MOLECULAR MECHANISMS OF PERSISTENT PAIN

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Molecular Mechanisms of Persistent Pain

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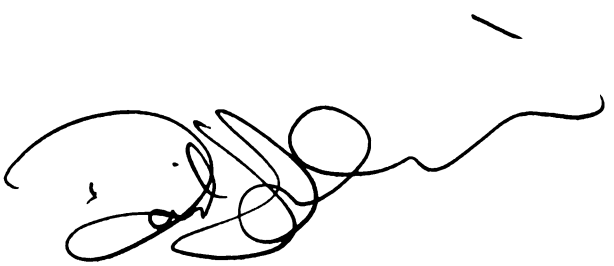
Abstract

Tissue injury not only activates primary afferent nociceptors to produce acute pain but it also initiates changes in the properties of nociceptors and spinal cord nociresponsive neurons. These changes, typically referred to as sensitization, can in turn establish a persistent pain state that is characterized behaviorally by ongoing pain, enhanced responses to noxious stimuli (hyperalgesia), and the production of pain by non-noxious inputs (allodynia). The work in this thesis identifies three distinct molecular mechanisms that contribute to the persistent pain associated with tissue injury.

Using a combination of behavioral and anatomical techniques, we demonstrated that serotonin released at the site of tissue injury acts via ionotropic 5-HT_3 receptors expressed by a unique complement of nociceptors to produce ongoing pain following tissue injury. A similar experimental approach revealed that disrupting the function of the second messenger α-calcium-calmodulin dependent protein kinase type II (α-CaMKII), a serine-threonine kinase expressed in both primary afferent nociceptors and spinal cord neurons, also has a profound effect on injury-induced persistent pain. Specifically, the ongoing pain resulting from tissue injury was eliminated in transgenic mice in which α-CaMKII could not achieve calcium-independent activity. Interestingly, neither the 5-HT_3 receptor nor α-CaMKII contributed to noxious stimulus evoked acute pain or to the hyperalgesia or allodynia associated with injury. Taken together, these observations highlight a dissociation between the mechanisms underlying ongoing/spontaneous pain and stimulus-evoked pain behaviors produced by tissue injury.

Finally, we assessed the contribution of another second messenger molecule, namely the gamma isoform of protein kinase C (PKCγ), to changes in nociceptive processing that occur under the condition of morphine tolerance. Previous work suggested that, like injury, chronic morphine treatment can sensitize spinal cord neurons, the consequence of which is heightened injury-evoked pain. Using a behavioral approach, we demonstrated that PKCγ contributes to the changes in response to tissue injury associated with morphine tolerance as well as to the development of morphine tolerance itself.

The studies of second messenger systems indicate that the molecular mechanisms that contribute to injury-evoked changes in nociceptive processing are similar, but not identical, to those that mediate activity-dependent synaptic plasticity in other regions of the central nervous system. Taken together, the data presented in this thesis emphasize the molecular complexity that underlies the development and maintenance of persistent pain conditions.



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Chapter 1

Introduction

Acute pain results from direct thermal, mechanical, or chemical activation of particular subsets of fine afferent C- and Aδ-fiber nociceptors. Under conditions of injury, the persistent component of the pain response is associated with the production and release of multiple inflammatory factors, including neurotransmitters, eicosanoids and protons. These act in concert not only to maintain activity of primary afferent nociceptors, but also to heighten nociceptor sensitivity. Additionally, increases in primary afferent activity in response to injury and inflammation can lead to prolonged functional changes in the spinal cord resulting in a further enhancement/sensitization of nociceptive processing. The behavioral manifestations of both peripheral and central sensitization include spontaneous pain behaviors, hyperalgesia (exaggerated pain in response to noxious insult), and allodynia (painful sensations produced by normally innocuous stimuli). The work described here characterizes molecular mechanisms of sensitization and the contribution of these mechanisms to injury-induced persistent pain behaviors.

