

# PAIN GENES?: Natural Variation and Transgenic Mutants

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■ **Abstract** Like many other complex biological phenomena, pain is starting to be studied at the level of the gene. Advances in molecular biological technology have allowed the cloning, mapping, and sequencing of genes, and also the ability to disrupt their function entirely (i.e. via transgenic knockouts). With these new tools at hand, pain researchers have begun in earnest the task of defining (*a*) which of the 70,000–150,000 mammalian genes are involved in the mediation of pain, and (*b*) which of the pain-relevant genes are polymorphic, contributing to both natural variation in responses and pathology. Although there are only a few known examples in which single gene mutations in humans are associated with pain conditions (e.g. an inherited form of migraine and congenital insensitivity to pain), it is likely that others will be identified. Concurrently, a variety of genes have been implicated in both the transmission and control of “pain” messages in animals. The present review summarizes current progress to these ends, focusing on both transgenic (gene→behavior) and classical genetic (behavior→gene) approaches in both humans and laboratory mice.

## INTRODUCTION

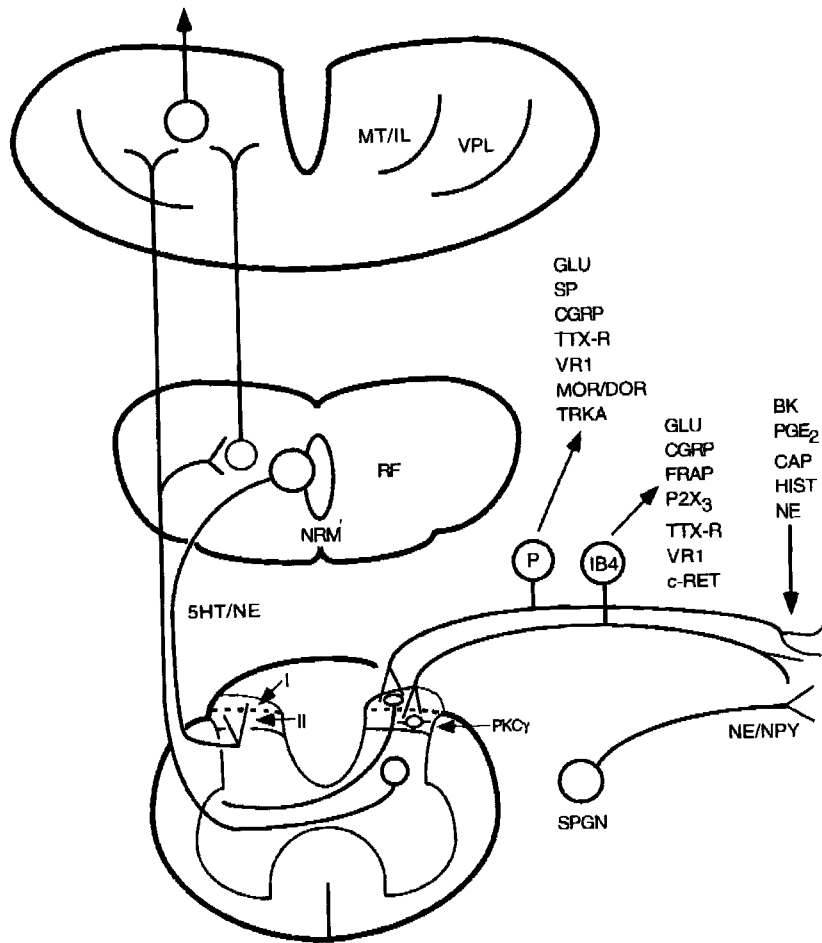
A modern understanding of pain signaling recognizes the distinction between three pain conditions. First, acute pain signaling provides a strong warning of injury; its loss is devastating and occurs in several conditions of congenital insensitivity to pain (for one of which a specific genetic mutation has recently been identified). Importantly, the small diameter primary afferent nociceptors (C-fibers) express a unique population of transducers, channels, and receptors that are involved in the transmission of “pain” messages. Several of the genes that encode these molecules have recently been identified. Second, in the setting of injury, there is a heightened sensitivity, such that pain can be produced by normally

innocuous stimuli (allodynia); this ensures that the injured part of the body is protected. This process involves molecules that sensitize the primary afferent and second order spinal cord neurons to innocuous stimuli. Finally, there are conditions in which pain is maladaptive; in these conditions, pain can be considered a disease of the nervous system. For example, after nerve injury, there may be intense, spontaneous pains, and various stimuli can evoke abnormally intense pain. These persistent “neuropathic pains,” which do not respond well to traditional aspirin-like or opioid drugs, are thought to arise from the development of long-term changes in the processing of pain messages by spinal cord and brainstem neurons. Many of these changes are associated with the induction of genes, the disruption or mutation of which might significantly affect the incidence of pain as a disease.

Our aim in this review is to consider the involvement of genes in the mediation of pain. In addition to highlighting a few profound examples in which a particular pain syndrome in humans has been linked to single genes, we review evidence for the hypothesis that variation in pain and analgesic responsiveness among different animals (especially strains of mice) can be attributed to genetic differences. Finally, we examine a host of recent studies that have used knockout and transgenic methodologies to implicate specific genes in nociceptive and analgesic processing. Because one of our laboratories has recently published a more comprehensive review of alterations in pain behavior produced by knockouts of a wide variety of molecules, including many less traditionally associated with pain (Mogil & Grisel 1998), we have been selective in this discussion. We begin with a brief review of the neurobiology of pain, highlighting regions within the “pain” transmission system where the manifestation of specific genes is most likely to influence how pain is generated.

## A BRIEF OVERVIEW OF THE NEUROBIOLOGY OF PAIN

The traditional view of the “pain” pathway consisted of a primary afferent C-fiber primary contacting a second-order dorsal horn neuron at the origin of the spinothalamic or spinoreticular pathway. The third third-order neuron projected to some unknown region in the cortex, ultimately producing pain. Figure 1 illustrates a more contemporary view of the key neuroanatomical and neurochemical features that underlie the transmission of nociceptive messages from the periphery to the spinal cord, brainstem, thalamus, and cortex. The illustration is far from complete. Notably, it omits the contribution of large diameter afferents to the control of dorsal horn nociresponsive neurons, greatly oversimplifies the ascending pathways that derive from the spinal cord, and it completely omits information that has recently been gathered on cortical mechanisms that are involved in the perception of pain. Although we have not labeled particular genes that contribute



**Figure 1** Contemporary view of the "pain" pathway. Details are in text.

to the neurochemistry highlighted by this figure, clearly alterations in the expression of the genes coding for these proteins would dramatically affect the transmission and control mechanisms that influence pain.

There are two major categories of unmyelinated primary afferent nociceptor (Snider & McMahon 1998). One is characterized by its expression of a host of peptides (P), including substance P (SP), and one that expresses fewer peptides, but which can be identified by its binding of the IB4 lectin and its expression of a fluoride-resistant acid phosphatase (FRAP). Although the major excitatory neurotransmitter of both populations is glutamate (GLU) and although several common receptors [e.g. the vanilloid/capsaicin receptor (VR1)] and channels [e.g. a

TTX-resistant Na<sup>+</sup> (TTX-R)] are expressed, there are many distinct features. For example, almost all of the peptide, but only a subset of the IB4 population synthesizes the neurotransmitter calcitonin gene-related peptide (CGRP). Furthermore, the peptide and IB4 populations express the neurotrophin receptors, TrkA and c-ret, respectively, and these are differentially responsive to nerve growth factor (NGF) and glial-derived neurotrophic factor (GDNF). Finally, the peptide population expresses the mu and delta opioid receptors (MOR/DOR), whereas a subset of the IB4 population uniquely expresses the P2X3 subtype of purinergic receptor. Transport of these and other receptors to the central and peripheral terminals of the primary afferent accounts for their activation by mediators released in the setting of injury. A few of these are noted, including bradykinin (BK), prostaglandin E2 (PGE2), capsaicin (CAP), histamine (HIST), and norepinephrine (NE). The illustration also includes a norepinephrine (NE) and neuropeptide Y (NPY)-containing sympathetic postganglionic neuron (SPGN), which probably comes into play in nociceptive processing in the setting of tissue and nerve injury.

