) found a significant sex difference in pain threshold to electrical stimuli but not to hot stimuli.

Likewise, in rodent studies divergent responses have been reported between the sexes both in nociceptive sensitivity tests and antinociceptive measures. The type of nociceptive assay may affect whether sex differences are revealed. Female rats and mice tend to exhibit lower nociceptive threshold than males on acute thermal tests, such as the hot-plate, but not on prolonged chemical assays such as the writhing and formalin assays (Romero and Bodnar, 1986; Sternberg et al., 1993). Some investigators find an effect of gonadectomy on sex differences found for morphine- and stress-induced antinociception (Baamonde et al., 1989; Mogil et al., 1993; Kepler, et al., 1989; Wong, 1987) while others do not (Cicero et al., 1996).

Another factor to consider in rodent studies is whether the genotype of the animals used affects sex differences. It has been suggested that inconsistent sex difference findings across laboratories may be due to differences in genotype (Sternberg, 1995; Mogil and Kest, 1999; Kavaliers and Innes, 1987). Recent evidence has shown that in certain strains of inbred mice, there are no sex differences in central morphine-

induced antinociception (Kest et al., 1999). Sex-specific quantitative trait loci (QTL), chromosomal regions thought to influence a behavior, have been described for a few nociceptive behaviors (Mogil et al., 1997, 1997a). If a sex-specific QTL is discovered, it suggests a gene or genes exert greater influence on a behavior in one sex of mice than the other. Thus, it remains unclear what aspects of the male and female organism are causing observable sex differences in nociceptive and antinociceptive responses in some studies but not others.

Genetics of Nociception and Antinociception

As stated above, the genotype of subjects could affect the outcome of experiments. One way of discovering more about nociception and antinociception is to search for genes that drive these processes which might explain individual differences in a population and variances between tests for nociceptive responses. Genetic mouse models provide a *in vivo* method to investigate genes affecting nociceptive and antinociceptive processes. A major advantage of using mouse models for genetic experiments is that the mouse genome has over 80% synteny with the human genome (Copeland et al., 1993). That is, the distribution of genes on chromosomes has been largely conserved between the two species. Thus, genes found to be associated with behaviors in the mouse may be mapped easily in the human genome.

Inbred strains are one of the most commonly used mouse models in behavioral research. The C57BL/6 and DBA/2 inbred strains have been found to be among the most genetically divergent, making them an excellent choice for studying the genetics of behavior and physiological measures (Taylor, 1972). A strain is defined as inbred when

it has been subjected to full sibling (brother by sister) mating for a minimum of 20 generations. Almost all commonly used inbred strains have been maintained for over 60 generations of full sibling mating (Silver, 1995). Inbred strains are used in genetic studies because they are virtually genetically identical at all loci; therefore, within-strain phenotypic variance can be attributed to environmental causes and between-strain phenotypic variance can be attributed to genetic causes. Numerous inbred strains of mice are available allowing many comparisons across different genotypes.

The C57BL/6 and DBA/2 strains have been found to differ on nociceptive related traits including stress-induced and opioid-induced antinociception on the hot-plate and tail withdrawal assays. Specifically, DBA/2 mice exhibit a higher baseline nociceptive sensitivity and are more sensitive to the antinociceptive effects of morphine than C57BL/6 mice on the hot-plate and tail-withdrawal assays (Belknap and O'Toole, 1991; Mogil et al., 1996). On the writhing and formalin assays, however, no difference is observed in baseline nociceptive sensitivity or morphine-induced antinociception between these two strains (Brase, et al., 1977; Mogil et al., 1996; Elmer et al., 1998).

Quantitative Trait Loci Mapping in the Mouse

A method of discovering possible genes that influence nociception and antinociception is to use quantitative trait loci mapping. Quantitative trait loci (QTLs) are chromosome sites containing alleles that influence a continuously distributed or quantitative trait (Lander and Botstein, 1989). Such traits are usually polygenic in nature and may have multiple environmental determinants. Typically, chromosomal mapping of QTLs is accomplished by searching for linkage or significant associations between

genetic markers and the phenotype of interest. Several drug-related behaviors have been examined using QTL analyses (Belknap et al.,1993, 1995, 1997a; Miner and Marley, 1995). Isolating and investigating individual QTLs has become feasible with the development of technologies to generate a high distribution of markers throughout the genome. Many of these markers are naturally occurring DNA sequences called simple sequence length polymorphisms (SSLPs), or microsatellites (Silver, 1995). These microsatellites are usually DNA sequences with repeated two-base motifs of variable-length surrounded by unique sequences. Because microsatellites are highly polymorphic and relatively small (75-600 bases), they are readily amplified with oligodeoxynucleotide primers by polymerase chain reaction (PCR; Dietrich et al., 1992; Sambrook et al., 1989). In the mouse, QTL mapping methods have utilized PCR techniques to genotype individual mice in a genetically segregating population to determine which alleles each mouse possesses at particular marker loci. There are over 5000 microsatellite markers identified that cover nearly the entire genome of the mouse, ~1600 of which are polymorphic between C57BL/6 and DBA/2 strains (Silver et al., 1998). This makes it possible to screen the genome for evidence of linkage between a QTL and a marker of known chromosomal location. If such linkage can be established, then the QTL is mapped to the same chromosomal region as the marker.

BXD Recombinant Inbred Strains

The BXD recombinant (RI) strains are the fully inbred descendants of an F2 intercross between the C57BL/6 and DBA/2 inbred strains (Taylor, 1978). Of the original 32 strains derived from the F2 intercross, 25 remain and have been inbred for at

least 70 generations. Inbreeding for more than 20 generations insures that the genetic variance exists between strains and not within strains; each strain is homozygous at all loci in its genome. The BXD RI mice have been used for a multitude of QTL mapping projects, since a relatively large number of BXD RI strains are available (Taylor, 1978; Gora-Maslak et al., 1991; Belknap et al., 1993, 1995, 1997a). Several BXD RI QTL analyses examining nociception and antinociception have been done or are in progress including nitrous oxide-induced antinociception on the writhing assay and baseline sensitivity and morphine-induced antinociception on the hot-plate (see Quock et al., 1996; Belknap et al., 1995, unpublished data).

Using the BXD RI strains for QTL mapping presents many benefits to researchers. More than 1500 polymorphic markers have been genotyped for these 25 strains (Silver et al., 1994; Manly and Cudmore, 1994), so they do not have to be genotyped with every behavioral project; this saves researchers time and money. The RI strains are often advantageous even in cases when the phenotypic difference between the C57BL/6 and DBA/2 mice is small or nonexistent, such as the case for acetic acid-induced writhing. This is because different combinations of alleles in the DBA/2 and C57BL/6 strains that increase and decrease the phenotype could mask a genetic difference in the phenotype. Recombination in the BXD RI strains could lead to a broad distribution of strain means and reveal a genetic difference (Gora-Maslak et al., 1991). Additionally, RI strains have a four-fold greater cross-over density than an F2 intercross population (see below), which extends the linkage map by about four-fold.

Some disadvantages are apparent with using BXD RI strains. Due to the

extensive inbreeding of these strains, some of the strains have low litter sizes or are prone to health defects such as hydroencephalitis. Maintaining the RI collection is more costly than maintaining a more productive genetically segregating mouse population for those reasons. Because every locus is homozygous in the RI strains, any effect of allele heterozygosity is masked. Also, the BXD RI strains only have two allelic possibilities, a C57BL/6 or DBA/2 allele; this limits the generalization of results to a population containing other allelic possibilities.

If 20 BXD RI strains are used in a QTL analysis, only markers accounting for 20% or greater of the genetic variance of a trait can be reliably detected as provisional QTLs ($p<0.05$, two-tailed; Cohen, 1988). Only large effect QTLs are detected due to the relatively small number of strains (genotypes) being analyzed . Additionally, in order to accommodate the multiple correlations used in the BXD RI strain analysis and avoid Type I errors, the threshold for significance must be elevated (Belknap et al., 1996). A QTL must reach a significance level of $p<0.0001$ or a logarithm of the odds (LOD) score of 3.3 (df=1) to be considered a "true" QTL (Lander and Kruglyak, 1989). Most QTLs mapped in this strain set, therefore, are considered provisional QTLs until they can be confirmed in one or more other populations.

F2 Intercross Population

An F2 intercross population is a genetically segregating mouse population created from an F1 cross of two inbred strains. An F2 intercross of the C57BL/6 x DBA/2 inbred strains (B6D2F2) often is used to confirm provisional QTLs detected in the BXD RI strain QTL analysis. Each B6D2F2 mouse is a unique genotype, and thus the population

provides a greater number of recombinant genotypes to increase the power of the experiment. Together, the BXD RI and B6D2F2 populations provide a powerful tool for mapping QTLs in the mouse. Many QTL analyses have been conducted in this way and significant QTLs have been revealed for many traits, including those pertaining to nociception and antinociception. For example, two QTLs revealed in the BXD QTL analysis for morphine-induced antinociception on the hot-plate were further confirmed in an B6D2F2 population (Belknap, et al., 1995).

Short-term Selection Lines

Short-term selection lines have been used more recently as another tool to confirm provisional QTLs. For a confirmation population to a BXD RI QTL analysis, these lines are created by using the B6D2F2 mice as a foundation population and the highest-scoring mice of each sex are mated with each other to form a 'high' line, while the lowest-scoring mice of each sex are mated to create a 'low' line. Individual (mass) selection with no sibling matings are used to attain a rapid selection response, which increases the power to detect QTLs (Belknap et al., 1997b). At each successive generation offspring are tested. Again, the high scorers are intermated as well as the low scorers to serve as the breeders for the next generation of the high and low lines respectively. Only three to five generations of selection are used to minimize inbreeding and fixation of alleles irrelevant to the trait of interest.

Because only two possible alleles (C57BL/6 or DBA/2) at equal frequency can exist at each chromosomal locus, changes in allele frequency due to the two-way selection process can be monitored easily. The response to selection is at or near the

maximal compared to populations with lower or higher initial allelic frequencies, because each QTL contributes maximally to the additive genetic variance (Falconer and Mackay, 1996). The probability of producing divergent high and low lines in a few generations, therefore, is increased. Likewise, the frequencies of alleles at marker loci inferred to be linked to QTLs affecting the trait under selection, as suggested by BXD QTL analyses, may be monitored to see whether they co-segregate with the selected trait. If a close linkage between a marker and a QTL affecting the trait exists, the allelic frequencies for the marker should diverge in the two oppositely selected lines roughly in parallel with the divergence of the trait (Lebowitz et al., 1987; Keightley and Bulfield, 1993).

Genetic Correlation Studies

Inbred/Recombinant Inbred Strains

Genetic correlations measure the extent of pleiotropic effects of sets of loci or QTLs influencing traits. In other words, genetic correlation studies examine whether a gene, or group of genes, is affecting two or more traits. Because the genetic loci are fixed in inbred strains, phenotypic correlations across inbred strain means estimate genetic correlations across these strains (Blizard and Bailey, 1979). Furthermore, the lack of a significant genetic correlation between two traits suggest marker loci associated or linked with one trait are not likely to be linked to the other trait (Plomin et al., 1991). The genes of inbred strains are fixed without respect to any particular trait; this may provide an unbiased comparison of traits for common genetic etiology. Another advantage of using inbred strains is that an estimation of genetic correlation can be done

in one generation. Because inbred strains have specifiable, replicable, and relatively stable genotypes, strain data collected in various laboratories and at different times may be used in correlation analyses (but see Crabbe et al., 1999). For example, a cumulative database for the BXD RI strains has allowed researchers to analyze possible significant correlations between previously studied behaviors and behaviors presently being assessed in these strains. Using a larger number of strains reduces the likelihood that chance associations result in falsely significant estimates of genetic correlation (Hegemann and Possidente, 1981). A potential problem in using RI strains is that genetic variance can be examined only at loci that are fixed differentially in the progenitor inbred strains. This may preclude the generalization of the correlation results to heterogenous mouse populations. Also, a genetic correlation found using inbred strains would be transient or changing in a heterogenous, randomly mated mouse population because the gene frequency changes.

Some correlation experiments have been done with inbred strains to compare nociception and antinociception between different nociceptive assays. A study of eight commonly used inbred strains examined the relationship of morphine antinociception in the hot-plate and writhing assays and did not find a significant correlation between responses on the two tests (Elmer et al., 1998). Likewise, in a study examining baseline nociceptive sensitivity on several nociceptive measures in eleven commonly used inbred strains, no significant correlation was found between responses on the hot-plate assay and the writhing assay (Mogil et al., 1999). These lines of evidence may support the conclusion that different genes influence nociceptive and antinociceptive responses on

the hot-plate versus the writhing assays. Both of these studies used a relatively small number of strains. Using more strains, such as the 25 BXD RI strains, would increase statistical power in experiments examining whether a zero correlation exists between responses on the hot-plate assay and writhing assay.

Selectively Bred Lines

Selectively bred lines have been used to estimate genetic variability of the selected trait and as a tool for genetic correlation studies. Specifically, the successful development of selectively bred lines demonstrates the importance of genetic influences on the selected trait. Through the selection process, a segregating gene causes simultaneous variation in all the traits it affects. Therefore, these lines can be used to identify genetic correlations between the selected trait and other traits of interest (Crabbe and Belknap, 1992). A limitation to selected lines is that some gene(s) irrelevant to the selected trait may have become fixed due to random changes in gene frequency (genetic drift) rather than the selection process. This may lead to false conclusions as to whether a trait is significantly correlated with the selected phenotype if the 'irrelevant' gene(s) is important to that particular trait. One way to avoid this is by testing selected lines early in the selection process while the effect of genetic drift is small. Another way is to create and test replicated lines to detect inconsistencies in genetic correlation estimates.