Nociceptive Pathways

The traditional view of the "pain pathway" consists of a primary afferent nociceptor that detects noxious stimuli, a second order neuron in the spinal cord dorsal horn that projects to the thalamus, and finally, a third order neuron that conveys the information to the cortex where the perception of pain is produced. Anatomical, electrophysiological and behavioral studies have revealed that this model is clearly an oversimplification. In addition to the modulation of information that undoubtedly occurs

at many levels (Basbaum and Fields, 1984), the populations of neurons that contribute to nociception are both neurochemically and functionally diverse.

Primary afferent nociceptors

Primary afferent nociceptors are responsible for conveying information regarding noxious mechanical, thermal, and/or chemical stimuli from the periphery to the central nervous system. Many nociceptors are polymodal in that they respond to multiple modalities of noxious stimuli, others are far more selective (responding only to intense mechanical stimulation), while "silent" nociceptors are only responsive in pathophysiological conditions (Handwerker et al., 1991). By definition, a neuron innervating intact, uninjured tissue qualifies as a nociceptor if it is activated by thermal stimuli $\geq$ 45°C, mechanical forces $\geq$ 10mN, and/or capsaicin, the pungent ingredient in hot peppers (Fields, 1987). Importantly, capsaicin is not the only chemical sufficient to activate a nociceptor. Indeed, a large number of endogenous molecules, such as histamine, ATP, and protons, among others, are capable of exciting nociceptors, suggesting that within the functional category of chemical nociceptors, there are likely many molecularly and functionally distinct subpopulations.

From an anatomical perspective, nociceptors innervating cutaneous tissue have traditionally been divided into two classes: (1) thinly myelinated Aδ-fiber nociceptors and (2) unmyelinated, slowly-conducting C-fiber nociceptors (Fields, 1987). C-fiber nociceptors have subsequently been assigned to one of two major classes based primarily on the neurochemistry of their cell bodies in the dorsal root ganglion (DRG) (Snider and McMahon, 1998). The so-called peptidergic population contains the neuropeptide

substance P (SP) and expresses the high affinity nerve growth factor (NGF) receptor trkA (Molliver et al., 1997a). These neurons retain responsiveness to NGF in the adult. The second nociceptor population does not contain SP, but can be selectively labeled with isolectin B4 (IB_4) and expresses fluroide-resistant acid phosphotase (FRAP) (Nagy and Hunt, 1982; Silverman and Kruger, 1988). This subset of afferents also upregulates expression of the tyrosine kinase receptor c-ret during the postnatal period, making the neurons responsive to glial-derived neurotrophic factor (GDNF) in the adult (Molliver et al., 1997b). Importantly, many, but not all, nociceptors belonging to both the peptidergic and non-peptidergic classes have been demonstrated to express the capsaicin/vanilloid receptor (VR1) suggesting some level of functional similarity between the two populations (Tominaga et al., 1998).

The spinal cord dorsal horn

The central branches of primary afferent nociceptors terminate primarily in the superficial laminae of the dorsal horn of the spinal cord. Interestingly, the aforementioned C-fiber nociceptor populations terminate in distinct regions of the dorsal horn. Specifically, SP-containing afferents project to lamina I and the outer segment of lamina II (IIo) whereas the IB_4-binding neurons terminate in the inner portion of lamina II (IIi) (Coimbra et al., 1974; Silverman and Kruger, 1990). Furthermore, Aδ-fiber nociceptors have been demonstrated to terminate primarily in lamina I and less frequently in lamina V (Light and Perl, 1979). The functional significance of these different projection patterns is not clear. However, as described below, the distinct laminae of the spinal cord

are comprised of equally distinct groups of neurons suggesting that the processing of primary afferent input is likely affected by termination site.