Although both the peptide and the IB4 populations of C-fiber terminate in the superficial dorsal horn, they target distinct laminae and thus distinct groups of neurons. The peptide population terminates almost exclusively in laminae I and outer II; the IB4 population primarily targets the inner part of lamina II, a region that contains a distinct subset of interneurons that synthesize the gamma isoform of protein kinase C (PKC $\gamma$ ) (Malmberg et al 1997b). Two major projection neurons are identified, namely those in laminae I and V. Although the former can be directly influenced by primary afferents, the latter are probably largely affected via polysynaptic circuits that involve interneurons of laminae I and II. The output of the projection neurons is highly simplified. Only projections to the thalamus and reticular formation (RF) are included. Among the targets of the spinothalamic and spinoreticular pathways are the ventroposterolateral (VPL) and medial thalamic (MT) nuclei, including the intralaminar nuclei (IL). No information is provided on the cortical targets of these thalamic regions.

Finally, Figure 1 denotes the presence of descending pathways that regulate the outflow of dorsal horn neurons. The precise termination of axons that arise from the brainstem, including the serotonergic (5-HT) neurons of the midline nucleus raphe magnus (NRM), are not specified. However, projections to laminae I and II of the dorsal horn have been established. Descending noradrenergic (NE) pathways are also included in Figure 1, but their cells of origin in the locus ceruleus, subceruleus, and A5 and A7 cell groups of the lateral brainstem are not illustrated.

Also highlighted are likely sites where clearly alterations in the function or expression of known and as yet unknown genes could induce long-term changes (or interindividual differences) in nociceptive transmission, dramatically altering how innocuous and noxious stimuli are perceived. Such alterations could also affect the modulation of pain by analgesic drugs or endogenous control mechanisms.

## SINGLE GENE POLYMORPHISMS OF RELEVANCE TO PAIN AND ANALGESIA IN HUMANS

Because gene mutations are well known as a cause of disease in humans, the possibility that certain painful pathologies have a simple genetic basis has been considered for some time. By convention, differential alleles at a genetic locus are known as mutations if they are present in <1% of the population, and as polymorphisms if present in a larger percentage. To date, a handful of mutations/polymorphisms affecting pain in humans have been elucidated.

### Congenital Insensitivity To Pain With Anhidrosis

Over 40 reported cases of congenital insensitivity to pain (CIP) have appeared in the literature since the 1932 description of "The Human Pincushion" (Dearborn 1932). He was a carnival performer with an act made possible by the absence of pain sensation over his entire body, despite the preservation of other sensory modalities and reflexes (see Thrush 1973). Although some early work implicated overproduction of endogenous opioids in this condition (Dehen et al 1977), the loss of small myelinated fibers in the peripheral nervous system represents the modal pathological finding (see Larner et al 1994).

One form of CIP, CIP with anhidrosis (CIPA; hereditary sensory and autonomic neuropathy, type 4; MIM 256800), has recently been fully elucidated on the molecular genetic level (Indo et al 1996). Interestingly, this effort was inspired by the demonstration that *TrkA* (now called *Ntrk1*) knockout mice (Smeyne et al 1994) displayed a mutant phenotype resembling CIPA. Indo and colleagues (1996) sequenced the analogous human gene, *TRKA* (now called *NTRK1*). In four affected patients, three separate, catastrophic single-gene mutations were identified: a single base deletion causing a frameshift, an A-C transversion causing RNA splicing errors, and a G-C transversion causing a Gly-to-Arg substitution in the third exon. The contribution of the nerve growth factor (NGF)/tyrosine receptor kinase system to the survival and regulation of small-diameter afferents carrying nociceptive information (see below) can explain much of the mutant phenotype in both CIPA patients and *Ntrk1* knockout mice. However, the considerable clinical and genetic heterogeneity among congenital sensory neuropathies (see Dyck et al 1993) renders the generalizability of this finding uncertain. Indeed, attempts to associate hereditary sensory and autonomic neuropathy type 1 and type 2 with various genes encoding neurotrophins and their receptors have not been successful (Davar et al 1996, Nicholson et al 1996).

### Familial Hemiplegic Migraine

Considerable excitement has surrounded the recent elucidation of this rare form of a common pain problem, migraine. The gene for familial hemiplegic migraine (FHM), an autosomal dominant inherited subtype of migraine with aura, was

assigned by linkage mapping to human chromosome 19p13 (Joutel et al 1993). Using a technique called exon trapping, Ophoff and colleagues (1996) cloned a P/Q-type calcium ( $\text{Ca}^{2+}$ ) channel subunit gene in this region, one recently found by another group and named *CACNLIA4* (now known as *CACNA1A*) (Diriong et al 1995). All 47 exons and flanking intronic regions were screened for mutations in 20 individuals with FHM or a related disorder, episodic ataxia type 2. These investigators identified four different missense mutations at highly conserved amino acid residues in affected individuals: one (R192Q) in the putative voltage-sensing domain, another (T666M) likely affecting ion selectivity, and all likely causing gains-of-function (Ophoff et al 1996).

P/Q-type  $\text{Ca}^{2+}$  channels have been recently associated with serotonin release, magnesium levels, and the phenomenon of cortical spreading depression, all thought to contribute to the pathophysiology of migraine (see Ophoff et al 1996). Although FHM is rare, idiopathic migraine has a definite (but small) genetic component, as demonstrated by twin studies (see Peroutka 1998), and the strong possibility exists that more subtle polymorphisms in  $\text{Ca}^{2+}$  channel subunit genes could underlie this component. This contention is controversial, with both positive (May et al 1995) and negative (Hovatta et al 1994, Kim et al 1998, Peroutka et al 1997) findings reported.

## Codeine Metabolism

Currently, only one pain-relevant genetic polymorphism affecting a large number of people has been fully described. The highly polymorphic gene in question codes for a particular cytochrome P450 enzyme, P4502D6 (P450DB1; CYP2D6; debrisoquine/sparteine oxygenase) (see Eichelbaum & Evert 1996, Sindrup & Brosen 1995 for reviews). It has been known since the 1970s that 7%–10% of Caucasian patients were unable to metabolize debrisoquine or sparteine when given at standard doses. Such “poor metabolizers” (PMs) inherit, in an autosomal recessive fashion, one of at least 12 different identified mutations leading to partial or complete loss of P4502D6 functioning (e.g. Gonzales et al 1988). This enzyme affects drug response and toxicity of over 40 widely used drugs, including the popular opioid analgesic, codeine (methylnorphine), which is biotransformed by O-demethylation to morphine by P4502D6. Because the analgesic effect of codeine is mediated almost entirely by its metabolite, morphine, PMs experience no analgesia from codeine administration (e.g. Desmeules et al 1991, Poulsen et al 1996). It remains unclear to what extent PMs remain susceptible to codeine’s side effects.

The clinical implications of this genetic polymorphism are obvious, especially considering codeine’s role as the first opioid on the World Health Organization’s “analgesic ladder.” Extensive work is now being performed to determine the relevance of P4502D6 in the analgesic efficacy of other drugs (e.g. Desmeules et al 1999). Intriguingly, a role for this enzyme in tonic pain sensitivity has been suggested; PMs rated the cold pressor test as more painful than did extensive

metabolizers (EMs) (Sindrup et al 1993). Finally, other genetic polymorphisms that affect the pharmacokinetics of analgesic drugs are emerging, including an alternate allele of the gene coding for cytochrome P4503A4, which has been shown to affect the elimination clearance of the opioid analgesic, alfentanil (Yun et al 1992).

## POLYGENIC MEDIATION OF PAIN AND ANALGESIA

Human studies have revealed impressive individual differences in sensitivity to experimental (e.g. Chen et al 1989, Libman 1934) and clinical (e.g. Jacobs et al 1995) pain, and to opioid (e.g. Galer et al 1992, Lasagna & Beecher 1954, Levine et al 1981) and nonopioid (e.g. Walker et al 1994, 1997) analgesics. Despite evidence of familial aggregation of pain traits and reasonable heritability estimates obtained from twin studies (see, however, MacGregor et al 1997), shared environmental variance and/or familial modeling have been consistently invoked to explain individual differences (Mogil 1999).