Two sets of lines selectively bred for high and low antinociception on the hot-plate test have been developed; for one set (HAR/LAR), antinociception was induced by levorphanol, a drug similar to morphine, and for the other (HA/LA) by cold water swim-stress . These lines were created from two different heterogenous stocks of mice by

mating the most antinociceptive mice together to form a high antinociceptive line and mating the least antinociceptive mice together to establish a low antinociceptive line. Using these lines, responses to other nociceptive assays have been tested to determine whether these traits are genetically correlated to the selected trait (Crabbe et al., 1990; Crabbe and Belknap, 1992). For example, the lines with high and low morphine-induced antinociception on the hot-plate (HAR/HA and LAR/LA, respectively) also exhibit high and low antinociception on another thermal, acute nociceptive test, the tail-withdrawal assay (Mogil et al., 1996). The low lines (LAR and LA) also have lower baseline latencies to nociceptive thermal stimuli than the high lines (HAR and HA) in both the hot-plate and tail-withdrawal assays. Therefore, it appears that both baseline nociceptive sensitivity and morphine-induced antinociception on the hot plate are positively correlated to these responses on the tail-withdrawal assay.

In contrast to responses on the thermal assays, Mogil and colleagues (1996) demonstrated that the HAR/LAR and HA/LA lines had opposite responses on the writhing assay. The high antinociceptive responders to the hot-plate assay (HA and HAR) were the low antinociceptive responders to the writhing assay, and the reverse was true for the low responders (LA and LAR). This suggests a negative genetic correlation between the hot plate and writhing assays. Since these two sets of lines were created separately in different labs yet yielded the same results, the evidence can be regarded as strong for the negative genetic correlation. A negative correlation implies that similar mechanisms underlie these two antinociceptive responses, but these mechanisms act in an opposing fashion. Yet it is unclear whether this is in fact the case because of the non-

significant correlation between the writhing and hot-plate assays using inbred strains. Therefore, it would be advantageous to see if QTLs for nociceptive and antinociceptive responses on the writhing assay are similar to QTLs found using the hot-plate assay but opposite in their direction of effect.

HYPOTHESES

This thesis addressed two broad questions. The first question was: are there genetic influences on nociceptive and antinociceptive responses in the acetic acid-induced writhing assay? The second question was: do these genetic influences differ from nociceptive and antinociceptive responses of other pain assays? The following hypotheses describe how these questions were addressed.

Hypothesis One: The first hypothesis was that several QTLs (genes) affect morphine-induced antinociception on the writhing assay. To test this, a two-step QTL chromosomal mapping project was carried out for morphine-induced antinociception assessed by the writhing assay. As many of the BXD RI strains as possible were utilized as the initial screen for the detection and mapping of candidate QTL sites. Candidate sites were tested in a second step using an B6D2F2 population comprised of about 300 mice, using the same protocol used in the BXD RI strains. Both sexes in the BXD RI strains and B6D2F2 population were phenotyped to determine whether a sex x strain interaction was present for morphine-induced antinociception. Separate QTL analyses for each sex were utilized where appropriate.

Hypothesis Two: The second hypothesis was that several QTLs (genes) affect baseline nociceptive sensitivity in the writhing test. A two-step QTL chromosomal mapping

project analyzed this possibility in the same manner as for morphine-induced antinociception to test this hypothesis. Both sexes in the BXD RI strains and B6D2F2 population were phenotyped to determine whether a sex x strain interaction was present for baseline nociceptive sensitivity. Separate QTL analyses for each sex were utilized where appropriate.

Hypothesis Three: The third hypothesis was that morphine-induced antinociception on the writhing assay is not significantly genetically correlated to morphine-induced antinociception on the hot-plate assay. Previous studies have shown no significant correlation between these two nociceptive assays in a panel of inbred strains and a significant negative correlation between the two assays in two separate pairs of selectively bred lines. This was assessed by correlation analysis on the BXD RI strain means for the hot-plate and writhing assays and comparing the short-term selection lines HMOR/LMOR in both nociceptive assays.

Hypothesis Four: The fourth hypothesis was that baseline nociceptive sensitivity on the hot-plate is not significantly genetically correlated to baseline nociceptive sensitivity in the writhing assay. The short-term selection lines, HMOR and LMOR, were tested in the writhing assay to determine their nociceptive sensitivity response compared to their nociceptive sensitivity response on the hot plate. The BXD strain means were analyzed by Pearson correlation to determine whether the hot-plate and writhing assays were significantly correlated.

RESEARCH DESIGN AND METHODS

Experiment One: BXD RI Strains QTL Analysis

Subjects

Mice in the BXD RI strain set, C57BL/6J, and DBA/2J inbred strains were purchased from the Jackson Laboratory, Bar Harbor, Maine and bred at the Portland Veterans Affairs Medical Center Veterinary Medical Unit (PVAMC VMU). Offspring from these matings were used for testing. The sexes were separated at weaning (21-25 days of age) and housed 1-4 mice per shoebox cage. Singly housed mice were avoided when possible. Food and water was available ad libitum. The colony rooms were on a 12:12 h light/dark cycle and temperature varied between 19° to 22°C. All mice were allowed to acclimate to individual colony rooms at least one week before testing to minimize the possibility of stress-induced antinociception. In addition, testing was avoided for 24 hours after a clean cage change. Mice were 65 to100 days of age on the day of morphine scoring (see below). A minimum of 16 mice per strain was used (8 per sex) with the exception of males in strains 8, 13, 15, and 28 (n=6-7). A total of 24 BXD RI strains were phenotyped in approximately fourteen passes.

Drugs

Morphine sulfate was obtained from NIDA Research Technology Branch (Bethesda, MD) and dissolved in 0.9% saline. Fresh solution was made on an as needed basis and kept at 5°C. Solution was never more than 4 weeks old. Glacial acetic acid was purchased from EM Science (Gibbstown, NJ) and diluted with 0.9% saline to make a final concentration of 0.65%. Acetic acid solution was made fresh every week.

Writhing Assay

Twenty-four of the 25 BXD RI strains mice were available to test for morphine-

induced antinociception and baseline nociceptive sensitivity using the writhing assay. All mice were acclimated to the procedure room for at least one hour prior to testing. Testing was done between the hours of 10:00 and 16:00 to avoid increases in baseline nociceptive sensitivity due to the dark photoperiod (Kavaliers and Hirst, 1983). Methods used for the writhing test were first described by Koster et al. (1959). In this test, mice were placed on a hard surface counter within Plexiglas cylinders (29 cm high; 30 cm diameter) and allowed 30 minutes to habituate to the cylinder. At this point the mice were weighed, injected with morphine (0.5 mg/kg) or saline (s.c.; 10 ml/kg), and then placed back into the cylinder. At 20 minutes post-injection, a dilute solution of acetic acid (0.65%) was injected (i.p.; 10 ml/kg). Mice were placed in the cylinders once again and observed continuously for 30 minutes after injection of the acetic acid for the number of writhes. These writhes, defined as lengthwise constrictions of the torso with a concomitant concave arching of the back, are quite stereotypical and can be distinguished from other behaviors. One to four mice were observed and scored at a time. A maximum of five sets of mice (n=20) were observed per day. Each mouse of the BXD RI strains was scored twice, once with a morphine injection and once with a saline injection, to obtain both a morphine antinociceptive and baseline nociceptive score. The morphine score test day was followed by a saline test day 7 to 9 days later.

QTL Mapping Analysis

Percent maximum possible effect (%MPE) of morphine-induced antinociception scores were calculated as [(saline score - morphine score)/(saline score)] x 100 for each individual mouse. This transformation takes into account and corrects for strain and

individual differences in baseline nociceptive sensitivity (saline) scores and may give a more accurate characterization of morphine's antinociceptive effect. These %MPE scores were used as a measure of morphine-induced antinociception. Because a cut-off of 100% MPE is imposed on the data, an arbitrary cut-off of -100% was used to better approximate a normal distribution. Any individual mouse with a score < -100% was given a score of -100%. The total number of constrictions in the saline condition was used as a measure of baseline nociceptive sensitivity.

Split-half reliability tests were run on the baseline nociceptive sensitivity and morphine-induced antinociception data separately. The most common reliability estimate is based on the split-half method (McNemar, 1962) for the total sample based on the correlation (r) between the two half-samples. The data were sorted by strain and sex and then numbered '1' or '2', consecutively, to yield two halves. A phenotypic correlation analysis was run between the two halves to obtain r for both baseline nociceptive sensitivity and morphine-induced antinociception. Reliability coefficients index the degree to which a given measure yields consistent or replicable results. This coefficient represents an estimate of the correlation coefficient expected between the presently observed and replicated strain means if this experiment is repeated at a future date. The coefficient was corrected by the Spearman-Brown correction factor.

The data from the BXD RI strains were subjected to QTL analysis (Gora-Maslak et al., 1991; Belknap et al., 1993) using two different programs, one developed by John Belknap and the Map Manager QT program designed by Ken Manly. For the BXD RI data, a regression of phenotype (strain means) on gene dosage at each marker, which is

equivalent to the correlation coefficient when there are only two genotypic classes, was used (Belknap et al., 1996). Both baseline nociceptive sensitivity and morphine-induced antinociception were examined. Provisional QTLs meeting the $p<0.05$ criterion (single test) were analyzed in a B6D2F2 population to confirm their validity.

The heritability of each trait was estimated by using the R^2 value of a one-way analysis of variance (ANOVA) on baseline and %MPE scores. The R^2 value is the proportion of the total variance for each trait due to genotype (strain). The R^2 value was calculated as SS_{strain}/SS_{total} which provides an estimate of the narrow sense (additive) heritability. A high value indicates a high 'signal' (genetic variation) to 'noise' (environmental variation) ratio, and the phenotypic strain means can be regarded as a more accurate predictor of genotypic value than if the R^2 value is low (Falconer and McKay, 1996). If R^2 is so low that it is not significant, then the trait is not significantly genetically determined.

Correlations between the writhing nociceptive assay and the hot-plate nociceptive assay were determined for the BXD RI strains on both morphine-induced antinociception and baseline nociceptive sensitivity. Unfortunately, only male mice have been completed in the BXD RI strains for morphine-induced antinociception on the hot-plate assay. Therefore, all correlations were made using only the data from male mice in both the writhing and hot-plate assays.

Experiment Two: B6D2F2 Population QTL Analysis

Subjects

(C57BL/6J x DBA/2J)F1 hybrid mice were purchased from the Jackson

Laboratory, Bar Harbor, Maine and bred at the PVAMC VMU to produce an F2 population (B6D2F2). The sexes were separated at weaning (21-25 days of age) and housed 2-4 mice per shoebox cage. Food and water were available ad libitum. The colony rooms were on a 12:12 h light/dark cycle. All mice were allowed to acclimate to individual colony rooms at least one week before testing. The mice were 65 to100 days of age on the day of morphine scoring.

The number (N) of the B6D2F2 mice tested depended on the heritability of the trait, or the proportion of the total variation due to genetic causes in the BXD RI strains. All calculations used the heritability of the baseline nociceptive sensitivity (combined sexes). This was calculated using the equation given by Soller et al. (1976) and Lander and Botstein (1989):

$$N_{(F2)}=(Z\alpha+Z\beta)^2(\sigma^2_{res}/\sigma^2_{exp})(1/\text{efficiency}).$$

$Z\alpha$ and $Z\beta$ are the normal variates for the desired α (0.0001, two-tailed, $Z\alpha$ =3.89) and β (0.2, one-tailed, $Z\beta$ =0.84), σ^2_{exp} is the variance explained by a QTL, σ^2_{res} is the residual (unexplained) variance in an F_2 population, and efficiency is the ratio of the amount of linkage information when selective genotyping is used versus when it is not used. Efficiency values are from Darvasi and Soller (1992) and Lander and Botstein (1989). The following assumptions were made: [1] α set at 0.0001 for any single marker, as recommended by Lander and Schork (1994); [2] a power (1-β), or the probability of correctly detecting a QTL, of 0.8; and [3] QTLs accounting for as little as 7% of the phenotypic variance (V_P). The efficiency value is 0.90 when 45% of the total mice are genotyped (Lander and Botstein, 1989). QTLs accounting for 7% of V_P correspond to

40% of the genetic variance (V_G) when the heritability (h^2_{F2}) is approximately 0.2. From the expression given above, the required N under these conditions is just under 300.

Writhing Assay

B6D2F2 mice were tested for morphine-induced antinociception and baseline nociceptive sensitivity using the writhing assay as described for the BXD RI strains in Experiment One. Each mouse of the B6D2F2 population was scored twice, once with morphine and once with saline, to obtain both a morphine antinociceptive and baseline nociceptive score. A morphine test day was followed by a saline test day 7 to 9 days later.

QTL Analysis

Both baseline nociceptive sensitivity and morphine-induced antinociception were analyzed in the B6D2F2 population. As before, percent maximum possible effect (%MPE) of morphine-induced antinociception scores were calculated as [(saline score - morphine score)/(saline score)] x 100 for each mouse. These %MPE scores were used as a measure of morphine-induced antinociception. Because a cut-off of 100% MPE is imposed on the data, an arbitrary cut-off of - 100% was used to better approximate a normal distribution. Any individual mouse with a score < - 100% was given a score of - 100%. The total number of constrictions in the saline condition was used as a measure of baseline nociceptive sensitivity.

DNA Isolation

The method for isolating DNA has been adapted for spleen using the protein salting-out method of Miller et al. (1988). B6D2F2 mice were sacrificed by cervical dislocation, and their spleens collected. Each spleen was cut in half and put in a 5 ml

capped tube containing 1 ml of saline. These tubes were stored in a -80°C freezer prior to spleen processing. Half of each spleen was placed in 10 ml of HBSS solution and strained. This extract was poured into a 50ml tube and spun at 1000 rpm at room temperature for 10 min. The pellet was removed and resuspended in 5 ml of lysis buffer (10 mM Tris-HCl, 400 mM NaCl and 2 mM Na_2EDTA, pH 8.2). RNAse (20ul of 500 uM stock solution) was added and the tissue incubated at 37°C while the tube was shaken gently. Immediately afterwards, 335 µl 10% SDS and 250 µl 10 mg/ml proteinase K solution were added to the suspension and incubated overnight at 45-55°C with gentle shaking. The samples were allowed to equilibrate to room temperature after the incubation. Then 1.65 ml of a saturated NaCl solution (6M) was added to each tube and shaken semi-vigorously for 15 sec. The tubes were spun at 3650 rpm at 5°C for 20 min. The supernatant was transferred to a new 50 ml tube and spun again. After the supernatant was equilibrated to room temperature, 2 volumes of room temperature absolute ethanol were added and the tubes inverted until the DNA precipitated. The precipitated strands were removed with a sterilized Pasteur pipette (previously flame-bent into a "J" shape), washed with 70% ethanol, and allowed to air dry until the DNA became transparent (4-5 min). Each pipette with DNA was transferred to a new 15 ml tube containing 4 ml of TE' buffer (5.6 mM Tris-HCl, 4.4 mM Tris Base, 0.1 mM Na_2EDTA, pH 8.0). After 5 min, the DNA was gently shaken from the pipette and was allowed to dissolve overnight at room temperature before quantitative analysis.

