A Genetic Analysis of Opioid-induced Hyperalgesia in Mice

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Background: Opioid-induced hyperalgesia (OIH) is a syndrome of increased sensitivity to noxious stimuli, seen after both the acute and chronic administration of opioids, that has been observed in humans and rodent models. This syndrome may reduce the clinical utility of opioids in treating acute and chronic pain.

Methods: In these studies, the authors measured the propensity of 15 strains of inbred mice to develop mechanical manifestations of OIH. These data were subjected to in silico genetic analysis, which resulted in the association of haplotypic blocks within or near several known genes. Both pharmacologic agents and transgenic mice were used to confirm the functional association of the most strongly linked gene with OIH.

Results: Both baseline mechanical noiceptive thresholds and the percentage changes in these thresholds after 4 days of morphine treatment were found to be highly strain dependent. The haplotypic blocks most strongly associated with the mechanical OIH data were located within the β2 adrenergic receptor gene (β2-AR). Using the selective β2-AR antagonist butoxamine, the authors observed a dose-dependent reversal of OIH. Furthermore, deletion of the β2-AR gene sharply reduced the mechanical allodynia present after morphine treatment in the wild-type mouse strain. Analysis of the associated β2-AR haplotypic block identified single nucleotide polymorphisms potentially explaining in part the strain specific differences in OIH.

Conclusions: Genetic variants of the β2-AR gene seem to explain some part of the differences between various strains of mice to develop OIH. The association of this gene with OIH suggests specific pharmacologic strategies for reducing the impact of OIH on patients consuming opioids.

ACCUMULATING evidence suggests that the administration of opioid analgesics leads not only to analgesia, but to a paradoxical sensitization to noxious stimuli that is particularly evident after the abrupt cessation of opioid administration or at the time of serum opioid nadir between regular doses. This phenomenon is referred to as opioid-induced hyperalgesia (OIH). Among the more important human studies documenting this effect are those demonstrating hyperalgesia in patients formerly addicted to opioids who are maintained on methadone when compared with matched controls not receiving methadone or other opioids.1–3 This hyperalgesia was most pronounced immediately before daily methadone administration but was measurable even at time points closer to peak methadone serum concentrations.4 A recent prospective trial in which sustained-acting morphine was given to patients with chronic low back pain demonstrated measurable hyperalgesia within 1 month of beginning therapy.5 Other human data suggest that the short-term infusion of opioids such as the μ-opioid receptor agonist remifentanil followed by abrupt cessation exacerbates preexisting hyperalgesia.6–8 Some evidence suggests this phenomena is due to the activation of N-methyl-D-aspartate receptors.7

More recently, rodent models have been used to study OIH. Many laboratories have reported mechanical allodynia, thermal hyperalgesia, or both after the acute administration of opioids such as heroin and fentanyl,9,10 the chronic (days) administration of intrathecal morphine,11,12 the local peripheral administration of morphine,13 or the chronic administration of systemic opioids of several types.14–16 Many mechanisms have been proposed to explain this type of sensitization, with some of the more commonly discussed possibilities including activation of N-methyl-D-aspartate receptors,14,17,18 activation of facilitative descending pathways from the rostral ventromedial medulla (RVM),10 the decreased reuptake of neurotransmitters from primary afferent fibers,19 and the enhanced responsiveness of spinal neurons to nociceptive neurotransmitters such as substance P and glutamate.20,21 Although not previously linked to OIH, the enhanced expression of β2-adrenergic receptors (β2-ARs) have been identified as adaptive changes occurring during chronic exposure to opioids.22,23 Likewise, the functional enhancement of β2-AR signaling has been demonstrated after chronic morphine exposure in various nervous system tissues.24,25 These observations will become relevant to the current studies. Although traditional pharmacologic, electrophysiologic, biochemical, and molecular techniques have been useful in the exploration of OIH, we are now in position to use murine genetics to identify genomic loci linked to this phenomenon.