The successful identification of single gene polymorphisms of relevance to pain in humans described above was facilitated by the all-or-none nature of the traits in question. Variation of pain sensitivity, analgesic sensitivity, and even susceptibility to more common pain pathologies (e.g. low back pain) in the “normal” range, all quantitative traits, are unlikely to be mediated by single genes (Plomin 1990). Complex, quantitative pain traits can also be studied in humans, either by linkage analysis or association study (Lander & Schork 1994). However, linkage analyses of complex traits require truly prodigious sample sizes to detect genes of modest effect (Risch & Merikangas 1996), and association studies can be easily confounded. Indeed, there exist very few replicated findings by either technique in the neurogenetic literature. For these reasons and because of ethical restraints associated with pain research in humans, studies of the genetic basis of the normal range of variability in nociceptive and analgesic sensitivity have been carried out only in laboratory rodents. Although some excellent work has been done using rats (e.g. Devor & Raber 1990, Hoffmann et al 1998, Urca et al 1985, Vaccarino & Couret 1995; see Mogil 1999 for review), we will focus on studies carried out in the mouse (*Mus musculus*), because this species has been examined for genetic differences more systematically. Studies using spontaneous mutants (e.g. *Jimpy*, *beige<sup>J</sup>*, *sepia*, *gunmetal*) (Shuster 1989) and the much studied recombinant inbred strain, CXBK (whose outlier properties are probably also due to a single gene mutation) (Mogil et al 1996b), will be omitted because such models cannot be used to investigate polygenic inheritance.

### Pain Trait Variability In The Mouse

Although much variability is encountered when testing outbred mouse populations (e.g. Swiss, ICR) for nociceptive and analgesic traits, the study of defined

genetic models such as artificially selected lines and inbred strains is particularly advantageous. There are two bidirectionally-selected lines of specific relevance to pain in the mouse. The HA/LA (high analgesia/low analgesia) mouse lines were bred for high and low analgesia, respectively, induced by forced swimming in cold water (Panocka et al 1986). The HAR/LAR (high analgesic response/low analgesic response) mouse lines were bred for high and low analgesia, respectively, produced by the opioid analgesic, levorphanol (Belknap et al 1983). Extensive investigation of the HA/LA and HAR/LAR mice has determined that (a) these traits are highly heritable, as attested to by profound and quick selection; (b) the differential response of high and low lines in both cases is determined by a very restricted number of genes; (c) genes having been fixed in each selection project have pleiotropic effects on a number of pain-related traits; and (d) opioid receptor density has been altered in high lines of both projects, although in different neuroanatomical loci (medial thalamus for HA/LA; dorsal raphe nucleus for HAR/LAR) (see Mogil et al 1996b for review).

The investigation of the nociceptive and analgesic sensitivity of inbred mouse strains is especially useful for genetic analysis because they facilitate partitioning of trait variance into genetic and environmental components, and serve as excellent progenitors of segregating (i.e. backcross or F<sub>2</sub> hybrid) populations needed for gene mapping efforts. A small number of inbred strain surveys of relevance to pain have been performed (e.g. Brown & Hughes 1962, Elmer et al 1997), although there are numerous examples of documented differences between two or three strains (Mogil 1999). In the most systematic effort thus far, we tested 11 inbred strains for their sensitivity to 12 common dependent measures of nociception (Mogil et al 1999a,b). Considerable strain variability was demonstrated, corresponding to ranges of 1.2- to 54-fold differences between extreme-responding strains, and heritability estimates of 0.3 to 0.8 for each measure. A consideration of the correlations of nociceptive sensitivity of each inbred strain with each measure revealed that the nociceptive assays used could be grouped into three “clusters”: thermal, chemical, and mechanical/hyperalgesia. That is, strains sensitive to one nociceptive assay could be predicted to be sensitive to other assays within the same cluster, implying that clustered assays share genetic mediation. Because common genetic mediation directly implies common physiological mediation, we contend that these data reveal fundamental pain “types” in the mouse (Mogil et al 1999b).

## Gene Mapping Studies In The Mouse

Advances in statistical methodology—enabling the isolation of additive genetic effects from “noise” created by both environmental factors and the effects of other genes—have allowed the mapping of quantitative trait loci (QTLs) that account for even modest proportions of overall trait variability. QTL mapping involves correlating the inheritance of a trait with the inheritance of polymorphic DNA markers known as microsatellites (Lander & Schork 1994). The major limitation of the

technique is that it cannot identify genes directly, but rather only broad chromosomal regions that contain the relevant gene. Gene identification is achieved either by positional cloning (fine-resolution mapping followed by sequencing) and/or the testing of “candidate genes” already mapped to the same region.

QTL mapping of pain-related traits in the mouse has begun in earnest, and the findings are intriguing. Belknap and colleagues (Belknap & Crabbe 1992, Belknap et al 1995, Hain et al 1999) mapped QTLs associated with analgesic sensitivity to systemic morphine using segregating populations derived from C57BL/6 and DBA/2 mice. Thus far, two QTLs have been confirmed, together accounting for over half of the genetic variance in this trait. One QTL has been localized to the proximal end [0–20 cM (centimorgan, or 1 million base pairs)] of mouse chromosome 10; the second to the “dilute” region (40–60 cM) of chromosome 9. Importantly, excellent candidate genes exist in both regions. The *Oprm* gene, encoding the  $\mu$ -opioid receptor type, has been mapped to  $\approx 7$  cM on chromosome 10. Ample pharmacological and transgenic evidence has been marshaled to support the crucial role of  $\mu$  receptors in the mediation of morphine’s biological actions. The *Htr1b* gene lies at 46 cM on chromosome 9 encoding the serotonin-1B receptor subtype. We have recently demonstrated that a selective antagonist of this receptor can reverse and prevent morphine analgesia in DBA/2, but not C57BL/6 mice, strongly supporting *Htr1b* as the QTL in question (Hain et al 1999). This finding nicely illustrates the utility of the QTL mapping approach. Although it has long been known that serotonin contributes to morphine analgesia, pharmacological limitations have hampered efforts to differentiate the effects of each receptor subtype. The identification of the chromosome 9 QTL provided an important heuristic impetus for the focused investigation of the strain-dependent effects of serotonin-1B receptors.

Two recent QTL mapping studies have provided evidence for sex-specific genetic mediation of pain traits. Mogil et al (1997a) identified a QTL on chromosome 4 (50–80 cM), provisionally associated with variability in hot-plate nociception. The statistical evidence for association with this trait in male mice far exceeded that for females. A candidate gene in this region was apparent—the *Oprd1* gene encoding the  $\delta$ -opioid receptor maps to 65 cM on chromosome 4—and suggested a simple confirmatory experiment. We demonstrated that pretreating male and female mice of both progenitor strains (C57BL/6 and DBA/2) with  $\delta$ -opioid antagonists produced a strain- and sex-dependent effect; the largest decreases in hot-plate latencies were observed in DBA/2 males, and the smallest in C57BL/6 females (Mogil et al 1997a). Evidence for sex-specificity was even more striking in another effort concerning nonopioid stress-induced analgesia produced by forced cold-water swimming (Mogil et al 1997b). A QTL was identified on distal chromosome 8 (>50 cM). It accounted for well over half of the genetic variance in female mice, but was entirely irrelevant to the trait in males. Unfortunately, no obvious candidate gene in this region has been identified as yet.

Ongoing QTL mapping projects of relevance to pain include nitrous oxide analgesia (Quock et al 1996), abdominal constriction test of nociception (Hain et

al 1998), Hargreaves' test of nociception, formalin test of nociception, and acetaminophen analgesia (JS Mogil and SG Wilson, unpublished data).

## MOLECULAR MANIPULATIONS OF GENE ACTIVITY IN ANIMALS: ADVANTAGES AND DISADVANTAGES FOR PAIN RESEARCH

The previous sections have described models of naturally occurring genetic variation, where adaptive or random processes have mutated or altered the allelic frequencies of genes relevant to pain in various subpopulations. We now consider experimentally generated genetic alterations. A number of strategies are in use to manipulate genes in experimental animals, especially the mouse. These include the transgenic approach (Palmiter & Brinster 1985), in which a cloned gene, under the regulation of a cloned promoter sequence, is introduced into the mouse embryo. The gene is integrated randomly into the host cell chromosome, and the offspring of each founder mouse carry the "transgene" at a particular chromosomal position. In most cases, this approach leads to a gain of function because the endogenous gene is still in place and functional, and the newly added transgene also works. This transgenic approach was widely used in the 1970s and early 1980s and has provided valuable information about gene function. However, because the transgene is functional at a random chromosomal site (thus introducing variability due to positional effect), and also because the transgene activity is under the control of an exogenous promoter, a major concern is how much the observed phenotype truly reflects the endogenous gene function.

During the 1980s, the technique of homologous recombination-based gene targeting (Capecchi 1989, Koller & Smithies 1992) made it possible to alter a gene on the chromosome, generating mice that carry "designer" changes. This is often called knockout because most of the manipulations published to date disrupt the endogenous gene activity. In some respect, gene targeting is conceptually the same as early work in *Drosophila*, where one made random mutations and then examined phenotypic changes to deduce the relationship between a genetic locus and a phenotype. This approach has greatly enhanced our knowledge of the contribution of individual genes to animal physiology and behavior.