The neurons of lamina I have classically been defined as either large marginal cells or smaller cells of unknown type (Nartozky and Kerr, 1978). Importantly, axons of many lamina I neurons project to the nuclei of the thalamus via the spinothalamic tract. In addition, lamina I neurons have been demonstrated to project to the reticular formation of the midbrain. By contrast, few lamina II neurons project out of the spinal cord (Willis et al., 1978). Rather, the axons of these neurons primarily project to other dorsal horn laminae and spinal cord segments. The two major cell types found in lamina II, or the substantia gelatinosa, are stalked cells and islet cells (Cervero and Iggo, 1980). The cell bodies of stalked cells are localized to the outer portion of lamina II whereas islet cells are found in both the outer and inner divisions of this lamina. It has been proposed that islet cells whose dendrites are localized to lamina IIo are nociceptive specific and that islet cells with dendrites in lamina IIi are mechanoreceptive (Bennet et al., 1980; Woolf and Fitzgerald, 1983). Furthermore, there are clear neurochemical differences between neurons of lamina IIo and IIi including the localization of the gamma isoform of protein kinase C to a subset of neurons in inner lamina II (Malmberg et al., 1997). Importantly, this particular population of excitatory interneurons (Martin et al., 1999) has been demonstrated to contribute to changes in nociceptive processing associated with injury (Malmberg et al., 1997).

In addition to the nociceptive processing that occurs in the superficial dorsal horn, neurons whose cell bodies are localized to deeper laminae, namely lamina V, also respond to noxious inputs. In contrast to the majority of lamina I and II neurons, most

lamina V neurons are of the "wide dynamic range" category. In other words, these neurons respond to stimuli over a range of intensities, including innocuous and noxious inputs. Although many lamina V neurons have dendrites that extend into lamina I and II where they can receive direct input from primary afferent nociceptors (Ritz and Greenspan, 1985), it is likely that most of these neurons are influenced by polysynaptic circuits involving the interneurons of the superficial dorsal horn. Like lamina I, many neurons of lamina V are also projection neurons that synapse in the thalamus (Willis et al., 1979).

Changes in nociceptive processing associated with injury

Primary afferent nociceptors and injury

Following tissue injury, a variety of inflammatory mediators, including histamine, ATP, protons, eicosanoids, neurotransmitters, and neuropeptides, are released from damaged cells and tissues at the site of injury (Levine et al., 1993; Dray, 1995). In some cases, these chemical mediators act directly on protein receptors localized to the free endings of nociceptors and cause activation of the neurons. For example, histamine released from damaged mast cells can induce action potentials in primary afferent nociceptors via activation of histamine H1 receptors (Lang et al., 1990; Koda et al., 1996). Alternatively, components of the inflammatory milieu may act at G-protein-coupled receptors, which in turn activate intracellular pathways that can ultimately alter membrane excitability causing sensitization of the nociceptor terminal. Prostanoids such as PGE2 have been demonstrated to act in such a manner, contributing to sensitization of primary afferent nociceptors through a cAMP-dependent process (Taiwo and Levine, 1989; Taiwo and Levine, 1991).

In addition to the inflammatory mediators produced by non-neuronal cells, primary afferent nociceptors themselves can contribute to inflammation by releasing neurotransmitters and neuropeptides at the injury site. For example, activation of nociceptors containing the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) can lead to the release of these peptides from both central and peripheral terminals. In peripheral tissues, SP acts to increase plasma extravasation, thereby contributing to the edema component of inflammation (Cao et al., 1998), and CGRP promotes a long-lasting vasodilatation which underlies the flare or redness of inflammation (Brain et al., 1985). Both can contribute to the behavioral hyperalgesia associated with inflammation.

Clearly, the chemical milieu of an injury site is composed of a diverse array of molecules, each of which may differentially contribute to the associated pain condition. It is thus of interest to identify and characterize these components and their mechanisms of action. Chapter 3 describes a series of experiments aimed at identifying how serotonin (5-HT), a major neurotransmitter component of the inflammatory milieu, contributes to injury-induced pain. Serotonin is released from platelets, mast cells, and basophils that infiltrate an area of tissue damage (Dray, 1995) and interacts with a number of molecularly distinct cell surface receptor subtypes, including the ionotropic 5-HT_3 receptor (R), expressed by primary afferent nociceptors (Maricq et al., 1991; Martin, 1998; Hamon, 1999). Briefly, we found that the serotonergic contribution to nociceptive processing via the 5-HT_3R involves unique subsets of primary afferent nociceptors. Furthermore, our data suggest that the 5-HT_3R contributes to the pain but not the edema associated with tissue injury.