A substantial and growing body of literature supports the conclusion that genetics influence pain sensitivity and analgesic responses. With respect to the consequences of chronic morphine administration, several
reports explore the genetic basis of tolerance and dependence specifically. The genetics of OIH are not well explored in part because of the barriers posed by genetic studies. Traditional murine mapping experiments have advanced our understanding of the genetic basis of pain, but the techniques generally used are quite time-consuming and accessible only to laboratories with substantial expertise in genetics and molecular biology. More recently, in silico techniques have been introduced, which allow mapping to be performed in much expedited fashion. These techniques rely on the availability of high-resolution single nucleotide polymorphism (SNP) databases. The computational algorithms then compare phenotypic trait data for a series of inbred mouse strains with the SNP alleles for those strains as organized into either genomic segments of arbitrary size or, more recently, as organized into haplotypic blocks. These now well-described techniques have proven useful in identifying chromosomal regions and even specific genes involved in many traits, including bone metabolism, alcohol withdrawal, immune system function, susceptibility to pulmonary injury, the expression of specific genes, and several other traits. In these studies, we used in silico mapping to identify haplotypic blocks associated with OIH and confirmed a functional association for one haplotypic block corresponding to the β2-AR using independent techniques.

Materials and Methods

Animals

All animal experiments were done after approval of protocols by our Institutional Animal Care and Use Committee (Palo Alto, CA) and complied with the Guide for the Care and Use of Laboratory Animals available through the National Academy of Sciences (Washington, D.C.).

Inbred Mouse Strains. Inbred mouse strains were obtained from Jackson Labs (Bar Harbor, ME) at 7-8 weeks of age. Mice were kept a further 7–10 days from the date of arrival in our animal care facility before use to allow for acclimation. Mice were kept under pathogen-free conditions and were provided food and water ad libitum with a 12:12 h light:dark cycle. The strains used were 129/SvJ, A/HeJ, A/J, AKR/J, B10.D2-H2oSNJ, BALB/cByJ, BALB/cJ, C3H/HeJ, C57BL/6J, DBA/2J, LP/J, LG/J, MRL/Mpj, NZB/BinJ, and NZW/LaCj (15 strains).

Transgenic Mice. FVB and FVB β2-AR congenic null mutants were obtained from a local breeding colony. The generation of these mice is described by Chruscinski et al. These mice were individually genotyped and used in our experiments at 7-8 weeks of age. Animal husbandry was otherwise identical to that used for the inbred strains.

Drug Administration

Morphine Administration. After baseline nociceptive testing, morphine (Sigma Chemical, St. Louis, MO) was administered to mice subcutaneously 20 mg/kg twice per day on days 1-3. On day 4, the dose was increased to 40 mg/kg twice per day in 50- to 100-μl volumes of 0.9% NaCl similar to our previous protocols for generating opioid-induced hyperalgesia. For OIH determinations, mice were assessed 16 h after the final dose of morphine.

Butoxamine Administration. The selective β2-AR antagonist butoxamine was obtained from Sigma Chemical and diluted in 0.9% NaCl before use. After the measurement of baseline mechanical thresholds in C57BL/6J mice, butoxamine was injected subcutaneously. Behavioral testing was repeated in 30 min, and the next higher dose of butoxamine in the series was then injected into the same mice to obtain cumulative dose-response information. Control mice received saline injections at the same time points. Pilot data confirmed 30 min to be a time of maximal drug effect.

Local hind paw injections were performed by lightly restraining the mice and injecting 5 μl of drug containing 0.9% NaCl subcutaneously into the central plantar area of the hind paw. For these injections, a 30-gauge needle and a microsyringe were used. Mice recovered for 10 min in their testing enclosures, which was observed to be a time of maximal drug effect.

Behavioral Assays

Mechanical Allodynia. Mechanical nociceptive thresholds were assayed using nylon von Frey filaments according to the “up–down” algorithm described by Chaplan et al. as we have used previously to detect allodynia after chronic opioid administration. In these experiments, mice were placed on wire mesh platforms in clear cylindrical plastic enclosures of 10 cm in diameter and 30 cm in height. After 20 min of acclimation, fibers of sequentially increasing stiffness (0.2–2 g, seven fibers) were applied to the center of the plantar surface of a hind paw just distal to the first set of foot pads and left in place 5 s with enough force to bend the fiber slightly. Withdrawal of the hind paw from the fiber was scored as a response. When no response was obtained, the next stiffest fiber in the series was applied to the same paw; if a response was obtained, a less stiff fiber was next applied. Testing proceeded in this manner until four fibers had been applied after the first one causing a withdrawal response allowing the estimation of the mechanical withdrawal threshold using curve fitting of the response data. Our index of mechanical OIH was calculated as the percentage decrease in baseline mechanical nociceptive threshold resulting from chronic morphine administration.