One limitation of this approach is that because the gene mutation is passed on in germline cells, the entire animal carries a homogenous cellular genetic content; every cell is the same, genetically speaking. Therefore, for a gene with multiple functions in more than one part of the body/organ, the effect of gene mutation may be pleiotropic as well. More importantly, because the mutation exists throughout embryogenesis as well as in the adult animal, it may alter the animal's development if the gene product is involved in any way in developmental processes. Therefore, it is difficult to rule out the possibility that the observed phe-

notype in adult animals is a combined result of developmental changes (and compensatory alterations) as well as the loss of the gene function in the adult animal.

This concern is pertinent for genes that are important in early development, such as homeobox genes, deletion of which often results in severe defects during development; homozygous mutant animals seldom survive postnatally. Because of such embryonic lethality, mutant animals seldom reach the adult stage for studies of the behavioral effects of the gene mutation. In the field of pain research, however, it appears that many of the genes studied are not developmentally critical. To date, few have resulted in embryonic lethality. Thus, knocking out these genes often results in animals that either have minor or no developmental changes (for example, opioid receptor genes), or that display rather restricted changes in the sensory nervous system, some of which mirror certain human conditions (for example the *trkA* receptor). In a way, these gene knockouts are “cleaner” ones that do not overtly affect the animal’s development, thus allowing the study of gene knockout in otherwise normal animals. Consequently, the use of a gene knockout approach has been particularly informative in dissecting the contribution of individual pain genes.

The knockout approach is subject to other interpretational concerns that relate to the genetic background of the mutant mice. For example, the knockout phenotype may be highly dependent on the background strain, that is, dependent on epistatic interactions between the targeted gene and the specific alleles of other trait-relevant genes (e.g. Kelly et al 1998, Magara et al 1999, McNamara et al 1998, Threadgill et al 1995). A related problem is the “hitchhiking donor gene confound”: The targeted gene may be closely genetically linked to another gene affecting the trait, such that the knockout “phenotype” actually is due to alternate alleles of the linked gene in the wildtype versus knockout populations (Gerlai 1996). We have shown that these problems are extremely relevant to pain research, since 129 mice (the embryonic stem cell donor strain) and C57BL/6 mice (in common use as a fecund recipient strain) differ greatly in their nociceptive and analgesic sensitivities (Mogil & Wilson 1997, Mogil et al 1999a). Refer to Mogil & Grisel (1998) for a fuller discussion of these issues.

Compensatory changes as the result of gene knockout, on the other hand, offer clues about gene interaction and related gene functions. This opportunity is particularly attractive in light of the rapid improvement of microarray technology that allows simultaneous screening of altered activity of thousands of genes (Iyer et al 1999). Thus, by comparing knockout versus wild-type mice for differences in gene expression levels, it will soon be possible to identify all the genes that incurred “compensatory” changes, providing a framework for further studies on genes that interact with and influence the knocked-out gene.

Some of the limitations of the knockout approach may also be obviated by new technological advancements that now make it possible to spatially and temporally regulate the gene modification. One approach is to use the *cre/loxP*

recombination system. Key to this approach is the use of promoters that are well characterized with regard to their spatial and temporal expression activity, so that a targeted gene mutation can be restricted to a specific body region or tissue/organ (Gu et al 1994). With this approach prenatal changes during development can be avoided altogether (Tsien et al 1996). Another promising approach is the use of chemically inducible systems for gene alteration. For example, gene activity may be controlled in a temporally specific fashion by using antibiotics (Stark et al 1998); alternatively, it is possible to construct a synthetic chimera gene that can be activated by an unnatural ligand (Redfern et al 1999). An interesting advantage for pain research, for example, is that the antibiotic can be administered to the spinal cord, which can induce localized gene deletion, even though a promoter that is specific to the spinal cord does not yet exist.

Rather than manipulating chromosomal genes, it is possible to indirectly alter gene activity using an expression inhibition, or “knockdown,” approach. In these studies, which are increasingly popular in pain research, antisense oligonucleotides are used to suppress mRNA translation and decrease mRNA stability. This approach at the RNA level has the advantage that the experimenter has control over what stage of the adult animal the manipulation is initiated, and it is far less costly and less time consuming than generating a genetic knockout animal model. This approach has been successfully used to study the contribution of particular genes to pain behavior and to the analgesic action of particular molecules (e.g. Pasternak & Standifer 1995). Disadvantages include the uncertain mechanism of action of antisense molecules and the difficulty in interpreting negative experimental results. It is also puzzling how such profound behavior phenotypes can arise despite generally very limited knockdown of protein levels, an issue of “threshold protein effect” that is poorly understood. Finally, another approach comes from the field of gene therapy, where a number of useful viral vectors based on retrovirus, adenovirus, and adeno-associated virus have been developed. Using these vectors, a gene of interest can be delivered stereotaxically in experimental animals to a particular CNS region, influencing only the cells in that region (Kaplitt & Makimura 1997, Ye et al 1999).

It is often argued that many studies with knockout mice can be performed instead with pharmacological antagonists. The two approaches are complementary—they cannot replace each other. It should be remembered that antagonists are not without problems. The literature is replete with examples in which a supposedly “specific” antagonist turns out to work at multiple receptor subtypes. There are, in fact, no specific antagonists; there are only relatively selective antagonists. Furthermore, it is difficult, if not impossible, to specifically remove the action of a given neurotransmitter system for an extended period of time with antagonists; specificity and prolonged deletion are readily achieved with knockout technology. Prolonged antagonism is often required when studying genes that contribute to persistent pain conditions. Clearly, pharmacological and genetic methods should be used, and results from the different approaches constantly compared.

## GENES INVOLVED IN THE DEVELOPMENT AND SURVIVAL OF THE PRIMARY AFFERENT NOCICEPTOR

Alterations in the development and maintenance of the phenotype of small diameter nociceptors are critical to normal pain responsiveness. For this reason, some of the most interesting phenotypes have been identified using knockout technology to delete genes that encode for the neurotrophins or their receptors, which are required for normal sensory afferent development. Several neurotrophin receptors have been identified in dorsal root ganglia. TrkA is targeted by nerve growth factor (NGF), trkB by brain-derived neurotrophic factor (BDNF), and NT-4 and trkC by NT-3. The low affinity p75 receptor is targeted by all of the growth factors; its deletion produces a unique phenotype (see below). As noted above, natural mutations of trkA in humans underlie the loss of pain sensitivity in patients with congenital insensitivity to pain. Thus, the deletion studies in animals are of particular interest and importance. Animals with null mutations in almost all of the neurotrophins and their receptors have been studied.

### TrkA and the Nociceptor

The trkA receptor is expressed by all peptide-containing, small diameter nociceptors. By contrast, although the nonpeptide population requires NGF and the trkA for its survival during embryonic development, the receptor is not expressed in these neurons in adults (Silos-Santiago et al 1995). Rather, this population expresses *c-ret* and is responsive to glial-derived neurotrophic factor (GDNF) in the adult. Deletion of trkA produces a syndrome similar to the congenital pain insensitivity observed in children. Few small diameter sensory afferents survive in trkA null mice, and there is a profound loss of pain responsiveness to pin prick and heat. Interestingly, de Castro and colleagues (1998) found a residual corneal sensitivity to thermal and chemical noxious stimuli in these animals, which raises the possibility that a distinct population of polymodal nociceptors does not express trkA during development.

Smeyne et al (1994) also reported the presence of skin ulcerations and self-mutilation in the trkA null mice. This may reflect the loss of acute neurogenic inflammatory responses (which require peripheral release of peptides from small diameter afferents) or insensitivity to self-inflicted pain, respectively. Whether the self-mutilation was a futile attempt to eliminate an apparent source of abnormal, possibly painful sensations (as in phantom limb pain) cannot be determined. It is of interest, however, that Devor & Raber (1990), using selective breeding, were able to generate lines of rats that show high and low levels of denervation-induced self-mutilation (autotomy). Although the relationship of the autotomy that is observed in some clinical conditions (e.g. Lesch-Nyhan syndrome) to that observed in rodents is not known, these observations do raise the possibility that single gene defects can generate destructive behaviors that are driven by abnormal pain sensations.