Genotyping

Selective genotyping was done as a cost and labor saving measure. Of the 300

mice in the B6D2F2 population, only the highest and lowest scoring 64 mice (32 of each sex) of the phenotypic distribution were genotyped, for a total of 128 animals. As recommended by Lander and Botstein (1989), this protocol required only 45% of the genotyping expense and effort compared to genotyping all mice, yet it retained 90% of the linkage information needed to detect and map QTLs. This is because the extreme scoring mice have a greater influence on the association between phenotype and genotype and are, therefore, more informative regarding the presence of a QTL. Using 128 B6D2F2 mice samples allowed the entire data set to be run on a four-lane agarose gel for each marker locus along with samples from each progenitor strain, their F1 intercross (positive control), and a basepair ladder to verify the marker's alleles.

Genotyping was carried out using microsatellite marker loci developed and characterized by Dr. Eric Lander's group at MIT (Dietrich et al., 1992). Over 1500 loci polymorphic in B6D2F2 intercrosses are distributed throughout the mouse genome, and most can be genotyped using the same experimental protocol with oligonucleotide primer pairs specific to each marker. The primer pairs were purchased from Research Genetics, Inc. (Huntsville, AL). PCR genotyping was a modification of that of Dietrich et al. (1992) to use ethidium bromide staining and high resolution agarose gel electrophoresis (MetaPhor, FMC) in place of ^{32}P radiolabeling and polyacrylamide. This reduces the cost and risk of toxicity, though some of the sensitivity to discriminate PCR products representing alleles $\leq$2% in bp length is lost. Using ethidium bromide and agarose gel electrophoresis works well for ~90% of the available MIT series microsatellite markers.

Statistics

The χ^2 test was used as our preliminary screen of associations between phenotype and genotype in the B6D2F2 population. Data for the BXD RI and B6D2F2 were combined using Fisher's method (Sokal and Rohlf, 1981). Those meeting the suggestive QTL criterion (LOD≥2.1) were subjected to further analysis using the MAPMAKER and MAPMAKER/QTL programs developed by Dr. Eric Lander's group at MIT. First, for the B6D2F2 data, the MAPMAKER/EXP 3.0 program was used to construct the primary linkage map for at least three microsatellite markers flanking each provisional QTL region, and the MAPMAKER/QTL 1.1 program was used to determine the presence of a QTL within this framework (Lander and Botstein, 1989; Lincoln et al., 1993; Lander et al, 1987). The latter program approximates linear regression of phenotype on gene dosage, but adds several features not seen in conventional linear statistics. The most important of these are (1) both additive and dominance effects (df=2) of a QTL are assessed; (2) interval analysis using maximum likelihood estimation is used conferring greater power to detect QTLs that may be located between markers; (3) a genotyping error check routine is built-in (Lincoln and Lander, 1992); and (4) correction for missing genotyping data. This last feature was particularly critical for the present studies, since only the extreme ends of the phenotypic distribution were genotyped, leaving the middle of the distribution as 'missing' data (Lander and Botstein, 1989).

Experiment 3: HMOR/LMOR Short-term Selection Lines in the Writhing Test

Subjects

(C57BL/6J x DBA/2J)F1 hybrid mice were purchased from the Jackson

Laboratory, Bar Harbor, Maine and bred at the PVAMC VMU to produce B6D2F2 mice. The sexes were separated at weaning (21-25 days of age) and housed 1-4 mice per shoebox cage. Food and water was available ad libitum. The colony rooms were on a 12:12 h light/dark cycle. All mice were allowed to acclimate to individual colony rooms at least one week before testing. The age of the mice was from 56 to100 days on the day of morphine scoring.

Selection Protocol

The HMOR and LMOR short-term selection lines were created from this B6D2F2 stock by mating mice that were either high (HMOR) or low (LMOR) morphine-induced antinociceptive responders on the hot-plate assay, respectively. A single dose of morphine (12 mg/kg) was used to define high and low antinociceptive responders in generations one and two. Because many of the HMOR mice were reaching the maximum possible response to morphine (ceiling effect), the morphine dose was reduced by half (6 mg/kg) to test HMOR mice in the third selection generation. The LMOR mice continued to receive 12 mg/kg as in the previous generations. Individual selection was used to create the lines; sibling pairs were avoided. This selective breeding was carried out for four consecutive generations. Seven breeder pairs plus one alternate pair were bred in each generation. By the second generation of selection, the difference in antinociceptive response on the hot plate as measured by %MPE scores was two times higher in the HMOR line as compared to the LMOR line while the baseline nociceptive sensitivity did not differ (see Figs. 4 and 5).

Writhing Assay

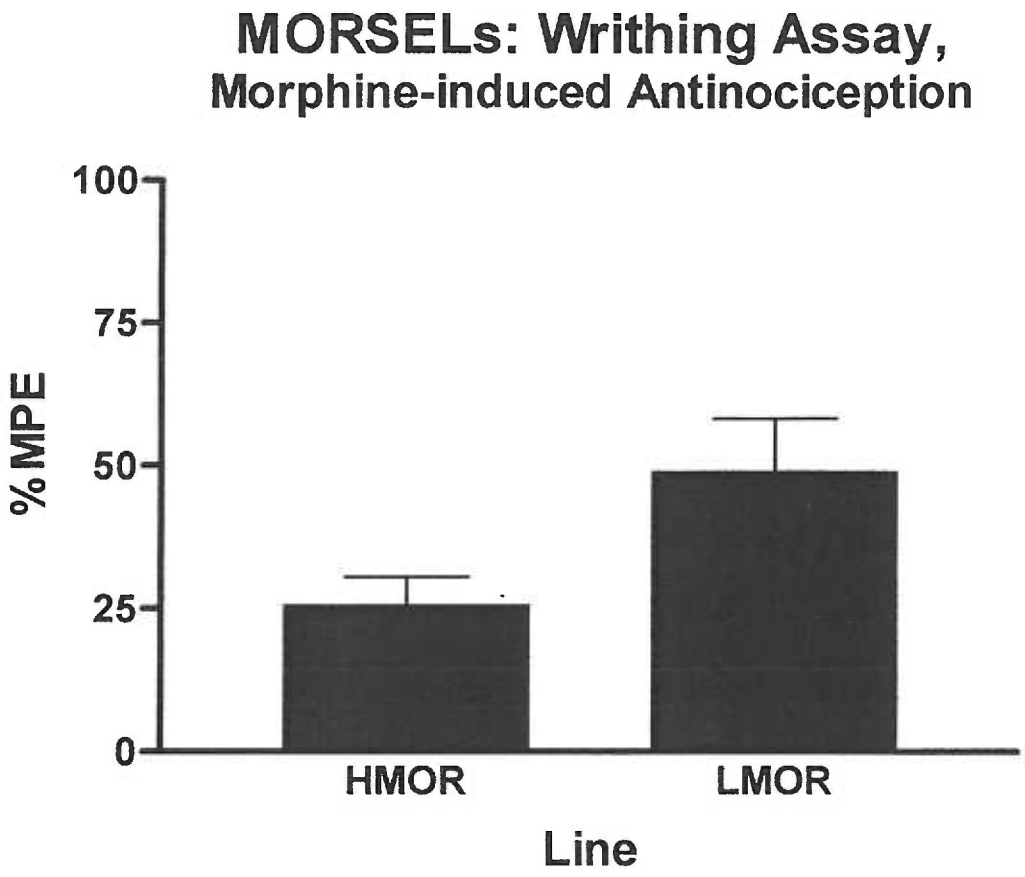

Figure 4. Selection response of HMOR and LMOR selection lines over generations to morphine-induced antinociception on the hot-plate assay. Symbols indicate the amount of morphine antinociception calculated as %MPE (see text) and error bars are standard error of the mean.

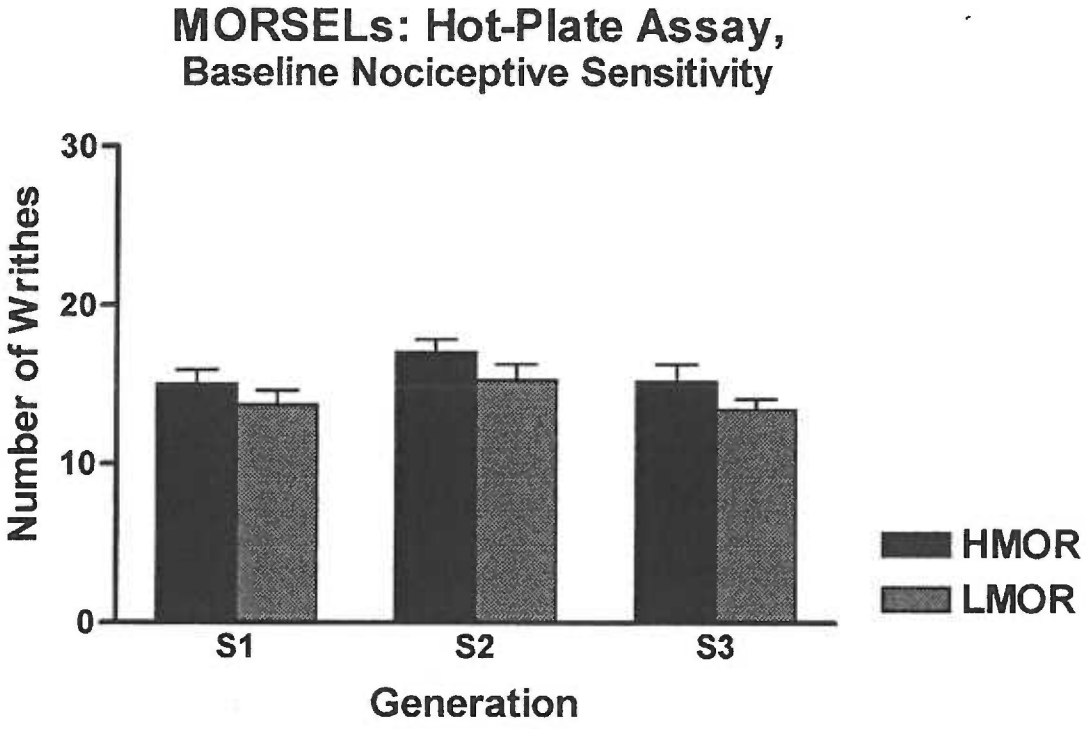

Figure 5. Selection response of HMOR and LMOR selection lines over generations to baseline nociceptive sensitivity on the hot-plate assay. Bars indicate the mean latency to response in seconds and error bars represent the standard error of the mean.

Fourth generation, naïve HMOR and LMOR mice were tested for morphine-induced antinociception and baseline nociceptive sensitivity using the writhing assay as described for the BXD RI strains and B6D2F2 hybrids. Each mouse of the HMOR and LMOR selected lines was scored twice, once with morphine and once with saline, to obtain both a morphine antinociceptive and baseline nociceptive score. A morphine score test day was followed by a saline test day 7 to 9 days later.

Statistical Analyses

As in Experiments One and Two, percent maximum possible effect (%MPE) of morphine-induced antinociception scores were calculated as [(saline score - morphine score)/(saline score)] x 100 for each individual mouse. These %MPE scores were used as a measure of morphine-induced antinociception. Because a cut-off of 100% MPE is imposed on the data, an arbitrary cut-off of - 100% was used to better approximate a normal distribution. The total number of constrictions in the saline condition was used as a measure of baseline nociceptive sensitivity.

Comparisons were made between the two nociceptive assays for the HMOR and LMOR selection lines. This was to address the issue as to whether nociceptive tests have the same underlying genetic components affecting the outcome of nociceptive behavior.

RESULTS

Experiment One: C57BL/6, DBA/2, and BXD RI Strains

Baseline Nociceptive Sensitivity

For baseline nociceptive sensitivity, a continuous distribution of strain means expressed as total number of constrictions was observed (see Fig. 6). An ANOVA

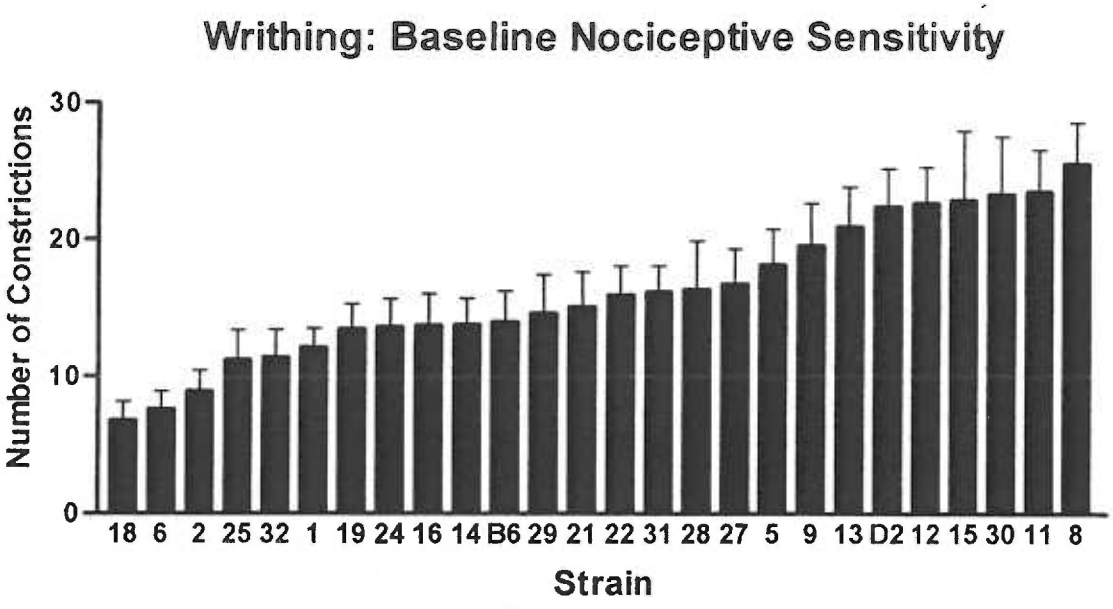

Figure 6. Baseline nociceptive responses of BXD RI, C57BL/6 (B6), and DBA/2 (D2) strains on the writhing assay. Each bar represents the mean of total number of constrictions in a 30 min period for each strain collapsed over sex. Error bars indicate the standard error of the mean for each strain mean. The estimated heritability was R^2=0.34.

revealed a significant main effect of strain ($F_{25,469}$=5.00, $p<0.001$) and sex ($F_{1,469}$=38.051, $p<0.001$) and a strain x sex interaction ($F_{25,469}$=5.00, $p<0.01$). That is, within some strains females had higher scores (more writhes) than males while other strains showed the reverse, and some strains had no difference between the sexes (see Fig. 7). Since approximately equal numbers of males and females were tested for each strain, the strain x sex interaction most likely is not due to sampling error.