Injury-induced sensitization of spinal cord neurons

Importantly, the persistent and/or increased activation of peripheral nociceptors following tissue injury can in turn initiate long-term functional changes in the dorsal horn of the spinal cord (Dubner and Basbaum, 1994). This activity-dependent plasticity is physiologically characterized by decreased firing thresholds, increased spontaneous activity, and enlarged peripheral receptive fields (Woolf, 1983; McMahon and Wall, 1984; Cook et al., 1987; Hylden et al., 1987; Hylden et al., 1989; Dubner and Ruda, 1992; Dubner and Basbaum, 1994). Collectively, these changes are often referred to as central sensitization (Woolf, 1983; Cook et al., 1987). As described above, the behavioral manifestations of central sensitization include hyperalgesia and allodynia.

Extracellular recordings of spinal cord neurons have demonstrated that activation of the N-methyl-D-aspartate (NMDA) receptor underlies the induction and maintenance of central sensitization produced by intense electrical or chemical stimulation of C-fibers (Woolf and Thompson, 1991). Importantly, injury-induced hyperalgesia and allodynia are inhibited by systemic or intrathecal administration of NMDA receptor antagonists (Davar et al., 1991; Coderre and Melzack, 1992; Mao et al., 1992; Yamamoto and Yaksh, 1992; Tal and Bennett, 1993; Chaplan et al., 1997). These results suggest that injury-induced hyperexcitability of the spinal cord is mediated by the NMDA receptor and, presumably, events downstream of receptor activation.

Because the NMDA receptor is highly permeable to calcium (Mayer and Westbrook, 1987), a number of calcium-dependent second messenger pathways, including those of protein kinase C (PKC), nitric oxide (NO), and the alpha isoform of calcium-calmodulin dependent protein kinase type II (α-CaMKII), are activated by

NMDA receptor activity (for review, see Bliss and Collingridge, 1993). These second messenger cascades can establish long-term changes in neurons throughout the CNS (Bliss and Collingridge, 1993; Coderre et al., 1993). PKC, for example, has been implicated in both activity-dependent potentiation of synapses in the hippocampus and injury-induced changes in spinal cord nociceptive processing (Malinow et al., 1989; Abeliovich et al., 1993; Coderre, 1992; Lin et al., 1996; Malmberg et al., 1997). Similarly, NO has also been suggested to contribute to hippocampal plasticity and it appears to mediate some changes in pain behavior following injury (Schuman and Madison, 1991; Meller et al., 1992; Meller and Gebhart, 1993; Meller et al., 1994). The work described in Chapter 4 reveals that a particular molecular feature of α-CaMKII, an enzyme that is expressed both in primary afferent nociceptors and in neurons intrinsic to the spinal cord dorsal horn, significantly contributes to ongoing pain behaviors associated with injury.

Opioid-induced sensitization of spinal cord neurons

Finally, although opioids such as morphine are frequently used to treat pain conditions, there is evidence to suggest that, like injury, prolonged opioid exposure sensitizes spinal cord neurons which in turn may alter nociceptive processing. For example, chronic morphine results in enhanced activation of spinal cord neurons following noxious stimulation, as evidenced by the expression of the biomarker c-Fos (Rohde et al., 1997). Interestingly, both the behavioral manifestations of injury-induced sensitization of spinal cord neurons as well as morphine tolerance and dependence can be reduced by NMDA antagonists (Davar et al., 1991; Tanganelli et al., 1991; Trujillo and

Akil, 1991; Coderre and Melzack, 1992; Mao et al., 1995). In Chapter 5, we provide further evidence in support of the hypothesis that chronic morphine can produce a central sensitization that enhances nociceptive processing and that it does so via a molecular mediator, namely the gamma isoform of PKC, that also produces such changes following injury.