## Neurotrophins and the Nociceptor: Developmental Abnormalities Establish a Key Role for NGF

Paralleling the results in *trkA* mutants are those derived from mice with deletions of the gene that encodes NGF. Not surprisingly, cells that express *trkA* were lost in the dorsal root ganglia (DRG) of the NGF null mice (there was no change in the numbers of *trkB*- or *C*-expressing neurons). Consistent with these results, immunostaining of the DRG and spinal cord for calcitonin gene-related peptide (CGRP), which is almost exclusively located in small diameter sensory afferents, was lost. Tests of pain sensitivity in the few null mutants that survived beyond one week revealed almost no response to noxious mechanical stimuli. Heterozygotes had slightly increased hot-plate latencies, suggesting that the pain phenotype was multimodal. In light of the results in the *trkA* mutants, these results are not surprising. By contrast, because there is evidence for phenotypic switches in the neurochemistry of large diameter afferents in the adult in the setting of injury [for example, substance P (SP) begins to be expressed by these afferents], perhaps the most interesting result in the *trkA* and NGF null mutants is that there was no compensatory response. Apparently, large diameter afferents develop properties of *C*-nociceptors when the latter are injured, but not in their absence.

## CONTRIBUTION OF THE SYMPATHETIC NERVOUS SYSTEM

Because sympathetic hyperactivity has been implicated in a variety of persistent clinical pain conditions, particularly those occurring in the setting of nerve injury, it is also significant that NGF regulates the growth and development of sympathetic postganglionic neurons. For example, peripheral nerve injury induces sprouting of sympathetic efferents in the DRG (McLachlan et al 1993). Because the sprouting is targeted to large diameter cell bodies, it has been hypothesized that this reorganization contributes to sympathetically maintained pain conditions in which stimulation of large diameter mechanoreceptive fibers triggers the pain (so-called A beta-mediated allodynia; Andersen et al 1995). In this regard, results in mice that overexpress NGF, driven off a keratin promoter, are of great interest (Davis et al 1994). In these animals, there is also hyperinnervation of the DRG by sympathetic efferents, but this occurs in the absence of peripheral nerve injury. The animals also had exaggerated responses to noxious mechanical and thermal stimuli, and nerve injury-induced mechanical and thermal allodynia was increased (Davis et al 1993). On the other hand, the sprouting in the mice that overexpress NGF was directed at DRG neurons that express *trkA*, that is, at the smaller diameter population (Davis et al 1998). What triggers the sympathetics

to target the non-*trkA*-expressing, large diameter DRG cells after nerve injury in the adult is not understood. Regardless of the mechanism, this result points again to the critical contribution of genes that code for NGF and *trkA* (whether deleted or enhanced) to nociceptive processing.

### Neurotrophins and Nociceptive Processing: Influence of the Genes in Postnatal and Adult Animals

Neurotrophins not only contribute to the survival of sensory afferents, but are also critically involved in the development of the nociceptor phenotype. Lewin & Mendell (1994) reported that removal of NGF during the first two weeks after birth alters the properties of C nociceptors. Apparently, many C nociceptors that respond to noxious mechanical and thermal stimuli were replaced by a population of very low-threshold C mechanoreceptors. Interestingly, when the treatment was started at birth, the loss of nociceptors was accompanied by loss of sensory ganglion cells; however, when the treatment began soon after birth the functional reorganization occurred without cell loss (Lewin et al 1992). Finally, although the downregulation of *trkA* expression in the nonpeptide population of small diameter afferents referred to above coincides with the time at which nociceptor phenotype can be altered by NGF, it does not appear that NGF is required for the reduced *trkA* expression.

The postnatal/adult contribution of NGF is particularly prominent in the setting of tissue and nerve injury. For example, levels of NGF are significantly increased in inflamed tissue, and this is associated with an upregulation of expression of neuropeptides (e.g. SP) in nociceptors. Consistent with this result, injection of NGF produces an immediate and profound thermal hyperalgesia, a delayed mechanical hyperalgesia (Lewin et al 1994), and an upregulation of SP and CGRP (Inaishi et al 1992, Lindsay 1996, Otten et al 1980). By sequestering NGF with a *trkA*-IgG, Bennett et al (1998) also demonstrated that there is a constitutive function of NGF. This treatment reduced the number of nociceptors that responded to noxious heat from 57% to 32%, without a change in the response to threshold or suprathreshold mechanical stimuli. The same treatment induced a persistent hypoalgesia to thermal and chemical noxious stimuli in the setting of carrageenan inflammation and a corresponding decrease of peptide in the sensory afferents. Although a direct action of NGF on primary afferent nociceptors is likely in these injury conditions, the fact that sympathectomy can prevent some of the hyperalgesic effects of NGF suggests that multiple routes are involved in the pronociceptive contribution of NGF.

Other studies have implicated NGF in the maintenance of nociceptor phenotypes. For example, Fjell et al (1999) recently reported that deletion of NGF by immunization in the adult reduces the expression of the tetrodotoxin (TTX)-resistant Na<sup>+</sup> channel, which is almost exclusively expressed by nociceptors, but only

in the *trkA*-expressing peptide population of C fibers. Finally, it is significant that NGF also regulates the expression of the vanilloid-1 (VR-1)/capsaicin receptor (Winter et al 1988). Because the latter responds to noxious heat and to decreased pH (Tominaga et al 1998), it is likely that NGF contributes to the sensitization of nociceptors to these stimuli in the setting of acidic inflammatory chemical milieu.

In summary, the developmental defects observed in null mutants are profound and have provided insight into the generation of congenital insensitivity to pain in children. The importance of neurotrophins in the adult, however, underscores the utility of studying inducible knockouts of these different genes in the adult, that is, under conditions in which the development of the sensory afferents has not been altered.

### Do Genes that Regulate the Development and Survival of Large Diameter Afferents Influence Pain?

Deletion of the other *trk* receptors, which are predominantly expressed by the larger diameter, nonnociceptive population of sensory afferent, does not obviously produce a pain phenotype. However, animals with such *trk* receptor deletions were not studied in models that might reveal the consequences of losing the regulatory role that large diameter afferents exert. Specifically, there is considerable evidence that the mechanical allodynia (i.e. decreased mechanical pain threshold) that occurs in the setting of tissue or nerve injury is generated in part by abnormal interactions between large diameter afferents and pain responsive dorsal horn neurons. This pathological coupling can arise from several mechanisms. For example, there is evidence for sprouting of the A-beta afferents and/or *de novo* expression of nociceptor-associated neurotransmitters (such as SP) leading to activation of nociresponsive neurons in regions previously occupied by C fibers (Neumann et al 1996, Noguchi et al 1995, Woolf et al 1995). Increased excitability of the "pain" transmission neurons, such that they can be activated by previously innocuous stimuli, has also been demonstrated (Hylden et al 1989), as has loss of inhibitory interneurons that normally suppress the input from the large diameter afferent (Zhang et al 1994). Bennett and colleagues (1996) recently demonstrated that spinal administration of NGF could block the A-beta sprouting seen after nerve injury, presumably because the NGF preserved the chemical integrity of the C fiber terminals. Interestingly, neither NT-3 nor BDNF counteracted the large fiber sprouting; this result indicated that damage to C fibers was indeed the critical factor for inducing sprouting. To what extent the neurotrophins that contribute to large diameter sensory afferent development and survival come into play in other injury conditions needs to be studied. These observations emphasize the critical importance of sharing the many mutants that have been generated with laboratories familiar with the study of phenomena as complex as pain. The routine screens through which knockout animals are put may not be sufficient to appreciate subtleties of a particular gene's contribution.

## CONTRIBUTION OF P75, A LOW-AFFINITY NEUROTROPHIN RECEPTOR

Although the contribution of *trkA* can be attributed to the changes that are induced when NGF binds, it is not clear which neurotrophin interaction underlies the contribution of *p75*, the low-affinity neurotrophin receptor. Deletion of *p75* has produced mice with reduced skin innervation and reduced pain responses to noxious heat (Lee et al 1992). On the other hand, the thermal hyperalgesia induced by NGF was not altered in *p75* null mice, indicating that *trkA* is sufficient (Bergmann et al 1998). Importantly, although there is an almost 50% loss of DRG neurons in *p75* null mice, the loss is broadly distributed across cell sizes (Bergmann et al 1997). Consistent with the anatomical changes, the functional abnormalities occurred in nociceptive and nonnociceptive afferents (Stucky & Koltzenburg 1997). A-delta mechanoreceptors, which respond to noxious mechanical and thermal stimuli, lost their responses to heat. By contrast, C mechanoheat nociceptors lost their response to mechanical stimuli. Finally, a surprising but interesting result was observed in the tooth pulp, which is thought to be exclusively innervated by nociceptors. Typically, CGRP levels in the tooth pulp decrease with age. Sarram et al (1997), however, found that CGRP levels were unusually high in the *p75* mutants and did not decrease over time. In fact, innervation in general was increased in these mice, just the opposite of what was observed in skin. The authors speculated that the increased innervation of the tooth was a response to an unusually rapid wearing-down of the molar crowns in the *p75* null mutants.