Heritability for baseline nociception response for combined sexes on the writhing assay was estimated at R^2=0.33. When analyzed separately, heritability for female mice was estimated at R^2=0.31 and for male mice was R^2=0.25. A split-half reliability estimate was calculated for this data as three different sets: combined sexes, females, and males. When used as a measure of baseline nociceptive sensitivity, the acetic acid-induced writhing assay was found to be a reliable measure, r=0.77 ($p<0.001$), r=0.84 ($p<0.001$), and r=0.67 ($p<0.001$) for combined sexes, females, and males, respectively.

Because a strain x sex interaction was found, three separate QTL analyses were completed: one using combined sexes strain means, one using only female strain means and one using only male strain means. A QTL analysis was done for combined sexes for a comparison to the morphine-induced antinociception data set, as no strain x sex interaction was found (see below). Tables 1, 2, and 3 outline the results from these analyses. Seven provisional QTLs ($p<0.05$) were found when sexes were combined, while separate analyses revealed ten and six provisional QTLs for females and males, respectively. Seven female-specific provisional QTLs and three male-specific provisional QTLs were discovered represented by '♀' and '♂' respectively.

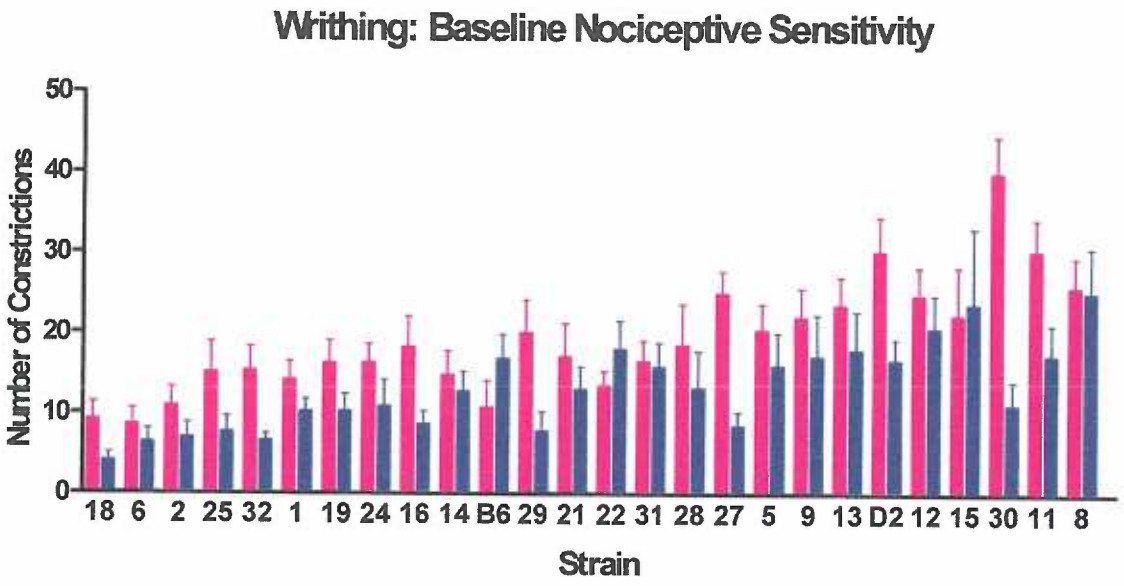

Figure 7. Baseline nociceptive responses of BXD RI, C57BL/6 (B6), and DBA/2 (D2) strains on the writhing assay, separated by sex. Red bars represent the mean of total number of constrictions in a 30 min period for female mice of each strain while blue bars represent the means for male mice. Error bars indicate the standard error of the mean for each strain mean. Heritability estimates were R^2=0.22 for males and R^2=0.33 for females.

Table 1. BXD RI QTL Analyses Results: Baseline Nociceptive Sensitivity, Combined Sexes

Analysis	Chr.	Range*	Peak Marker†	High Allele ‡	p level
Combined Sexes	3	29-50	D3Byu17 (29.6 cM)	D2	0.0016
	7	3-6	Mr66-2 (5 cM)	B6	0.0035
	9	28-31	D9Mit4 (29 cM)	B6	0.011
	10	60-69	D10Mit14 (65 cM)	B6	0.00038
	11	26-35	Rcvrn (35 cM)	B6	0.025
	14	1-3	D14Ncvs44 (1.5 cM)	B6	0.018
	16	3-11	D16J1 (10.7 cM)	B6	0.010

*range of markers $p<0.05$
†marker with greatest significance
‡allele conferring high baseline nociceptive sensitivity

Table 2. BXD RI QTL Analyses Results: Baseline Nociceptive Sensitivity, Females

Analysis	Chr.	Range*	Peak Marker†	High Allele ‡	p level
Female	1♀	70-99	D1Ncvs48 (79 cM)	D2	0.002
	2♀	69-71	D2Rik63 (69-71 cM)	D2	0.019
	3	29-50	D3Byu17 (29.6 cM)	D2	0.0037
	7♀	3-25	Mr66-2 (5 cM)	B6	0.0066
	9♀	28-31, 54-71	D9Mit4 (29 cM), D9Mit18 (71 cM)	B6, B6	0.019, 0.021
	10	60-69	D10Mit14 (65 cM)	B6	0.013
	11	26-32	D11Ncvs47 (26 cM)	B6	0.034
	14♀	1-3	D14Byu1 (0.5 cM)	B6	0.020
	16♀	25-32	Hmg1-rs7 (18.95 cM)	D2	0.024
	19♀	7-17	D19Ncvs20 (17 cM)	D2	0.0032

*range of markers $p<0.05$
†marker with greatest significance
‡allele conferring high baseline nociceptive sensitivity

Table 3. BXD RI QTL Analyses Results: Baseline Nociceptive Sensitivity, Males

Analysis	Chr.	Range*	Peak Marker†	High Allele ‡	p level
Male	2♂	83-91	D2Rik67 (83-91 cM)	B6	0.034
	3	29-56	D3Ncvs45 (55 cM)	D2	0.0047
	10	60-69	D10Mit14 (65 cM)	B6	0.00016
	11	0-35	D11Ncvs74 (0 cM)	B6	0.024
	16♂	3-11	Iapls3-6(10.7 cM)	B6	0.00038
	17♂	24-32	Upg1 (30 cM)	B6	0.013

*range of markers $p<0.05$
†marker with greatest significance
‡allele conferring high baseline nociceptive sensitivity

Morphine-induced Antinociception

A continuous distribution of strain means expressed as %MPE was observed using a dose of 0.5 mg/kg morphine (Fig. 8). Unlike baseline nociception, no sex difference or strain x sex interaction was revealed ($F_{1,447}$=1.08, n.s.; $F_{25,447}$=1.19, n.s., respectively). Both sexes were combined yielding a significant main effect of strain ($F_{25,447}$=2.39, $p<0.001$). The estimated heritability was R^2=0.14. Although this is a low heritability estimate, a QTL analysis was completed for this trait because a significant main effect of strain was revealed by ANOVA, and the trait was found to be significantly reliable by the split-half reliability test (r=0.467, $p<0.05$). A QTL analysis also allowed a comparison between this trait and the BXD QTL analysis of morphine-induced antinociception on the hot-plate. The low heritability and reliability for this trait, however, were not ideal for successful QTL detection and mapping. Seven provisional QTLs emerged from the BXD RI QTL analysis (see Table 4).

Genetic Correlations

Baseline nociceptive sensitivity on the writhing assay was significantly, negatively correlated with morphine-induced antinociception (%MPE) on the writhing assay but only in the female mice (r=-0.46, $p<0.05$;see Fig. 9). However, an influence analysis indicated BXD strain 30 was an outlier in these data. Without this strain, the correlation is not significant (r=-0.35, p=0.08). No significant correlation was found in the male mice as shown in Fig. 10 (r=0.027, n.s.). This is unlike results using the hot plate assay in which a significant positive correlation was found between baseline nociceptive sensitivity and morphine-induced antinociception (data not shown).

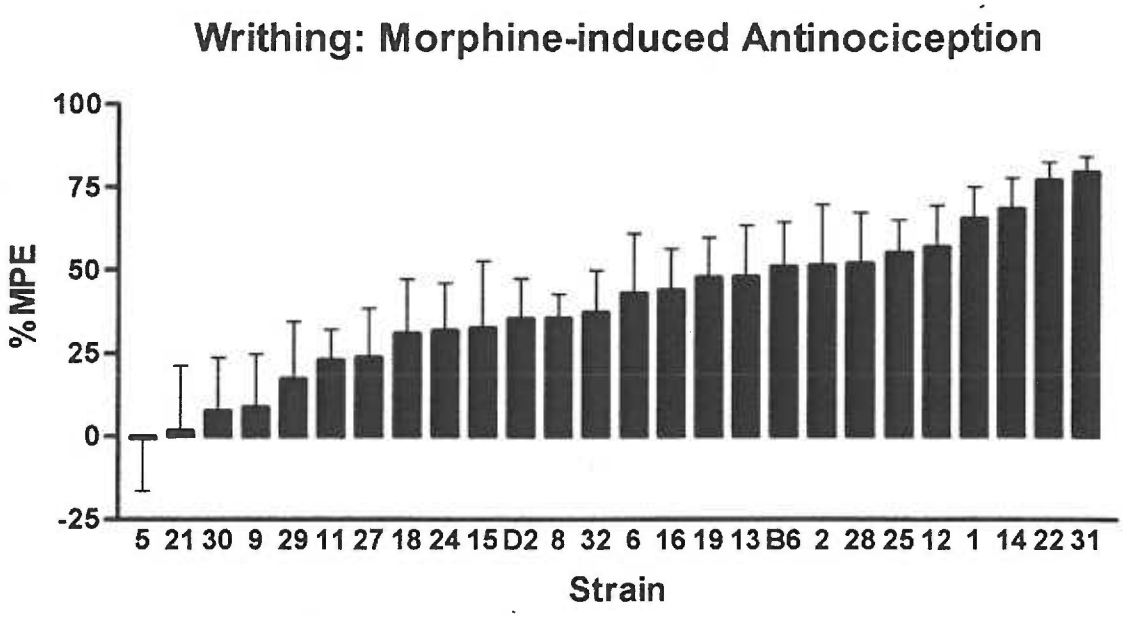

Figure 8. Morphine-induced antinociception of the BXD RI, C57BL/6 (B6), and DBA/2 (D2) strains on the writhing assay. Each bar represents the %MPE (see text) for each strain collapsed over sex. Error bars indicate the standard error of the mean for each strain mean. The estimated heritability was R^2=0.14.