Thermal Withdrawal Latency. Response latencies to noxious thermal stimulation were measured using the...
method of Hargreaves et al. as we have modified for use with mice. In this assay, mice were placed on a temperature-controlled glass platform (29°C) in a plastic enclosure as described above. After 20 min of acclimation, a beam of focused light was directed toward the same area of the hind paw as described for the von Frey assay. The time to purposeful withdrawal of the foot from the beam of light was measured to 0.1 s. A 20-s cutoff was used to prevent tissue damage. In these experiments, the light beam intensity was adjusted to provide an approximate 10-s baseline latency for the C57BL/6 index strain before morphine treatment, and the same light intensity was used for all subsequent experiments. Two measurements were made per animal per test session.

In Silico Mapping

Association Studies Using Haplotypic Mapping. Using HapMapper software developed by Roche Bio-science (Palo Alto, CA) and a 158,000 SNP database organized into haplotypic blocks for all strains tested, we attempted to determine associations between our OIH trait data and individual blocks. Briefly, this approach identifies haplotypic blocks whose pattern of genetic variation correlates with the distribution of trait values among the inbred strains. The actual correlation between trait values (mechanical OIH in this case) and the strain groupings for each haplotype block is determined using analysis of variance–based modeling. The resulting P values are used to rank the strengths of correlation for each block in the database. This technique has been used recently to identify genes associated with a number of different murine phenotypic traits. At the time of analysis, this haplotypic map contained blocks corresponding to 2,171 genes.

Statistical Analysis

All data are displayed as mean ± SEM unless otherwise noted. Dose–response data were fitted using a sigmoidal function with variable slope (Prism 4; GraphPad Software, San Diego, CA). Where repeated measures were used, analysis of variance was applied with post hoc t testing.

Results

Strain Differences for OIH

Figure 1 displays the mechanical baseline nociceptive responses and those observed after 4 days of morphine treatment. The differences in baseline nociceptive responses for the various strains were large, with the most mechanically sensitive strain having a baseline nociceptive threshold 16% of the least sensitive strain. As can also be seen in figure 1, the degree of mechanical allodynia acquired during morphine administration varied for the different strains. Table 1 lists the mechanical allodynia caused by chronic morphine administration as a percentage change in baseline thresholds. Although some strains displayed only small mechanical changes, six strains had a greater than 80% reduction in mechanical nociceptive threshold. There was no correlation between baseline mechanical nociceptive thresholds and the degree of OIH developed by the individual strains.

In Silico Mapping Based on Haplotypic Analysis

HapMapper software and an expanded SNP database organized into haplotypic blocks were then used to analyze the data. In this analysis, the distribution of the

Table 1. Strain-specific Mechanical Sensitization

<table>
<thead>
<tr>
<th>Strain</th>
<th>OIH—Mechanical</th>
</tr>
</thead>
<tbody>
<tr>
<td>129/SvImJ</td>
<td>28.5</td>
</tr>
<tr>
<td>A/HeJ</td>
<td>49.5</td>
</tr>
<tr>
<td>A/J</td>
<td>52.0</td>
</tr>
<tr>
<td>AKR/J</td>
<td>74.8</td>
</tr>
<tr>
<td>B10.D2-H2/oSNJ</td>
<td>82.0</td>
</tr>
<tr>
<td>BALB/cByJ</td>
<td>60.6</td>
</tr>
<tr>
<td>BALB/cJ</td>
<td>63.8</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>74.2</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>87.3</td>
</tr>
<tr>
<td>DBA/2J</td>
<td>86.5</td>
</tr>
<tr>
<td>LP/J</td>
<td>40.7</td>
</tr>
<tr>
<td>LG/J</td>
<td>82.6</td>
</tr>
<tr>
<td>MRL/MpJ</td>
<td>89.0</td>
</tr>
<tr>
<td>NZB/BinJ</td>
<td>54.6</td>
</tr>
<tr>
<td>NZW/LaGJ</td>
<td>85.0</td>
</tr>
</tbody>
</table>