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Chapter 2

A brief overview of the rodent formalin test

The formalin test is a unique model of injury-evoked persistent pain. Dubuisson and Dennis were the first to describe the behaviors, namely guarding, licking, and flinching, exhibited by a rat following the injection of a dilute formalin solution into the paw (1977). Specifically, they reported that formalin evoked a biphasic pain response with a first (acute) phase lasting approximately 5 minutes. This was followed by a brief quiescent period, and finally, a second (tonic) phase of pain behavior that persisted on the order of 45 minutes. Thus, in addition to producing acute chemical-evoked pain behaviors, the formalin stimulus also resulted in spontaneous/ongoing behaviors that were independent of transiently applied stimuli, a feature not found in most traditional pain models. After the original description of the rodent formalin test, much effort has been aimed at identifying the molecular mediators and neuronal mechanisms underlying the pain behaviors associated with the first and second phases. As Chapters 3, 4, and 5 describe experiments and results related to the formalin model of persistent pain, a brief review of the present understanding of this test is outlined in more detail below.

Mechanisms contributing to Phase 1

After an injection of formalin, rodents exhibit a burst of pain-like behavior directed at the injured paw. This activity lasts for 3 to 5 minutes and is generally presumed to result from the direct activation of primary afferent nociceptors (Dubuisson and Dennis, 1977; Puig and Sorkin, 1996). Electrophysiological recordings from primary afferent neurons, including Aβ–, Aδ- and C-fibers, provide evidence that nociceptors are

indeed activated following formalin application (Heapy et al., 1987; McCall et al., 1996; Puig and Sorkin, 1996). Furthermore, studies of spinal glutamate levels in formalin-treated rats indicate that release of this neurotransmitter is substantially increased during the first phase (Malmberg and Yaksh, 1995a,b). Importantly, AMPA receptor antagonists suppress Phase 1 behavioral responses (Hunter and Singh, 1994) as well as formalin-evoked activity of dorsal horn neurons (Chapman and Dickenson, 1995), suggesting that first phase behaviors arise largely from glutamate acting at AMPA-type glutamate receptors. By contrast, blockade of NMDA-type glutamate receptors has no effect on either Phase 1 behavioral responses or dorsal horn neuronal activity (Haley et al., 1990; Coderre and Melzack, 1992). This indicates that although these receptors are likely activated during the first phase (see below), they are not necessary for maximal expression of acute responses to formalin.

Pharmacological and release studies have also identified the neuropeptide SP as a contributor to formalin-evoked behaviors (McCarson and Goldstein, 1991; Chapman and Dickenson, 1993). Genetic evidence implicating SP has been provided by behavioral examination of mice lacking the PPTA gene, which encodes for both SP and the tachykinin, neurokinin A. Consistent with pharmacological studies, first phase formalin-evoked licking is reduced in PPTA mutant mice relative to wild-type littermates (Cao et al., 1998; Zimmer et al., 1998). Interestingly, this phenotype is observed only when intermediate concentrations of formalin are used (Cao et al., 1998), highlighting the importance of glutamate as the primary mediator of the formalin response.

Formalin-evoked inflammation begins immediately following injection (Taylor et al., 2000) and there is evidence to suggest that inflammatory mediators are necessary for

the full expression of first phase behaviors. For example, acute formalin-evoked behaviors are significantly reduced in mice lacking histamine H1 receptors (Mobarakeh et al., 2000) as well as in mice deficient for the ATP-gated channel, P2X3 (Cockayne et al., 2000; Souslove et al., 2000). Similarly, bradykinin B1 receptor null mutant mice exhibit decreased first phase behaviors when compared to wild-type littermates (Pesquero et al., 2000). By contrast, nonsteroidal anti-inflammatory drugs (NSAIDs) do not diminish Phase 1 responses suggesting that the inflammatory mediators produced by cyclooxygenase (COX) enzymes do not contribute to the acute pain behaviors associated with the formalin test (Malmberg and Yaksh, 1992). In summary, Phase 1 can be attributed to the direct chemical activation of primary afferent nociceptors and the subsequent release of glutamate from central terminals of these afferents.