Table 4. BXD RI QTL Analyses Results: Morphine-induced Antinociception

Analysis	Chr.	Range*	Peak Marker†	High Allele‡	p level
Combined Sexes	1	64-96	D1Ncvs46 (71 cM)	B6	0.0073
	2	47-71	Mdk (49 cM)	B6	0.0042
	3	29-47	D3Byu17	B6	0.015
	7	3-29	Tstap 198-7 (25 cM)	D2	0.00049
	8	52-56	Zfp4 (52 cM)	B6	0.0016
	11	26-32	D11Ncvs47 (26 cM)	D2	0.0050
	15	57-65	Nfe2 (58.1 cM)	B6	0.0050

*range of markers $p<0.05$
†marker with greatest significance
‡allele conferring high morphine-induced antinociception

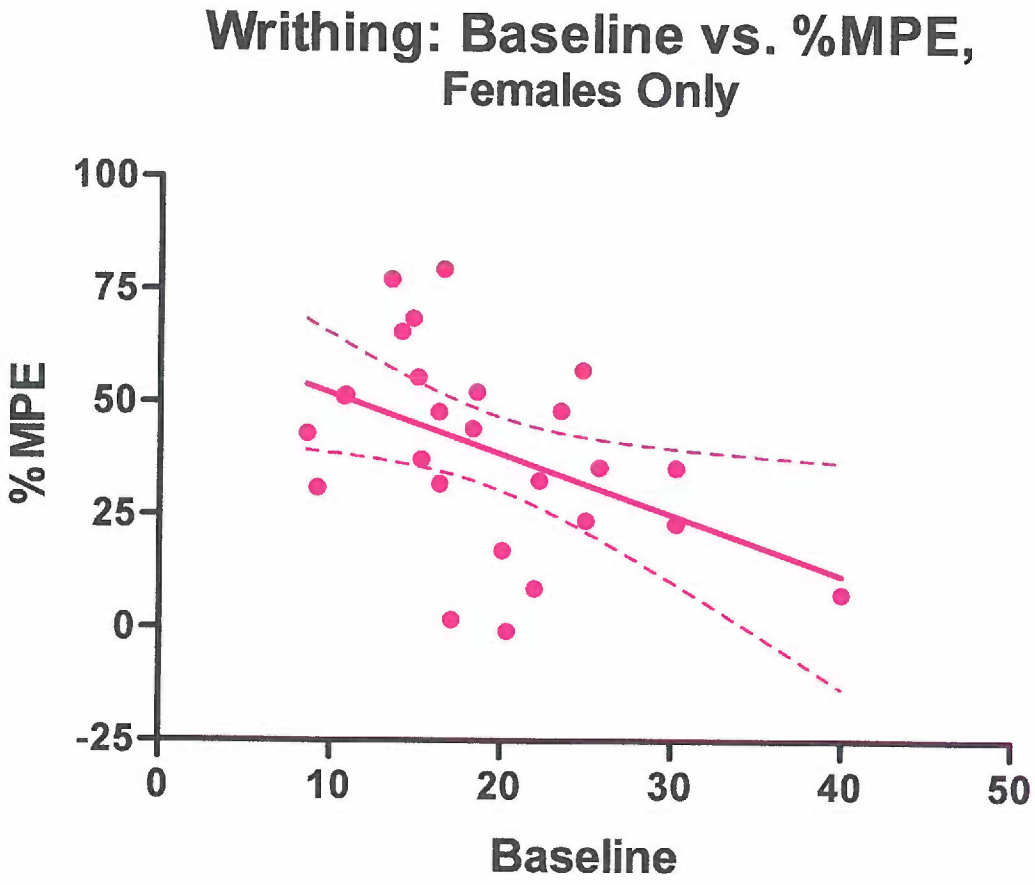

Figure 9. Scatterplot and regression line of baseline nociceptive sensitivity with morphine-induced antinociception in the writhing assay for female mice of the BXD RI, C57BL/6, and DBA/2 strains. A significant inverse correlation was found suggesting high nociceptive sensitivity correlates with low antinociceptive responses in female mice ($r = -0.46$, $p<0.05$). However, influence analysis suggests strain 30 is an outlier, and the correlation is not significant without this strain ($r = -0.352$, $p=.085$).

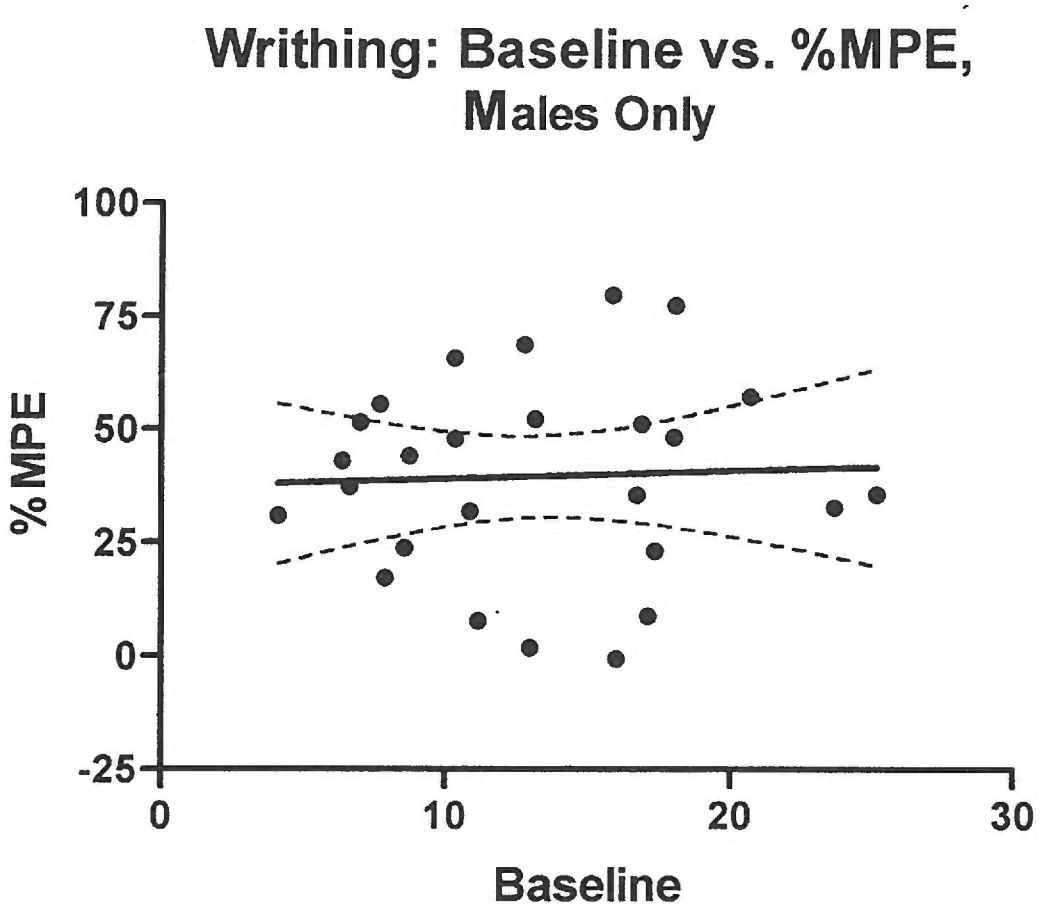

Figure 10. Scatterplot and regression line of baseline nociceptive sensitivity with morphine-induced antinociception in the writhing assay for male mice of the BXD RI, C57BL/6, and DBA/2 strains. No significant correlation was found (r= 0.027, n.s.).

Baseline nociceptive sensitivity between the hot-plate and writhing assays was not significantly correlated (r=0.247, n.s.) as shown in Fig. 11. The lack of a significant correlation supports findings in at least one previous study of inbred mice (Mogil et al., 1999). As seen in Fig. 12, morphine-induced antinociception between the hot-plate and writhing assays also was not significantly correlated (r=-0.183, n.s.). This result also supports previous findings (Elmer et al., 1998).

Experiment Two: B6D2F2 Intercross Population

Baseline Nociceptive Sensitivity

As in the BXD strain analysis, analyses were run on three sets: combined sexes, males, and females. Each analysis corresponded to the previous BXD analysis. Markers used for QTL analysis are shown in Table 5. Only chromosomes with three or more markers attaining $p<0.05$ in the BXD RI data set were analyzed by PCR genotyping markers in the corresponding chromosomal region in the B6D2F2 population. A minimum of three markers were used per QTL region found in the BXD RI QTL analysis. Combining BXD RI and B6D2F2 data (χ^2 analysis) using Fisher's method (Sokal and Rohlf, 1981), several suggestive QTLs were found for baseline nociceptive sensitivity (see Tables 6, 7, and 8). These were subjected to interval analysis using MapMaker and MapMaker QTL.

Interval analysis on the combined sexes data detected one suggestive QTL on chromosome 10 (Fig. 13) when data from the BXD RI and B6D2F2 populations were combined. One suggestive QTL on chromosome 19 was observed in the females only analysis (Fig. 14). This QTL was not found in the males only data and, hence, a

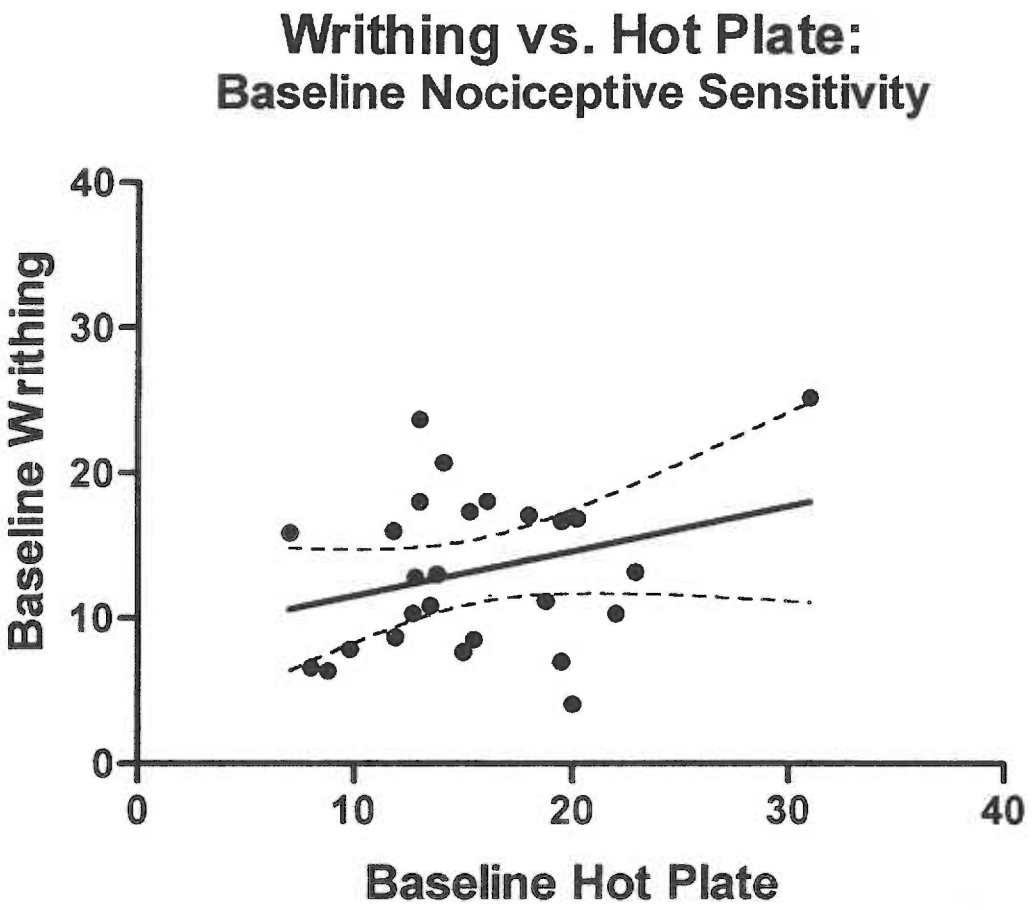

Figure 11. Scatterplot and regression line of hot-plate baseline nociceptive sensitivity with writhing assay baseline nociceptive sensitivity mice of the BXD RI, C57BL/6, and DBA/2 strains. No significant correlation was found ($r = 0.25$, n.s.).

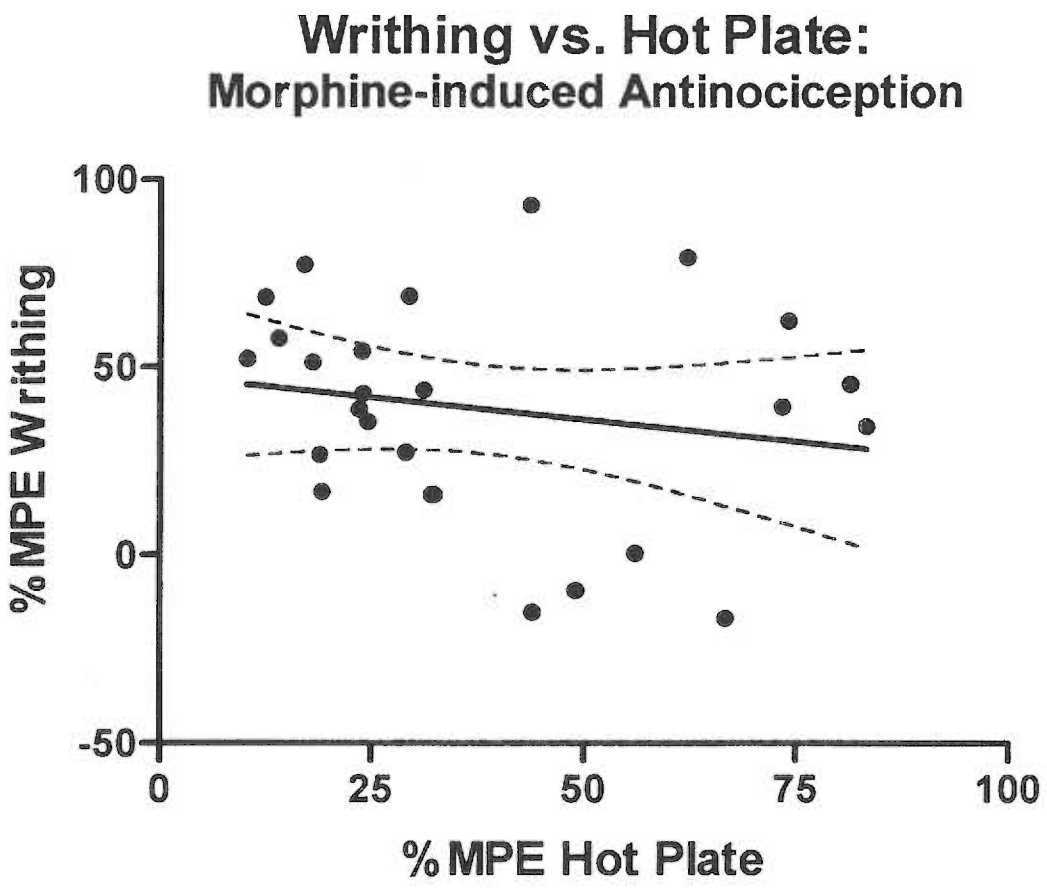

Figure 12. Scatterplot and regression line of hot-plate morphine-induced antinociception with writhing assay morphine-induced antinociception of the BXD RI, C57BL/6, and DBA/2 strains. No significant correlation was found (r= −0.18, n.s.).