In summary, it is not possible to pinpoint the conditions under which *p75* comes into play. Because of the rather global effect of *p75* deletion, it is likely that the phenotype resulted from reduced function of all neurotrophins; that is, *p75* may somehow facilitate the action of neurotrophins at their respective high-affinity receptors. Regardless of the mechanism, it is obvious that attempts to regulate expression of these genes in the adult can profoundly influence the processing of acute pain messages and provide an approach to altering the persistent pains that can arise in the setting of tissue and nerve injury.

## GENES THAT ENCODE PRIMARY AFFERENT NEUROTRANSMITTERS

As noted above, when individuals cannot sense acutely noxious stimuli, the consequences are devastating. In addition to the phenotype arising from a defect in the synthesis of neurotrophins or their receptors, acute signaling could be significantly affected by a failure to synthesize neurotransmitters of the nociceptor or of the receptors that they target. In fact, several studies have established unique phenotypes that are associated with loss of subclasses of primary afferent neu-

rotransmitters or their receptor. Recently we evaluated the effect of deleting the preprotachykinin (PPT-A) gene, which codes for the tachykinins, SP and NKA. PPT-A null mice appeared normal and their responses to mild to moderate noxious stimuli were intact. The loss of the tachykinins was only apparent in response to intense noxious stimuli. Importantly, responses were reduced for all modalities of intense stimuli, including thermal, mechanical, and chemical. Given the residual polymodal nociceptive sensitivity of the cornea in *trkA* mutants described above, our results raise the possibility that some SP/NKA-containing afferents do not require *trkA*/NGF for their survival. Another laboratory that generated a PPT-A mutant found somewhat different results, particularly in studies of visceral pain (Zimmer et al 1998); it is not clear whether these differences reflected variations in genetic background (see below) or differences in methods of testing pain behavior.

Because there is evidence that tachykinins contribute to central sensitization (i.e. to the lowering of pain thresholds in the setting of injury), in part through their facilitation of NMDA-mediated sensitization of dorsal horn neurons (Dougherty et al 1993, Rusin et al 1992), we expected to observe significant defects in the PPT-A mutants in different injury models. In fact, neither the magnitude nor the time course of tissue or nerve injury-induced mechanical and thermal allodynia was altered in these animals. We therefore concluded that SP/NKA only comes into play when the stimulus is intense. We further hypothesized that glutamate release from the primary afferent mediates the low stimulus intensity-induced pain condition. Interestingly, McLeod et al (1999) recently described heightened thermal and mechanical allodynia in an NGF-overexpressing mouse that was driven off of a myelin basic protein promoter. In these animals, NGF expression is enhanced in oligodendroglia, and there is an abnormal primary afferent-derived SP input to the lateral white matter of the spinal cord. Whether the different phenotype reflects the fact that the abnormal SP input in the NGF-overexpressing mice contacts neurons not normally activated by SP-containing afferents needs to be assessed. These differing results emphasize that identifying the phenotype produced by gene deletion or overexpression is but the first step in discovering the mechanisms through which a particular gene exerts an effect on the processing of pain messages.

## PRE-PROTACHYKININ PRODUCTS AND NEUROKININ RECEPTORS

Phenotypic differences between the behavior of the PPT-A mutant mice and of mice in which the neurokinin-1 receptor (NK-1) was deleted proved to be particularly illuminating. Because both SP and NKA have high affinities for the NK-1 receptor (Maggi 1995), it is not clear which of these endogenous tachykinins is more critical to the production of pain. It was thus of interest that in contrast

to the PPT-A mice, the response to acute noxious stimuli appears to be intact in the NK-1 receptor mutant (De Felipe et al 1998). Furthermore, although the NK-1 receptor mutants showed signs of persistent pain in the formalin test, the PPT-A mutant mice only had reduced first phase behavior, which is indicative of acute pain responsiveness.

More interesting perhaps is the insight that these animals provided into the possible contribution of an additional neurokinin receptor, namely the NK-2 receptor. There is considerable pharmacological evidence for a second neurokinin receptor in the spinal cord. Surprisingly, however, the receptor cannot be detected in Northern blots, by *in situ* hybridization, or by immunocytochemistry with antisera directed against the cloned NK-2 receptor. One possibility is that the spinal cord NK-2 receptor is pharmacologically similar, but not identical to the peripheral NK-2 receptor. Because NKA has a higher affinity for the NK-2 receptor than does SP, a contribution of the NK-2 receptor could account for some of the differences between the PPT-A and the NK-1 receptor null mice. To address this possibility, we are presently generating mice in which either SP or NKA, but not both, are deleted.

## SECOND MESSENGER MOLECULES: IDENTIFYING GENES THAT CONTRIBUTE TO ABNORMAL PAIN CONDITIONS

As described above, major features of clinical pain are its persistence, the fact that innocuous stimuli are pain-producing, and that the magnitude of the pain experience is not proportionate to the injury. Long-term changes in the organization of dorsal horn circuitry are clearly a major contributor to these problems. Here again, knockout technology has provided important insights into the underlying mechanisms. In these studies gene deletion is particularly valuable because antagonists against different second messenger molecules are notoriously non-selective. They not only do not distinguish among different isoforms of a particular kinase, but they may also block very different kinases.

Our laboratory has studied two mice, one with a deletion of the R1 $\beta$  subunit of protein kinase A (PKA) (Malmberg et al 1997a) and one with a deletion of the gamma isoform of protein kinase C (PKC $\gamma$ ) (Malmberg et al 1997b). There was already considerable evidence for a contribution of cAMP-dependent PKA to the peripheral sensitization process. Studies in the laboratory of Levine and colleagues (Taiwo et al 1989), in particular, had implicated PKA in the lowered threshold of nociceptors that occurs in inflamed tissue. More recently, their laboratory provided evidence that the TTX-resistant Na<sup>+</sup> channel is modulated/sensitized via PKA (Gold et al 1998). In many respects, the results we found in the R1 $\beta$ -PKA knockouts were consistent with those observations. Specifically, although the response to acutely painful stimuli was not altered in the R1 $\beta$ -PKA

null mice, there was a significant decrease in the magnitude of the allodynia that developed in the setting of tissue injury. The magnitude of the second phase of the formalin test (which provides a measure of persistent tissue injury-induced pain) was also significantly reduced in these mice. Because this isoform is expressed in DRG and because the protein product is transported to the peripheral terminal, it is likely that the phenotype we observed involves changes in the peripheral sensitization process. On the other hand, we also found that the sensitization produced by direct spinal injection of the prostaglandin, PGE<sub>2</sub>, which acts via PKA, was also reduced. This indicates that both the central and peripheral terminals of the nociceptor are targets of PKA-mediated sensitization. Finally, we found no changes in nerve injury-induced pain in these mice. That result is particularly important in light of the dramatic changes we observed in the PKC $\gamma$  null mutant mice.

We chose PKC $\gamma$  for several reasons. First, it is only expressed in the CNS. Second, because it is not expressed by DRG neurons, it was likely that any phenotype observed could be attributed to changes in the spinal cord or brain. The plethora of possible explanations involving peripheral loci (primary afferent terminals, mast cells, immunocompetent cells, etc) could be ruled out. Third, in contrast to other PKC isoforms, PKC $\gamma$  first appears postnatally; thus, developmental defects could be avoided. As for the R1 $\beta$ -PKA study, we found no difference in acute pain responsiveness between the wild type and PKC $\gamma$  null mice. The most intriguing result was observed in a neuropathic pain model. In this model, we cut approximately one half to two thirds of the diameter of the sciatic nerve. Such partial nerve injuries often result in persistent pain conditions, in animals as well as in humans. Within 24 hours of cutting the sciatic nerve, we observed a profound thermal and mechanical allodynia in the partly denervated hindpaw of the wild-type mice (Malmberg et al 1997b). In wild-type mice the thermal allodynia persists for months and the mechanical allodynia appears to be permanent. By contrast, there was only a minimal change in the PKC $\gamma$  null mice, and this resolved quickly.

To address the locus of the "defect" in the null mice, we have recently recorded from dorsal horn nociceptive neurons after injury and followed the development of central sensitization (Martin & Basbaum 1998). Consistent with the behavioral results, we found equivalent responses to acute noxious stimuli; indeed, the large barrage produced by an intensely noxious stimulus (mustard oil) was comparable. Great differences, however, were observed when we assessed the magnitude of mustard oil-induced central sensitization. The wild-type mice developed a prolonged central sensitization (the threshold for driving the lamina V neuron dropped and persisted for hours), but we observed only a transient sensitization in the mutant mice. Within two hours, the thermal and mechanical thresholds had returned to pre-mustard oil levels.