Listed are the specific strains of mice used in these experiments along with the degree of opioid-induced hyperalgesia (OIH) observed after chronic morphine administration. These data were calculated as the percent reduction in mechanical nociceptive thresholds obtained using von Frey fibers after 4 days of exposure to morphine.
propensity of the strains to develop mechanical hyperalgesia after morphine administration was compared with the partitioning which would be predicted for the strains given the allelic possibilities for each available haplotypic block. Higher correlation resulted from the strain-specific trait data more closely matching the pattern predicted by the haplotypic alleles for any given block. Figure 2A displays the results of the haplotypic mapping as a cartesian plot with the inverse of the P value plotted against the chromosomal location of the blocks. Figure 2B displays the tabular results of the HapMapper program. Note that each of the color coded blocks under “Haplotype” represents the haplotype for one strain of mice, with the leftmost strain being the one with the lowest degree of mechanical OIH (129/SvJ) and the rightmost strain being the one displaying the greatest degree of mechanical OIH (MRL/MpJ). Our most highly correlated block overall, which corresponded to the β2-AR gene (Adrb2), possessed three possible haplotypic alleles represented as either a red, green, or blue block in figure 2B. One of the alleles was possessed by only one strain (NZW/LaCJ). The seven strains having the least tendency to develop mechanical sensitization were of one haplotype, whereas the seven more extensively sensitized strains had the other haplotypic allele. This particular haplotypic block was defined by a group of eight SNPs.

**Effects of Selective β2-AR Antagonists on OIH**

We next hypothesized that if mechanical allodynia after chronic morphine administration was supported by β2-AR, the administration of a β2-AR antagonist should
reduce that allodynia. Control mice and mice rendered allodynic by the administration of morphine for 4 days were injected with various doses of the selective $\beta_2$-AR antagonist butoxamine. The C57BL/6J strain were used after 4 days of morphine treatment to induce opioid-induced hyperalgesia or after 4 days of saline administration as a control. In A, data representing the measurement of mechanical withdrawal thresholds after the simultaneous administration of butoxamine are presented. In B, control mice or mice treated with morphine were administered a just-maximal dose of butoxamine to determine the effects on the thermal manifestations of opioid-induced hyperalgesia. Data are presented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Eight mice were used in each group.

OIH in $\beta_2$AR Null Mutant Mice
We next sought a pharmacologically independent approach to confirming or refuting a role for $\beta_2$-AR in supporting the mechanical and thermal manifestations of OIH. In these experiments, $\beta_2$-AR null mutants and littermate FVB wild-type mice were used. Figure 4 presents data demonstrating that the wild-type and null mutant strains had similar baseline mechanical and thermal withdrawal thresholds but that after morphine treatment, the null mutant mice developed no discernible mechanical allodynia or thermal hyperalgesia. The wild-type mice, on the other hand, displayed statistically significant reductions in both the mechanical and thermal indices of OIH. Not all morphine-induced changes were
different in the β2-AR null mutants, however. The weight loss which characterizes the chronic treatment of mice with morphine was similar for null mutants and wild-type mice (7.5% vs. 7.8%; no statistical difference).

Effects of Local Injections of β2-AR Ligands

Because reports in the literature suggest that peripherally expressed β2-ARs support states of enhanced mechanical nociceptive sensitivity,39,40 we undertook studies directed at determining whether the peripheral administration of butoxamine could reduce mechanical allodynia after local injection, supporting the hypothesis that this receptor is an important regulator of nociceptive sensitivity.

Structure of the β2-AR Haplotype Block Associated with Morphine-induced Allodynia

Figure 6 provides a diagram of the haplotype block associated with morphine induced mechanical allodynia. This block begins on the 5′ side of the β2-AR gene on chromosome 18 and extends through the coding region to the 3′ side of the gene. The block contains eight SNPs. The single SNP located within the coding region of the gene is associated with a silent mutation and thus does not change the receptor’s amino acid sequence.