Table 5. B6D2F2 Marker Analysis Results: Baseline Nociceptive Sensitivity

Chromosome/ Marker	cM position	Comb. Sex p level	Female p level*	Male p level*
Chromosome 1				
D1M101	73	0.36	0.11	0.23
D1M110	87.9	0.31	0.24	0.50
D1M150	100	0.022	0.233	0.017
Chromosome 2				
D2M102	62	0.44	0.052	0.074
D2M223	76.7	0.19	0.292	0.039(D2)
D2M285	86	0.085	0.34	0.012(D2)
Chromosome 3				
D3M69	29.5	0.36	0.43	0.36
D3M9	38.3	0.49	0.29	0.37
D3M12	49.2	0.35	0.35	0.43
Chromosome 7				
D7M57	4	0.23	0.15	0.50
D7M246	15	0.31	0.19	0.44
D7M84	28.4	0.36	0.43	0.19
Chromosome 9				
D9M205	18	0.13	0.24	0.19
D9M232	31	0.053	0.24	0.056
D9M8	42	0.017	0.055	0.081
D9M182	55	0.019	0.13	0.039

* '( )' indicate when B6D2F2 high allele is in opposite direction as BXD RI high allele

Table 5. Continued

Chromosome/ Marker	cM position	Comb. Sex p level	Female p level*	Male p level*
Chromosome 10				
D10M11	50	0.056	0.20	0.082
D10M162	59	0.13	0.15	0.30
D10M297	70	0.022	0.010	0.30
Chromosome 11				
D11M82	14	0.003	0.024	0.028
D11M86	28	0.02	0.18	0.027
D11M179	52	0.16	0.44	0.11
Chromosome 14				
D14M1	3	0.49	0.23	0.22
D14M54	12.5	0.12	0.023(D2)	0.40
Chromosome 16				
D16M9	4	0.11	0.19	0.19
D16M165	10.3	0.22	0.098	0.42
D16M138	31	0.22	0.17	0.44
Chromosome 17				
D17M198	16	0.00022	0.083	0.00022
D17M10	24.5	0.0014	0.026	0.012
D17M53	38.5	0.030	0.15	0.056

* '( )' indicate when B6D2F2 high allele is in opposite direction as BXD RI high allele

Table 5. Continued

Chromosome/ Marker	cM position	Comb. Sex p level	Female p level*	Male p level*
Chromosome 19				
D19M61	9	0.28	0.035	0.49
D19M16	15	0.49	0.098	0.094
D19M40	25	0.45	0.18	0.24

* '( )' indicate when B6D2F2 high allele is in opposite direction as BXD RI high allele

Table 6. BXD and B6D2F2 Confirmation Combined Results: Combined Sexes Baseline Nociceptive Sensitivity

Analysis	Chr.	Range*	BXD High Allele	BXD p level†	B6D2F2 p level‡	Combined p level	Combined LOD
Combined Sexes	1♀	70-99	D2	--	0.31	n.s.	
	2♀	69-71	D2	--	0.44	n.s.	
	2♂	83-91	B6	--	0.085	n.s.	
	3	30-55	D2	0.0016	0.35	n.s.	
	7	0-6	B6	0.0035	0.23	n.s.	
	9	28-31	B6	0.011	0.019	n.s.	
	10	61-67	B6	0.00038	0.022	0.00011	3.26
	11	26-35	B6	0.025	0.02	n.s.	
	14	1-3	B6	0.018	0.12	n.s.	
	16	3-25	B6	0.010	0.11	n.s.	
	17♂	25-32	B6	--	0.00022	0.00022	2.96
	19♀	7-24	D2	0.044	0.28	n.s.	

*range of markers $p<0.05$
†marker with greatest significance
‡marker with greatest significance
'( )' indicate when B6D2F2 high allele is in opposite direction as BXD RI high allele

Table 7. BXD and B6D2F2 Confirmation Combined Results: Female Baseline Nociceptive Sensitivity

Analysis	Chr.	Range*	BXD High Allele	BXD p level†	B6D2F2 p level‡	Combined p level	Combined LOD
Female	1♀	70-99	D2	0.002	0.11	n.s.	
	2♀	69-71	D2	0.019	0.052	n.s.	
	2♂	83-91	B6	--	0.34	n.s.	
	3	29-50	D2	0.0037	0.35	n.s.	
	7♀	3-25	B6	0.0066	0.15	n.s.	
	9♀	28-31, 54-71	B6	0.019, 0.021	0.054, 0.13	n.s., n.s.	
	10	60-69	B6	0.013	0.01	0.0013	2.25
	11	26-32	B6	0.034	0.024	n.s.	
	14♀	1-3	B6	0.020	0.023(D2)	n.s.	
	16♂	3-11	B6	--	0.19	n.s.	
	16♀	27-32	D2	0.024	0.098	n.s.	
	17♂	24-32	B6	0.024	0.083	n.s.	
	19♀	7-17	D2	0.0032	0.035	0.0011	2.30

*range of markers $p<0.05$
†marker with greatest significance
‡marker with greatest significance
'( )' indicate when B6D2F2 high allele is in opposite direction as BXD RI high allele

Table 8. BXD and B6D2F2 Confirmation Combined Results: Male Baseline Nociceptive Sensitivity

Analysis	Chr.	Range*	BXD High Allele	BXD p level†	B6D2F2 p level‡	Combined p level	Combined LOD
Male	1♀	70-99	D2	--	0.23	n.s.	
	2♀	69-71	D2	--	0.074	n.s	
	2♂	83-91	B6	0.034	0.012(D2)	n.s.	
	3	29-56	D2	0.0047	0.31	n.s.	
	7♀	3-25	B6	--	0.19	n.s	
	9♀	28-31, 54-71	B6	--	0.056, 0.039	n.s, n.s	
	10	60-69	B6	0.00016	0.082	0.00016	3.09
	11	0-35	B6	0.024	0.027	n.s.	
	14♀	1-3	B6	--	0.22	n.s	
	16♂	3-11	B6	0.00038	0.19	0.00076	2.46
	16♀	27-32	D2	--	0.44		
	17♂	24-32	B6	0.013	0.012	0.0015	2.18
	19♀	7-17	D2	--	0.24	n.s	

*range of markers $p<0.05$
†marker with greatest significance
‡marker with greatest significance
'( )' indicate when B6D2F2 high allele is in opposite direction as BXD RI high allele

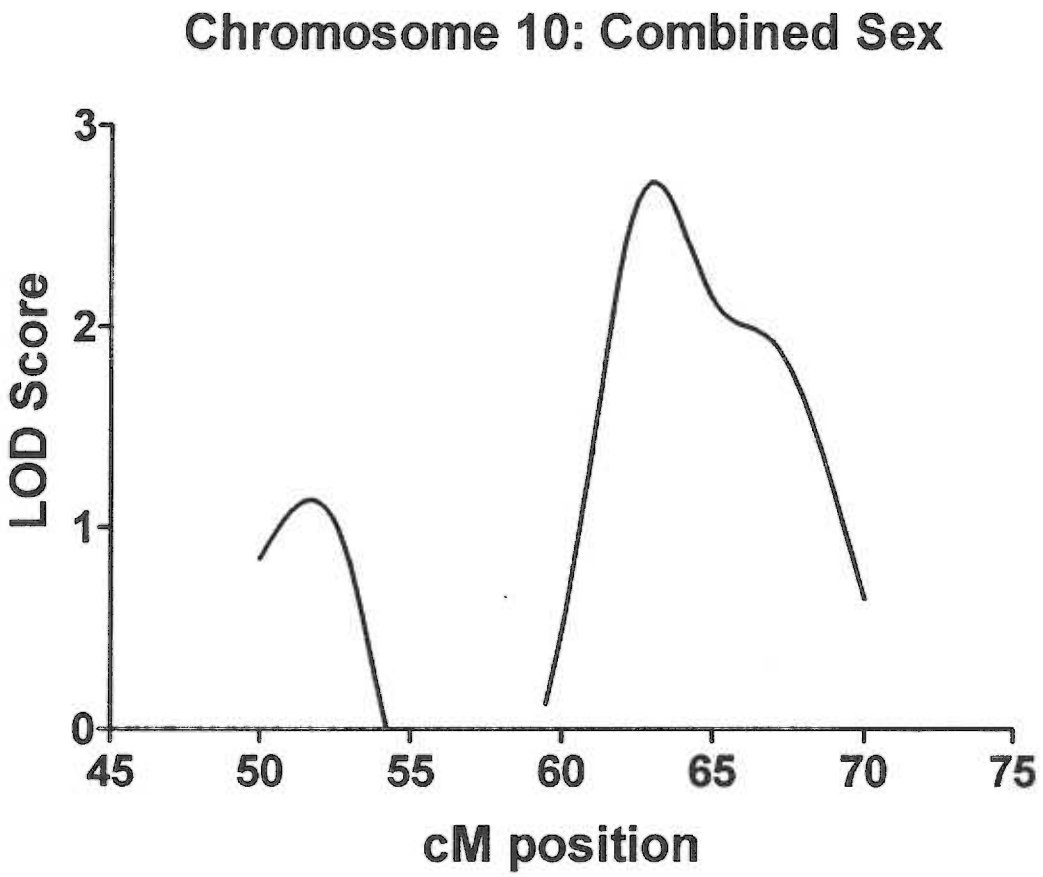

Figure 13. Range of suggestive QTL on chromosome 10 for baseline nociceptive sensitivity, combined sexes analysis. Data were combined from BXD RI and B6D2F2 QTL analyses.

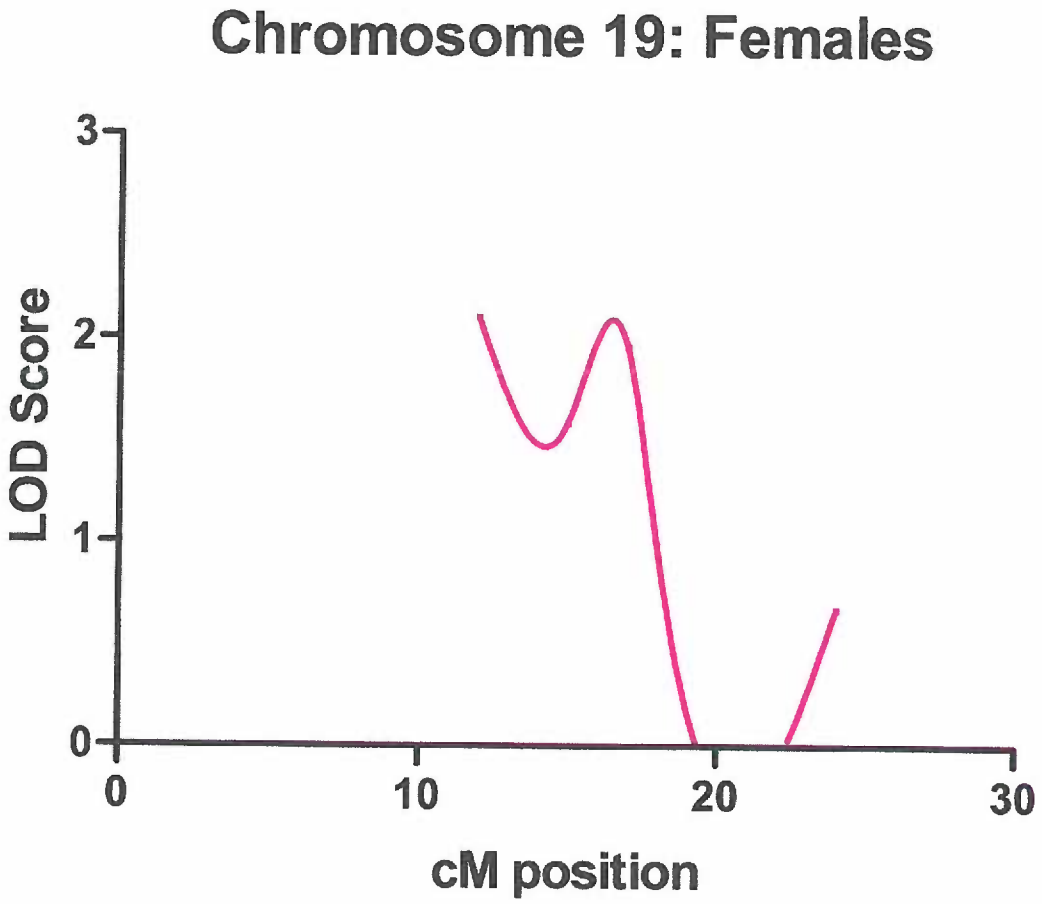

Figure 14. Range of suggestive QTL on chromosome 19 for baseline nociceptive sensitivity, females only analysis. Data were combined from BXD RI and B6D2F2 QTL analyses.

significant sex difference emerged (χ^2=9.28, p<0.01). Interval analysis on the males only data detected two suggestive QTLs on chromosomes 10 and 17 (Fig. 15 and 16). Neither of these was significantly different from data analyses using females only (χ^2=3.91, n.s.; χ^2=4.04, n.s., respectively).

Morphine-induced Antinociception

Markers used for the QTL analysis are shown in Table 9. No QTLs were confirmed to be significant in the B6D2F2 population (Table 10), but one suggestive QTL did appear on chromosome seven with χ^2 analysis. Interval analysis, however, did not detect this provisional QTL as suggestive. The low rate of confirmed QTLs is most likely due to the low heritability and reliability of this trait.

Experiment 3: HMOR/LMOR Short-term Selection Lines

Baseline Nociceptive Sensitivity

A significant main effect of line (data combined over sex) was found with ANOVA ($F_{1,36}$=5.08, p<0.05) in the writhing assay (Fig. 17). This is in contrast to the hot-plate baseline nociceptive sensitivity in which HMOR and LMOR mice did not differ in baseline response in selection generations one through three. This suggests different genes underlie baseline nociceptive sensitivity in the hot-plate versus baseline nociceptive sensitivity in the writhing assay.