Mechanisms contributing to Phase 2

Following a quiescent period that lasts approximately 5 minutes, rodents exhibit a second bout of formalin-evoked behavior. In contrast to Phase 1, the second phase is presumed to result from the continued stimulation of nociceptors by inflammatory mediators as well as from a first phase-induced spinal cord hyperexcitability, also termed central sensitization. Recordings from primary afferent neurons during the time in which second phase behaviors are exhibited indicate that there is ongoing activity in Aδ- and C-fibers, but not Aβ-fibers (McCall et al., 1996; Puig and Sorkin, 1996). Furthermore, the importance of primary afferent activity during the second phase was revealed by studies in which a local anesthetic injection following Phase 1 effectively eliminated second phase behaviors (Taylor et al., 1995; but see Coderre et al., 1990). This indicates that

primary afferent activity throughout the duration of the formalin test is necessary for the persistence of the second phase.

Presumably, primary afferent activity associated with the second phase is largely driven by inflammatory mediators produced at the site of formalin injury. Similar to first phase, the inflammatory mediators histamine, bradykinin, and ATP have been implicated in second phase behavior and/or dorsal horn activity (Chapman and Dickenson, 1992; Cockayne et al., 2000; Mobarakeh et al., 2000; Pesquero et al., 2000; Souslove et al., 2000). Additionally, serotonin, a major component of the inflammatory soup, has been demonstrated to contribute to second phase pain behaviors via an action at the ionotropic 5-HT_3 receptor (Chapter 3). Interestingly, in contrast to the other agents described, the 5-HT_3 receptor does not appear to contribute to Phase 1 behaviors (Chapter 3). Finally, peripherally administered NSAIDs decrease second phase activity in dorsal horn neurons, suggesting that prostaglandins may contribute to the activation of primary afferent neurons and subsequent second phase behaviors (Chapman and Dickenson, 1992).

Although primary afferent activity is clearly necessary for expression of second phase behavior, the levels of primary afferent activity during Phase 2 are markedly reduced when compared to the neural activity associated with first phase (McCall et al., 1996; Puig and Sorkin, 1996). This observation gives rise to the hypothesis that a first phase-evoked sensitization of spinal cord neurons is necessary to produce maximal Phase 2 behaviors. Indeed, formalin-evoked activity in wide-dynamic range dorsal horn neurons is characterized by a barrage of first phase activity followed by second phase activity that is more robust than that recorded from primary afferent neurons (Dickenson and Sullivan, 1987). Further evidence in support of a change in spinal nociceptive

processing is provided by the observation that spinal glutamate release is not significantly increased during the second phase (Malmberg and Yaksh, 1995a,b). It is thus possible that spinal sensitization processes result in an increased responsiveness of spinal neurons such that less glutamate is required to produce the same behavioral responses during Phase 2 as seen in Phase 1 of the formalin test.

The barrage of primary afferent input associated with Phase 1 has been suggested to produce an NMDA receptor-dependent sensitization of dorsal horn neurons similar to that seen in other models of persistent pain. Consistent with this hypothesis, administration of NMDA receptor antagonists prior to, but not after Phase 1 significantly reduce Phase 2 behavioral responses and/or the activity of dorsal horn neurons (Haley et al., 1990; Coderre and Melzack, 1992). Furthermore, second messenger systems downstream of NMDA receptor activation, including those utilizing PKC and CaMKII, have been demonstrated to contribute to Phase 2 behaviors (Malmberg et al., 1997; Chapter 4). In particular, the gamma isoform of PKC has been implicated in tonic formalin-evoked behaviors (Malmberg et al., 1997). Importantly, this isoform of PKC is not found in primary afferent neurons, but rather, its expression is restricted to a subpopulation of dorsal horn neurons (Malmberg et al., 1997), highlighting the importance of changes in intrinsic spinal cord neurons for the generation of second phase behavior. Similarly, activation of extracellular signal-related protein kinase (ERK), localized to dorsal horn spinal cord neurons, has been implicated in the enhanced nociceptive processing underlying second phase behaviors (Ji et al., 1999; Karim et al., 2001).