These results are important not only because they implicate a specific PKC isoform in a major pain condition, but also because they point to the spinal cord

as the locus of action of this gene product. In the course of our studies we noted that PKC $\gamma$ , in spinal cord, is confined to a subpopulation of interneurons in the inner part of the substantia gelatinosa, lamina II. Importantly, the gamma isoform is not expressed in lamina V projection neurons (i.e. in the neurons that transmit the nociceptive message to brainstem and thalamus). It follows that the phenotype observed must have resulted from an alteration in the circuit that includes primary afferent nociceptors, PKC $\gamma$  interneurons, and lamina V projection neurons. Based on double-label studies for markers of neurotransmitters, we believe that the PKC $\gamma$  interneurons are probably excitatory (Martin et al 1998). Whether injury-induced activation of PKC $\gamma$  results in increased excitatory drive from non-nociceptive inputs to the lamina V projection neuron, via the interneurons of inner lamina II, needs to be assessed.

Recently, Khasar et al (1999) provided evidence for an independent contribution of the epsilon isoform of PKC (PKC $\epsilon$ ) to nociceptive processing by peripheral afferents. Their previous studies revealed that overexpression in PC12 cells of PKC $\epsilon$ , which is diacylglycerol- but not Ca<sup>2+</sup>-dependent, enhances NGF-induced neurite outgrowth (Hundle et al 1995). They reasoned, therefore, that the pain phenotype of the PKC $\epsilon$  null mice is related to alterations in NGF regulation of the nociceptor population.

Finally, because of the evidence for dramatic colocalization of nitric oxide synthase (NOS) in GABAergic neurons of the spinal cord (Valtschanoff et al 1992) and because pharmacological studies have implicated this enzyme in the development of injury-induced persistent pain (Meller & Gebhart 1993), several laboratories evaluated mice with a deletion of the gene that encodes NOS. These studies did not reveal a pain phenotype that was predicted from the pharmacological studies. For example, pain behavior in the acute and persistent phases of the formalin test was intact in these mice (Crosby et al 1995). More surprisingly, perhaps, NOS inhibitors blocked the pain behavior in the knockout! This suggested that although NOS contributes to these behaviors in the wild type, there was a compensatory response to deletion of the NOS, resulting in normalization of the pain behavior, presumably via an alternate pathway. This dramatic example of compensatory responses illustrates the uncertainties that may arise in the analysis of knockout mice (see below). Caution must always be used when these animals are evaluated.

## GENES INVOLVED IN MODULATION OF PAIN SENSATION IN ADULT ANIMALS

Recent studies, especially those using the gene targeting approach, have also unraveled the contribution of a number of genes to the modulation of pain; both the "usual suspects" and some unsuspected ones have been studied.

## Opioids and Their Receptors

Opioids are among the most efficacious pain-relieving drugs used for clinical management of pain (Basbaum & Fields 1984, Pasternak 1993). Indeed, the analgesic utility of opioids has been exploited for several thousand years (Brownstein 1993), making opioids possibly the longest used drugs that are still widely prescribed in clinical medicine today. There is now considerable information on the mechanism through which opioids exert an analgesic action. Importantly, however, (and possibly relevant to the side effect profile produced by opioid analgesics), opioid peptides and alkaloids also affect a number of physiological functions, including hormone secretion, neurotransmitter release, feeding, gastrointestinal motility, and respiratory activity (Pasternak 1988).

There are three well-characterized peptide groups of endogenous agonists: endorphins, enkephalins, and dynorphins. Each is processed from large protein precursors (Akil et al 1998). The biological effects of these peptides are mediated via three well-studied receptor classes: mu, delta, and kappa (Goldstein 1987, Pasternak 1993). A new peptide group, the endomorphins, has been recently identified (Zadina et al 1997). Because the gene(s) for endomorphins have not yet been cloned, it remains to be seen whether these small peptides are also processed from larger protein precursors.

All endogenous opioid peptides produce analgesic effects when applied experimentally, and activation of all three classes of opioid receptors results in pain relief under certain experimental conditions (Pasternak 1993). However, most studies have concentrated on the mu opioid receptor because it is the main cellular target for clinically relevant opioid drugs, including: (a) naturally occurring drugs such as morphine and codeine; (b) synthetic compounds, such as fentanyl and methadone; and (c) the major metabolites of heroin. Gene targeting studies directed at opioid receptors have provided evidence that each class is relevant to pain control (for review, see Kieffer 1999). Most importantly, perhaps, deletion of the mu opioid receptor gene generated homozygous knockout mice that completely lost their ability to respond to morphine in several tests of pain behavior (Matthes et al 1996, Sora et al 1997). Despite this, these animals displayed near-normal levels of sensitivity to noxious stimuli and retained the ability to respond to analgesic effects of other, non-mu reagents, including delta and kappa opioids (Loh et al 1998, Matthes et al 1996, Schuller et al 1999, Sora et al 1997, Tian et al 1997). Similarly, when the kappa opioid receptor gene was inactivated by gene targeting, mu and delta opioid agonists retained their ability to induce analgesia in the homozygous mutant mice (Simonin et al 1998). Furthermore, when the precursor gene for either beta-endorphin (Rubinstein et al 1996) or enkephalins (Konig et al 1996) was knocked out, mutant mice showed only limited alterations in pain processing. These cases highlight the parallel and redundant nature of the opioid system in pain modulation, perhaps reflecting the need for ensuring functional nociception (i.e. preserving acute pain processing), which is necessary for an animal's self-protection and survival.

## The Issue of Compensatory Changes in Knockout Mice

As described above, a major concern in the generation of knockout mice is the potential for compensatory changes. Specifically, because the protein of the targeted gene is missing throughout embryogenesis, developmental changes may occur to compensate for the missing protein function. Knockout mice involving the opioid system, however, appear to exemplify the other side of the story; that is, complete elimination of a peptide ligand or a receptor causes limited compensatory change. For example, when the preproenkephalin gene was deleted, no detectable change in the levels of the other two endogenous opioid peptide families, beta-endorphin and dynorphin, was observed (Konig et al 1996). The same was true when the mu opioid receptor was eliminated; all the other components of the opioid system, including the endogenous peptides, as well as the delta and kappa opioid receptors, showed very little change (Kitchen et al 1997, Matthes et al 1996). Similarly, very few changes of the other opioid system components were observed in kappa opioid receptor knockout mice (Simonin et al 1998, Slowe et al 1999). These results highlight the relatively independent nature of components of the opioid system, or they may be indicative of significant redundancy of this system. It should be noted, however, that examples of functional compensation in beta-endorphin knockout mice have been noted (Rubinstein et al 1996; JS Mogil, JE Grisel, JR Bales, MD Hayward, M Rubenstein, JK Belknap, and M Low, unpublished data), as has regional upregulation of mu and delta receptor binding in enkephalin mutants (Brady et al 1999). In addition, the opioid system can respond to alterations in the expression of other, nonopioid genes. For example, the opioid peptide dynorphin showed a reduced expression in the basal ganglia in dopamine D1 receptor knockout mice (Xu et al 1994), suggesting a feedback modulation by the dopamine system, which is in turn modulated by the opioid system.

Studies of knockout mice also unraveled an important issue regarding compensatory changes, i.e. lack of substantial (in terms of experimentally measurable) change in related protein levels, yet subtle change in function as the result of knocking out a particular gene. For example, in mice deficient of beta-endorphin, where compensatory changes were not observed at the receptor binding level, subtle *functional* compensation took place: The lack of opioid stress-induced analgesia (SIA) was compensated for by upregulated non-opioid SIA (Rubinstein et al 1996). This highlights the need to carefully examine knockout mice for behavioral manifestations underlying functional modification, viz functional compensatory responses that are not manifest at the level of receptor number or binding affinity.

## Splice Variants and the Mu Receptor Controversy Vis-à-Vis Heroin Responsiveness

Studies that used antisense oligonucleotides to reduce expression of the mu opioid receptor gene suggested the existence of alternative slicing variants for this

receptor (Pasternak & Standifer 1995; Rossi et al 1995, 1997). Among the variants proposed was one missing coding exon 1, which corresponds to the N-terminal region and the first of the seven hydrophobic domains in this G-protein-coupled receptor. The conclusion that was drawn from those experiments was quite unorthodox at the time. Specifically, the authors proposed that the first  $\alpha$ -helix at the N-terminal region of the receptor, considered essential for receptor activity, could be spliced out, and yet the receptor remained functional. Subsequent gene targeting studies of the mu opioid receptor provided both further evidence (see below) and controversy.