Morphine-induced antinociception

No main effect of line (data combined over sex) was detected ($F_{1,36}$=5.00, n.s.; Fig. 18). This data was in contrast to the hot-plate data of the second selection generation in which a significant main effect of line (p<0.001) was discovered (see

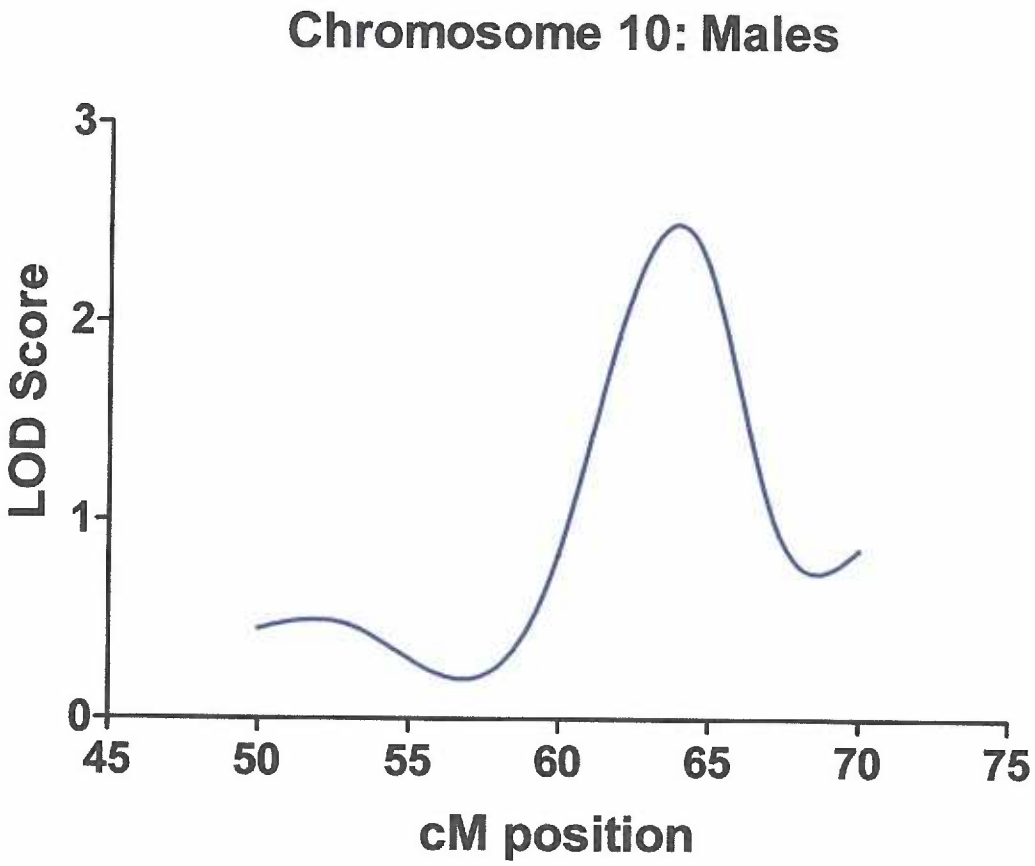

Figure 15. Range of suggestive QTL on chromosome 10 for baseline nociceptive sensitivity, males only analysis. Data were combined from BXD RI and B6D2F2 QTL analyses.

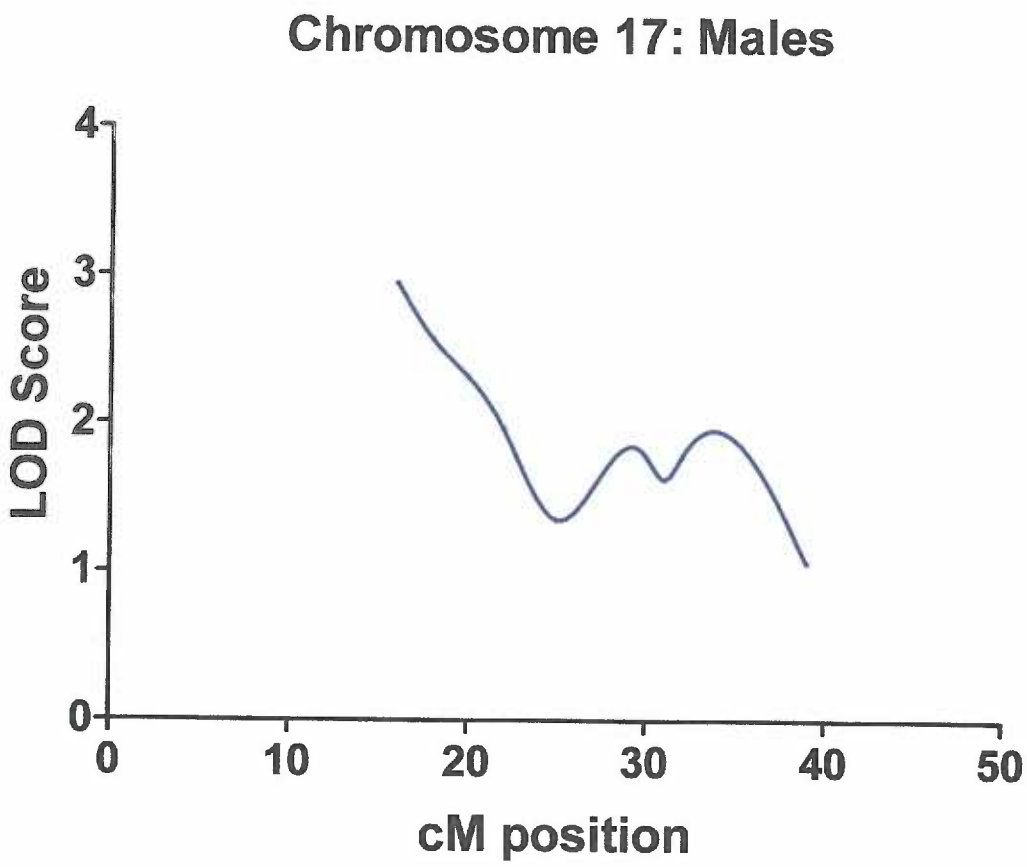

Figure 16. Range of suggestive QTL on chromosome 17 for baseline nociceptive sensitivity, males only analysis. Data were combined from BXD RI and B6D2F2 QTL analyses.

Table 9. B6D2F2 Marker Analysis Results: Morphine-induced Antinociception

Chromosome/ Marker	cM position	Combined Sex p level*
Chromosome 1		
D1M191	63.1	0.052 (D2)
D1M101	73	0.19
D1M110	87.9	0.037 (D2)
Chromosome 2		
D2M14	48.5	0.31
D2M102	62	0.37
D2M223	76.7	0.29
Chromosome 3		
D3M69	29.5	0.36
D3M9		
D3M12	49.2	0.26
Chromosome 7		
D7M57	4	0.36
D7M246	15	0.19
D7M84	28.4	0.11
Chromosome 8		
D8M312	45	0.22
D8M88	58	0.50

* '( )' indicate when B6D2F2 high allele is in opposite direction as BXD RI high allele

Table 9. Cont.

Chromosome/ Marker	**cM position**	**Combined Sex p level**
Chromosome 11		
D11M82	14	0.052 (B6)
D11M86	28	0.47
D11M4	37	0.40
Chromosome 15		
D15M189	48.5	0.50
D15M48	60	0.40
D15M161	69.2	0.35

Table 10. BXD and B6D2F2 Confirmation Combined Results: Morphine-induced Antinociception

Analysis	Chr.	Range*	BXD High Allele	BXD p level†	B6D2F2 p level‡	Combined p level	Combined LOD
Combined Sexes	1	64-87	B6	0.0073	0.037(D2)	n.s.	
	2	47-71	B6	0.0042	0.29	n.s.	
	3	29-47	B6	0.015			
	7	0-30	D2	0.00049	0.11	0.00058	2.57
	8	52-59	B6	0.0016	0.50	n.s.	
	11	26-32	D2	0.0050	0.052(B6)	n.s.	
	15	57-59	B6	0.0050	0.35	n.s.	

*range of markers $p<0.05$
†marker with greatest significance
‡marker with greatest significance
'( )' indicate when B6D2F2 high allele is in opposite direction as BXD RI high allele

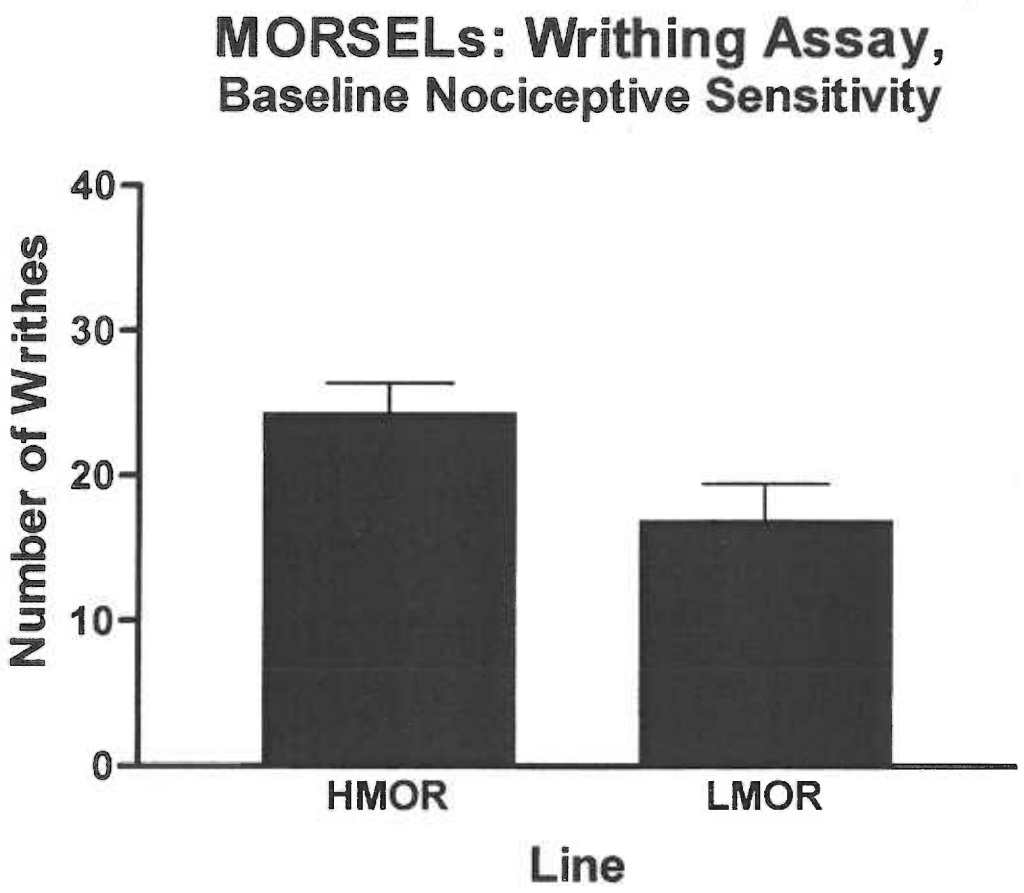

Figure 17. Baseline nociceptive responses of fourth generation HMOR and LMOR selection lines on the writhing assay. Each bar represents the mean of total number of constrictions in a 30 min period for each line collapsed over sex. Error bars indicate the standard error of the mean for each strain mean.

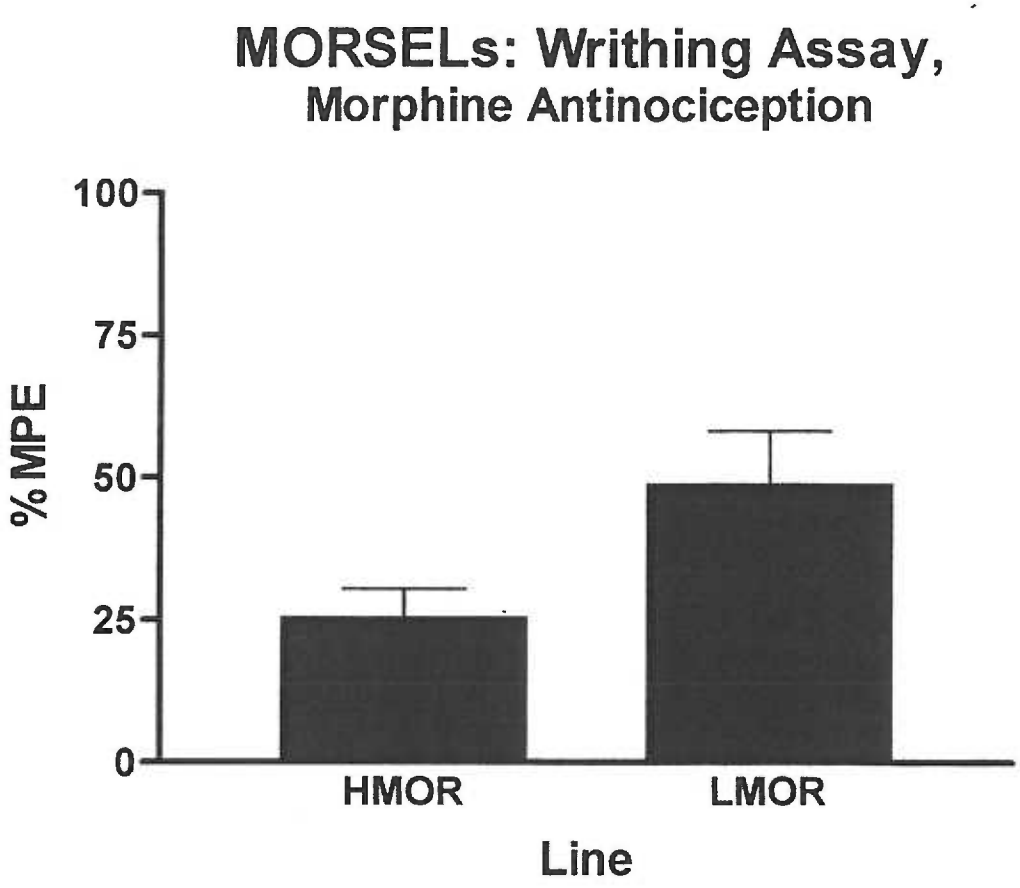

Figure 18. Morphine-induced antinociception of the fourth generation HMOR and LMOR selection lines on the writhing assay. Each bar represents the %MPE (see text) for each line collapsed over sex. Error bars indicate the standard error of the mean for each strain mean.

Fig.4). This is further evidence that different genes underlie morphine-induced antinociception in the hot-plate versus the writhing assay in this population of mice.