Discussion

The goals of this project were (1) to use in silico techniques to derive predictions as to what specific genes are associated with the propensity of mice to develop OH, and (2) to pursue one of these predictions to confirm or refute a functional role in supporting OH. The data presented in figure 1 as well as table 1 demonstrate strong interstrain differences in the propensity to develop mechanical sensitization. Subsequent in silico genetic mapping identified the β2-AR gene as a candidate gene involved in modulating mechanical OH in mice (fig. 2). Pharmacologic and β2-AR null mutant mouse experiments provided results consistent with the genetic association (figs. 3–5). Ours were not the first efforts to use genetics to investigate pain-related traits. Although specific genes were not identified, previous studies demonstrated influences of genetics on nociceptive measurements of many types41–43 and other consequences of chronic morphine administration, such as tolerance and dependence.26–28,44

The in silico mapping approach used a set of SNPs (158,000 at the time of analysis) organized into haplotypic blocks using an algorithm described by Wang et al.31 The significance of naturally occurring haplotype blocks with respect to genomics and genomic mapping strategies has been reviewed in recent publications.45,46 This technique took advantage of the naturally occurring linkage disequilibrium that exists for the SNPs corresponding to the 2,171 genes within the approximately 75 Mb of the murine genome for which SNP discovery had been undertaken. Be-
cause these blocks tend to be small in genomic terms, 39 Kb on average, linkage of phenotypic traits to these haplo-
typic blocks often results in the identification of specific
genes associated with the traits of interest. Figure 2 displays
our results from these studies. From the group of haplo-
typic blocks present in the HapMapper database, the one
corresponding to the $\beta_2$-AR was most strongly associated.
Although three haplotypes were represented in the 15
strains of mice, 14 strains had one of the two predominant
haplotypes. One of the reasons for the high degree of
association of this block with mechanical OIH was the
partitioning of the strains into nonoverlapping groups by
the two predominant haplotypes.

With a spectrum of data in hand implicating $\beta_2$-AR in
OIH, we must ask whether these observations fit with
existing literature. One set of observations suggesting
$\beta_2$-AR might be involved in opioid-induced hypersensi-
tivity states involves measurements of increased $\beta_2$-AR
density in the central nervous system after chronic ex-
posure of rats to morphine22–24 as well as the up-regu-
lation of guanosine triphosphate binding proteins, the
molecules used by $\beta$-AR to activate ion channels and
second-messenger systems.47 In cell culture systems,
chronic exposure to morphine enhances stimulated cy-
clic adenosine monophosphate production by a mecha-
nism involving an increased expression of $\beta_2$-AR.25 Al-
though the mechanism was not well described, it is
interesting to note that serum levels of cyclic adenosine
monophosphate were significantly increased in rats
treated chronically with morphine as well.48

Independently reported animal behavioral data sup-
ports possible roles for peripherally expressed $\beta_2$-AR in
states of enhanced pain sensitivity. These data lead us to
test the hypothesis that the hind paw injection of $\beta$-AR
antagonists could reduce mechanical OIH. For example,
Khasar et al.39 demonstrated that both epinephrine and
the more selective $\beta$-AR agonist isoproterenol caused a
mechanical hyperalgesia when injected into the hind
paws of rats. This sensitization was blocked by $\beta$-AR
antagonists. In this study, the authors went on to provide
evidence that the mechanical sensitization might be re-
lated to the sensitization of small diameter dorsal root
ganglion neurons. In a later study, Aley et al.40 repro-
duced the previous data and provided further evidence
that $\beta_2$-AR was the likely receptor subtype responsible
for the sensitization. In additional studies, cultured dor-
sal root ganglion neurons were found to respond to
$\beta_2$-AR stimulation with phosphorylation of extracellular
signal-related kinase.40 This type of phosphorylation has
been linked to enhanced nociception by many labora-
tories. In addition, $\beta_2$-ARs have been shown to enhance
inflammation in models of arthritis49,50 possibly involv-
ing enhanced production of tumor necrosis factor $\alpha$.51