Several groups have generated mu opioid receptor knockout mice (Loh et al 1998, Matthes et al 1996, Schuller et al 1999, Sora et al 1997, Tian et al 1997) and all showed that morphine was inactive in producing analgesia in these animals. When other opioid drugs were tested, however, it was observed that heroin, its metabolite 6-acetylmorphine, and a morphine metabolite, morphine-6 $\beta$ -glucuronide (M6G), were able to produce significant analgesia (Schuller et al 1999). In these homozygous mutant mice, the first coding exon was deleted. This should have produced a loss of the first membrane-spanning  $\alpha$ -helix, together with the entire N-terminal part of the mu opioid receptor. Despite this, binding studies indicated that low levels of M6G binding persisted (Schuller et al 1999), suggesting that mechanisms such as alternative splicing can generate different forms of the mu opioid receptor. These results also provide a potential way by which the proposed subtypes of the mu opioid receptor (Pasternak 1993) could be generated.

Controversy exists regarding this issue, however, as others found no residual heroin activity in another line of exon 1-deletion mutant mice (Kitanaka et al 1998). In this respect, it is of interest that studies using inbred strains of mice have shown that opioid receptor pharmacology is influenced by the genotype of the mouse strain. For example, the effect of heroin is mediated by the mu opioid receptor in ICR mice, but by the delta opioid receptor in Swiss Webster mice (Rady et al 1991). Thus, these data suggest that small differences in the genetic background may collectively be critical contributors to the pharmacological profile of the receptor for a particular drug. This view is supported by the finding that differences in genetic background in inbred mice can result in prodigious variation for a given nociceptive test (Mogil et al 1999a). In other words, subtle differences between the exact genetic makeup of each mu receptor knockout mouse model have the potential to appear as major phenotypic differences. In fact, close examination of the molecular strategies used by different groups for making the knockout constructs indicates that mutations engineered into the mu opioid receptor gene differ from one another. These differences may be still another source that contributed to the observed phenotypic difference in heroin metabolite responsiveness.

## NOCICEPTIN/ORPHANIN FQ AND ITS RECEPTOR (ORL): EVOLUTIONARILY-RELATED GENES

Cloning efforts in other neurotransmitter receptor systems have often identified many more receptor subtypes than were predicted from pharmacological studies. This is particularly true for serotonin receptors (Kroeze & Roth 1998). Mindful of such precedence, upon the cloning of the three opioid receptors (Kieffer 1995) many laboratories sought to clone "opioid receptor subtype" genes for which pharmacological evidence existed (Pasternak 1993). To date, however, the only new gene uncovered is an opioid receptor-like (ORL) "orphan" receptor that displays very poor affinity for conventional opioid compounds (e.g. Bunzow et al 1994, Chen et al 1994, Fukuda et al 1994). At the sequence level, this orphan receptor clearly belongs to the opioid receptor gene family. It possesses a high degree of amino acid sequence similarity to all three opioid receptors (~65%), which compares favorably with the ~70% sequence similarity among opioid receptors themselves (Chen et al 1994). Subsequent studies showed that this orphan receptor responds to dynorphin activation at concentrations that also activate the mu and delta receptors, albeit all in the nanomolar range compared to the sub-nanomolar affinity of kappa receptor for dynorphins. Most importantly, the gold standard antagonist, naloxone, is ineffective at blocking the effect of dynorphin at the ORL orphan receptor (Zhang et al 1998, Zhang & Yu 1995).

Search for endogenous ligands for the ORL orphan receptor led to the identification of a novel endogenous peptide, variously termed nociceptin (Meunier et al 1995) and orphanin FQ (Reinscheid et al 1995). Although its putative status as an "opioid" led researchers to predict analgesic properties for nociceptin/orphanin FQ (N/OFQ), the actual functional relevance of this peptide is still being debated. The original investigations concluded that N/OFQ was pronociceptive, producing hyperalgesia (Meunier et al 1995, Reinscheid et al 1995). Many—but not all—subsequent investigations have failed to replicate this finding, and considerable support exists for antianalgesic (including antiopioid) actions of this peptide when administered supraspinally (e.g. Mogil et al 1996a). The spinal actions of N/OFQ are similarly controversial, with analgesic (King et al 1997), allodynic (Okuda-Ashitaka et al 1996), and antiallodynic (Hao et al 1998) actions having been noted. However, when the ORL orphan receptor gene was knocked out, the mutant mice showed no change in baseline pain threshold or morphine analgesia (Nishi et al 1997); rather, they displayed a partial loss of morphine-induced tolerance (Ueda et al 1997), as well as enhanced long-term potentiation and memory retention (Manabe et al 1998).

These results further highlight the complexity of the opioid peptide/receptor system—in addition to the three major classes of opioid receptors, the ORL orphan receptor represents an expansion of the opioid receptor gene family and may underlie a modulatory function that is not yet fully understood. These studies also support the notion of gene duplication as a mechanism for generating evo-

lutionarily-related genes: Multiple members of a gene family likely arose from gene duplication events during evolution and subsequently diverged upon spontaneous mutations. The products of sequence level-related proteins have probably taken on additional and/or distinct physiological functions compared to those of the progenitor gene product.

## CANNABINOID RECEPTOR: MODULATORS OF PAIN CONTROL GENES?

A number of other neurotransmitter/neuromodulator systems have been linked to the control of pain. One such system includes the cannabinoids and their receptors (Calignano et al 1998). To date, two cannabinoid receptors have been identified by molecular cloning. The CB1 receptor is abundantly expressed in the brain and the periphery, whereas the expression of the CB2 receptor tends to be restricted to lymphoid organs. When the CB1 receptor gene was knocked out rather interesting phenotypes were observed, as reported in two recent independent studies (Ledent et al 1999, Zimmer et al 1999). One group found that homozygous mutant mice showed no changes in baseline nociceptive sensitivity, morphine-induced analgesia, or tolerance development with chronic morphine treatment (Ledent et al 1999). Another group showed that for baseline nociceptive sensitivity, homozygous mutant mice displayed unaltered response in tail flick test, but hypoalgesia in hotplate and formalin tests (Zimmer et al 1999). Furthermore, naloxone-precipitated withdrawal symptoms were greatly reduced (Ledent et al 1999), indicating that the CB1 receptor may regulate specific aspects of opioid analgesia. These results also suggest that pain genes may be a constituent of a more extensive gene network that includes genes that exert an indirect role on the processing of pain, by modulating the pain control genes.

## CONCLUSIONS AND FUTURE DIRECTIONS

Of course, there was never really a question as to the existence of pain genes. The question is simply whether we can identify genes that are particularly relevant to the generation, appreciation, and/or regulation of pain processing. Clearly, there are many. In the classical genetic sense too, as long as individual differences in pain responses are at least partially heritable, then genes will underlie some of the variability. However, investigation of pain on the genetic level has been deterred until recently by a number of factors, including (a) the complexity surrounding the study of pain, which is entirely subjective in humans and inferred from presumed nocifensive and recuperative behaviors in laboratory animals; and (b) the availability of physiological and pharmacological tools facilitating conventional protein-level experimentation. As a result, we know little at present

about pain genes. The recent explosion of interest in this field should rectify the situation in due course, and the impending completion of the human and mouse genomes and the increasing practicality of genomic technologies should further accelerate this effort.

A number of clinical benefits are likely to derive from the study of pain genetics. First, the discovery of novel genes and the confirmation of known genes as being pain-relevant should spur targeted drug-discovery efforts. Second, the elucidation of genetic polymorphisms rendering individuals with varied sensitivity to pain and its inhibition should allow idiosyncratic use of existing therapies, maximizing their effectiveness and minimizing side-effect liability. This is in addition to the promised land of individualized molecular medicine—physicians ordering gene chip-based lab tests for individual polymorphisms and prescribing medicine accordingly. Finally, because DNA sequence differences are the ultimate cause of gene-based variation in biological response, the use of genetic approaches theoretically allows us to distinguish causes from effects. It should be emphasized, however, that environmental factors, acting both alone and in concert with genotype, exert more overall influence on complex biological phenomena, such as pain, than do genes (see Crabbe et al 1999 for a dramatic reminder of this point). Thus, in addition to putting up with the pain required to decipher pain genes, the search for sociocultural and other nongenetic factors that influence pain susceptibility and response must be pursued with equal vigor.

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