DISCUSSION

QTL Analyses

Baseline Nociceptive Sensitivity

The results of the QTL analysis on baseline nociceptive sensitivity in the writhing assay provide further evidence that genetic factors influence the processing of pain. This trait was reasonably heritable and reliable in both sexes of the BXD RI mice. In all three sets of data that were analyzed (combined sexes, female, and male), several provisional QTLs were discovered in the BXD RI strains. While none of these were confirmed upon combining BXD RI and B6D2F2 data, four are considered suggestive QTLs based on the criteria set by Lander and Kruglyak (1995). This supports the second hypothesis that several QTLs affect baseline nociceptive sensitivity in the writhing test.

The confidence interval for the QTL on chromosome 3 in the BXD RI writhing analysis may overlap a QTL found in the hot-plate analysis. Although these two traits were not correlated (see below), it is possible a single gene is responsible for both of these QTLs, but that it affects a discrete mechanism in each assay. Another possibility is that one gene is affecting the same mechanism, but because other QTLs or genetic influences differ between the two traits, this mechanism has no detectable effect on the overall genetic correlation. An alternative conclusion is that the gene underlying the QTL found in the hot-plate assay is in fact not the gene responsible for the QTL seen in the writhing assay. Finer mapping techniques might resolve this issue.

Morphine-Induced Antinociception

Morphine-induced antinociception in BXD RI strains has a low heritability on the writhing assay (BXD R^2=0.14) which implies this trait is influenced more by environmental factors than genetic factors. No QTLs were confirmed in the B6D2F2 population. This does not support the first hypothesis that several QTLs affect morphine-induced antinociception. However, there was not enough power to detect QTLs in a trait with the low heritability estimate using only 300 mice. A much greater number of B6D2F2 mice would be needed. Although it appears one QTL was promising (chromosome seven), the low heritability and reliability suggest confirming provisional BXD RI QTLs for this trait may be difficult.

Neither of the two QTLs confirmed as significant in the analyses of morphine-induced antinociception on the hot plate were detected in the writhing assay BXD RI QTL analysis. Of the provisional QTLs detected in the hot-plate and writhing analyses, only the QTL on chromosome 11 appears in both BXD RI QTL analyses. The allele on this chromosome conferring higher antinociceptive responses was opposite between the two tests. Interestingly, QTL analyses of morphine-induced antinociception on neither the writhing nor the hot-plate assay confirmed this QTL as suggestive or significant. These results support the notion that antinociception is genetically influenced differently in these two assays and that each uses differing mechanisms to inhibit nociceptive processes.

Most intriguing, however, was the fact that the QTL analysis of morphine-induced antinociception on the writhing assay did not reveal the QTL on proximal

chromosome 10 that surfaced in the hot-plate antinociception and several other morphine-induced trait QTL analyses (see Belknap and Crabbe, 1992). This region contains the gene for the μ-opioid receptor, the primary receptor to which morphine binds, and a pharmacological binding assay has shown the C57BL/6, DBA/2, and BXD RI strains differ in maximal binding (B_{max}) capacity of [^{3}H]naloxone (Belknap et al., 1995). A QTL on proximal chromosome 10 may not have appeared in the antinociception writhing analysis for several reasons. Because morphine-induced antinociception on the writhing assay had a low heritability, there may be too much environmental 'noise' to detect a QTL on proximal chromosome 10. Other genetically influenced processes may override the genetic difference in the μ-opioid receptor. That is other factors, such as inflammatory mediators, may be more dominant to differences in inhibiting writhing responses than the μ-opioid receptor.

Because only one dose of morphine was used in the writhing assay, it is unclear whether these results are generalizable to all morphine-induced antinociception. Preliminary data using higher doses of morphine (0.75 and 1.0 mg/kg), however, resulted in a number of strains scoring at or near the 100% maximal response to morphine (data not shown). That is, the mice had no or very few writhes when an injection of morphine preceded the injection of acetic acid. Therefore, the dose of 0.5 mg/kg was chosen to maximize the difference seen between strains in morphine-induced inhibition of writhing just as the 16 mg/kg dose of morphine was chosen for the hot-plate analysis. As morphine may evoke different mechanisms depending on whether a strain is at the maximal responding level or elsewhere in the dose response curve, a QTL analysis at

various doses of morphine might have yielded different results. On the hot-plate assay, similar BXD RI QTL results were obtained across doses of 8, 16, and 32 mg/kg of morphine (Belknap, unpublished data). Another way to examine the effect of morphine inhibition of nociception might be to compare the 50% antinociceptive dose in each strain. More studies need to be conducted to determine whether any genetic influences on morphine-induced antinociception in the writhing assay are apparent across doses.

Candidate Gene Hypothesis

Once QTLs have been identified in a population, genes in the proximity of the QTL can be considered as candidates that might be influencing the trait. Interestingly, some of the genes in chromosomal areas of suggested QTLs found in the writhing assay are immunomodulatory factors. For example, a gamma interferon gene is located in the region where the suggestive QTL mapped on chromosome 10, two mast cell protease genes are found near the region of the chromosome 17 suggestive QTL (males), and a gene for a subunit of an IgEβ high affinity receptor is near the region of the suggestive QTL on chromosome 19 (females). These may be representative of the inflammatory conditions resulting from the injection of acetic acid. If these genes in fact have variations that confer functional differences between strains, this may have implications not only for inflammatory pain but for inflammatory-related phenotypes as well. Some other intriguing candidate genes related to pain processes include cation channels on chromosomes 10 and 17 and a somatostatin receptor on chromosome 17.

One must not discount possibly variances in more common aspects of nociceptive processing such as pH levels, space, and fat content in the abdominal cavity. Differences

in these 'trivial' items may affect baseline nociception or antinociception as well as other genetic factors.

Genetic Correlations

Baseline Nociceptive Sensitivity

In the BXD RI strains, baseline nociception responses on the writhing assay did not significantly correlate with baseline nociception on the hot-plate test, which supports Hypothesis Four. Evidence from the HMOR/LMOR short-term selection lines add support for this conclusion. These data further support previous data which suggests a non-significant correlation between the hot-plate and writhing assays (Mogil et al., 1999). Only male mice have been tested thus far in the BXD RI analysis on the hot-plate test. A comparison of correlations between these two nociceptive tests in BXD RI male and female mice would be of interest since a sex difference was suggested in the writhing assay.

The lack of a significant correlation between these two nociceptive tests suggests that different genes influence how diverse nociceptive information is processed and may indicate that different mechanisms are utilized in processing and responding to various noxious or nociceptive stimuli. A recent study looking at various types of nociceptive procedures and stimuli revealed subsets of nociception that were categorized into groups related to the stimulus used in the nociceptive test (Mogil et al., 1999). For example, results from thermal assays such as the hot plate and tail immersion clustered together while data from chemical assays such as the formalin test (footpad of the mouse) and writhing assays (intraperitoneal injections of acetic acid and magnesium sulfate) grouped

together in another subset. In fact, a preliminary analysis of another F2 intercross population (A x C57BL/6 and C57BL/6 x A, reciprocal intercrosses) on the formalin assay has generated similar preliminary QTL results to this study (Mogil, personal communication). The similarities of QTLs in divergent mouse populations using the two chemical assays, one being somatic (formalin) while the other is visceral (acetic acid), suggests that the genetic influences on the nociceptive responses may be due to the nature of the chemical stimuli. Both formalin and acetic acid spontaneously evoke nociception and cause inflammation after being injected.

Interestingly, a significant difference in baseline nociceptive sensitivity in the writhing assay was found between the HMOR and LMOR short-term selection lines at the fourth generation of selection while no difference was found on the hot-plate in generation one through three. This finding suggests different genes influence baseline nociception in these two assays. Because the HMOR/LMOR lines were selected for morphine-induced antinociception on the hot plate, the results may indicate a common genetic influence of this trait to baseline sensitivity on the writhing assay. The provisional QTL on chromosome 9 in the writhing assay study is in a region including the QTL found in a morphine-induced antinociception study using the hot-plate assay (Belknap et al., 1995). The same gene may or may not be influencing both of these traits; further studies using finer mapping techniques or other confirmation steps may elucidate whether a single gene influences both of these traits.

Morphine-Induced Antinociception

Morphine-induced antinociception in the writhing assay was not significantly

correlated to morphine-induced antinociception in the hot-plate assay. The estimation of heritability in the writhing assay (R^2=0.14) using the BXD RI strains was considerably lower than the heritability found in the BXD RI analysis on the hot plate (R^2=0.45). This low heritability in the writhing assay may mean that the phenotypic correlation typically used as an estimate of genetic correlation when employing inbred strains may actually be too small to detect amid the environmental influences (Falconer and McKay, 1996). That is, the phenotypic variance is not a good estimate of the genetic variance. Therefore, the absence of a significant correlation between responses on the hot-plate and writhing assays may be due simply to a greater environmental influence on the writhing assay. However, no significant correlation was found for morphine-induced antinociception between these two assays in a panel of eight inbred strains either, suggesting this is not a spurious finding (Elmer et al., 1998). Elmer's study used a different measurement for morphine antinociception as well. The low reliability in the present studies suggests replication of these experiments might result in a different outcome. The reliability was not mentioned in Elmer's study (1998).

No difference in the magnitude of morphine-induced antinociception on the writhing assay was found between the HMOR and LMOR selection lines in the fourth generation of selection at the dose of morphine used in BXD RI and B6D2F2 mice. In contrast, a two-fold difference in magnitude in morphine-induced antinociception on the hot plate was found between the HMOR and LMOR lines in the second generation of selection. These data provide further evidence that the hot-plate and writhing assays are not significantly correlated. Previous work demonstrated a significant negative

correlation between these assays using the selectively bred HAR/LAR and HA/LA pairs of lines (Mogil, et al., 1996a). The discrepancy between these results may be due to the fact the HAR/LAR and HA/LA lines were created from heterogeneous stocks containing more than two allelic possibilities for each loci, the Binghamton HET stock and the Swiss Webster stock respectively. Hence, the genetic pool is more varied and alleles that do not vary in the C57BL/6 and DBA/2 strains may differentiate in these populations. Therefore, other mechanisms may come into play affecting morphine's inhibition of nociception in these selected lines. These could include deviations in neurotransmitters or receptors that are involved in opioid pain inhibition such as GABA, serotonin, or norepinephrine. Another possibility is that because the HAR/LAR and HA/LA selected lines were in the 30-33rd generation of selection, genes irrelevant to antinociceptive responses on the writhing assay could have been fixed throughout the selection process, thus, affecting the outcomes. This is unlikely, however, as each pair of lines were created separately and from different stock populations yet yielded the same results.

Possible Sex Differences

The strain x sex interaction found in the baseline nociceptive sensitivity in the writhing assay suggests females and males may process basal visceral nociception differently. Other studies using similar procedures have found no difference between sexes on visceral nociception (Sternberg et al., 1993). The present experiments did not examine changes in the estrus cycle of the female mice which might alter their responses. However, the female data were as reliable as the male data as shown by split-half reliability coefficients. This suggests the estrus cycles were either similar across strains

or the sex differences seen in baseline nociceptive sensitivity on the writhing assay probably were not due to fluctuations in sex hormones. It would be interesting to see if sex differences occurred for the hot-plate assay. Since only male mice have been completed, I cannot make a direct comparison between the writhing and hot-plate assays as to whether the thermal stimulus affects the sexes differently.

Several of the provisional QTLs found in the BXD RI analysis of baseline nociceptive sensitivity on the writhing assay appeared to be sex-specific. A QTL is only considered sex-specific if it has a significantly larger effect in one sex versus the other. The suggestive QTL on chromosome 19 for the females only analyses seems to be sex-specific as no QTL emerged in the males only analyses. This may mean there is a gene on chromosome 19 that differentially affects baseline writhing responses in female mice but not in male mice. Further confirmation studies are needed to determine whether the suggestive QTL on chromosome 17 (male) is a significant sex-specific QTL as well.

Future Directions

Since several QTLs were discovered to be suggestive according to criteria of Lander and Botstein (1989) in the QTL analyses of baseline nociceptive sensitivity in the writhing assay, increasing the number of B6D2F2 mice, and therefore the number of recombinant genotypes, in each sex would increase the power to decipher whether these are significant QTLs. Likewise, this might allow us to determine whether significant sex-specific QTLs exist. In addition, increasing the number of B6D2F2 mice would increase the ability to detect QTLs accounting for a smaller proportion of the total variance attributed to baseline nociceptive sensitivity in the writhing assay.

Epistatic interactions, or QTLs that interact with other QTLs, might impact our ability to detect significant QTLs. Therefore, an analysis of epistatic interactions comparing the provisional QTLs in the BXD RI analysis to the genome of BXD RI strains might reveal an effect of other loci (modifiers) on those provisional QTLs and unmask a significant result. Epistatic interactions are currently being examined.

Congenic strains offer another population to confirm BXD provisional QTLs. Congenic strains are strains that have the complete genome of one progenitor strain except for a region containing a locus of choice which is the genotype of the other progenitor strain. These mice are created by first crossing two inbred strains and then backcrossing mice which have an allele of interest from one progenitor (donor) to the other progenitor strain (recipient). Several congenic strains are presently being created for other QTL projects that may be used to confirm suggestive QTLs found in the writhing assay.

SUMMARY

The studies presented indicate a genetic influence on baseline nociceptive sensitivity on the writhing assay. Several of the QTLs mapped for this trait were near genes for components of the immune system suggesting a link between the mechanisms of nociception and immune response caused by chemical stimuli evoking inflammatory, spontaneous nociceptive responses . Correlation studies demonstrated a non-significant correlation between baseline nociceptive sensitivity on the writhing assay versus the hot-plate assay. This evidence infers different genetic influences are affecting this trait in writhing versus on the hot plate.

Heritability for morphine-induced antinociception in the writhing was low which indicates the environment plays an exceptionally large role in this trait. Correlation studies comparing the antinociceptive response to morphine on the writhing assay and the hot-plate assay showed a non-significant correlation between the two assays. Different genes may be affecting antinociception in each of the two assays studied.

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