Our own data using the local hind paw injection of
butoxamine and terbutaline to decrease and increase
mechanical nociceptive sensitivity, respectively, are con-
sistent with the peripheral $\beta_2$-AR effects outlined above.
An additional factor perhaps amplifying the role of pe-
ripheral $\beta_2$-AR in supporting OIH is the increased levels
of circulating catecholamines present after the acute
administration of morphine and the increases during
opioid abstinence in rodents.52–54 Although we have focused
on the periphery, it is possible that in the setting
of OIH, $\beta_2$-ARs expressed in other locations participate
in modulating the nociceptive sensitization as well. Ulti-
ately, our findings will need to be integrated in a model
for OIH that includes the other brain and spinal cord
level mechanisms which have been described.12,16,19,21

Although the results we obtained are notable for the
association made with $\beta_2$-AR, they are equally notable
for the lack of association with genes coding for proteins
well demonstrated to modulate OIH. The protein per-
haps best associated with the modulation of OIH at this
point is the $\text{N-methyl-D-aspartate}$ receptor.7,9,10,12,14,17
Haplotype blocks pertaining to the genes coding for the
principal subunits of this receptor are represented in the
database used by the haplotype in silico mapping pro-
gram we employed. The lack of a high-strength associa-
tion with any of these subunits should not be interpreted
as inconsistent with the existing pharmacologic data,
however. It may be that the $\text{N-methyl-D-aspartate}$ recep-
tor is critical in the modulation of the OIH trait, but that
the genetic variants of receptor subunits that exist do
not lead to important functional differences in the result-
ning proteins and thus are not likely to be identified in this
type of mapping study.

It is also notable that this data set did not lead to the
identification of factors influencing the distribution or
elimination of morphine, although haplotype blocks per-
taining to various drug transporters and metabolic en-
zymes using morphine as a substrate were included.
Simple morphine brain level measurements after acute
administration did not predict mechanical OIH (data not
shown). It is possible that circulating or brain morphine
levels would influence other consequences of chronic
morphine administration, however. Similarly, we have
completed mapping studies using only morphine. Al-
though other opioids can cause OIH, it is not clear that
$\beta_2$-AR would always play as prominent a role.

A number of factors limit the strength and meaning of
these mapping results. The first is that although the sets of
SNPs and haplotypic blocks used here were relatively
large, they were not comprehensive. SNPs are the most
common type of genetic polymorphisms. Although one
of the advantages of haplotype analysis is that not all
SNPs need to be known to define the common haplo-
types, the map we used was far from complete. Our
haplotype mapping likely analyzed only approximately
one tenth of all murine genes at optimal resolution.
Therefore, it is possible if not likely that some genes
influencing OIH were not identified in these studies.
Also, while the number of strains used was relatively

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large, 15 more many strains and corresponding SNP data would be needed to have the power to identify all haplotypic blocks associated with complex traits such as OIH, opioid tolerance, or pain sensitivity. An analysis of the strain dependence of power in haplotypic analysis has been published recently.31 This analysis suggests that the use of 30–40 strains would very substantially enhance the power of the study and will be required for whole genome analyses. The use of 15 strains of mice would result in a power of approximately 0.8 in detecting causal genetic loci having genetic effects in the range of 0.5, i.e., explaining 50% of the genetic variance. Therefore, continuing to accumulate strain specific data may allow us to identify more genes involved in this complex trait.

A list of the most useful tools and techniques available for neuroscience research would likely include pharmacologic approaches, electrophysiology, molecular biology, the generation of transgenic models, immunohistochemistry, and others. We are poised to add in silico murine genetic studies to this list. In this set of studies, a relatively simple paradigm for measuring OIH in various strains of readily available inbred mice was used to obtain a data set that was directly subjected to in silico genetic analysis. Having demonstrated the ability of β2-AR blockade to reduce or eliminate OIH in mice, we are now in position to translate these findings to human studies. It may be possible to determine the ability of β2-AR blockade to reduce OIH in human models.5–8 Conceivably, the addition of β2-AR to a chronic opioid regimen might improve the long-term efficacy of this form of treatment.

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