Intrathecal administration of agents in addition to NMDA receptor antagonists has identified other central mediators of Phase 2 behavior. For example, spinal delivery of a neurokinin-1 (NK-1) receptor antagonist prior to, but not after, formalin injection reduces second phase behaviors (Yamamoto and Yaksh, 1991; Yashpal et al., 1993). Given that SP is the major ligand for NK-1 receptors, this suggests that SP contributes to the generation but not the maintenance of tonic formalin behavior (but see Cao et al., 1998). Consistent with this hypothesis is the observation that disruption of the NK-1 receptor gene in mice results in decreased Phase 2 behavior (DeFelipe et al., 1998). Similarly, spinal delivery of NSAIDs diminishes Phase 2 formalin responses (Malmberg and Yaksh, 1992b). Thus, in addition to acting at peripheral terminals of nociceptors, prostanoids may also act centrally to enhance nociceptive processing under injury conditions. In contrast to NMDA and NK-1 receptor antagonists, NSAIDs effectively reduce second phase behavior even when given after Phase 1.

Clearly, the mechanisms underlying the persistent phase of the formalin test are complex, requiring a number of molecular mediators that contribute to the activation of primary afferent nociceptors and/or alterations in the central processing of primary afferent input. The experiments described in this thesis further elucidate the processes that underlie formalin-evoked pain behaviors.

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Chapter 3

5-HT_3 receptors signal pain without inflammation via a unique population of myelinated and unmyelinated nociceptors

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Abstract

Serotonin is a major component of the inflammatory chemical milieu and contributes to the pain of tissue injury via an action on multiple receptor subtypes. Here we studied mice after genetic or pharmacological disruption of the 5-HT_3 receptor, an excitatory serotonin-gated ion channel. We demonstrate that tissue injury-induced persistent, but not acute pain is significantly reduced following functional elimination of this receptor subtype. Specifically, in the setting of tissue injury, the 5-HT_3 receptor mediates the activation, but not the sensitization of nociceptors and does not contribute to injury-associated edema. This result is explained by the localization of 5-HT_3 receptor transcripts to a previously uncharacterized population of unmyelinated and myelinated afferents, few of which express the proinflammatory neuropeptide substance P. Finally, we provide evidence that central serotonergic circuits modulate nociceptive transmission via a facilitatory action at spinal 5-HT_3 receptors. We conclude activation of both peripheral and central 5-HT_3 receptors is pronociceptive and that the contribution of peripheral 5-HT_3 receptors involves a unique complement of primary afferent nociceptors.

Introduction

An understanding of the mechanisms that underlie pain requires an explanation of both the immediate response to injury (acute pain) and the persistence of pain in the setting of tissue injury (Dubner and Basbaum, 1994). Acute pain results from direct thermal, mechanical or chemical activation of particular subsets of primary afferent neurons (nociceptors). The persistent component of the pain response, by contrast, is associated with the production and release of multiple inflammatory factors, including neurotransmitters, eicosanoids and protons (Rang et al., 1994). These act in concert not only to maintain activity of primary afferent nociceptors and sustain pain, but also to heighten nociceptor sensitivity, such that innocuous stimuli produce pain. Serotonin (5-hydroxytryptamine; 5-HT), a major neurotransmitter component of the inflammatory chemical milieu, may be released from platelets, mast cells, or basophils that infiltrate an area of tissue damage (Dray, 1995). Once released, serotonin is free to interact with a number of molecularly distinct receptor subtypes expressed by primary afferent nociceptors, including the 5-HT_3 receptor (5-HT_3R) (Martin et al., 1998; Hamon and Bourgoin, 1999).

Unlike all other known serotonin receptor subtypes, which are G protein-coupled, the 5-HT_3R is a member of the nicotinic acetylcholine superfamily of excitatory ligand-gated ion channels (Maricq et al., 1991). Functional homopentameric serotonin-gated channels are formed when a single cDNA encoding the 5-HT_3R-A subunit is expressed in heterologous systems (Maricq et al., 1991). The recent identification of a second subunit gene, 5-HT_3R-B (Davies et al., 1999) provides molecular evidence that some native 5-HT_3Rs may exist as heteromeric complexes. However, because 5-HT_3R-B subunits

cannot form functional ion channels on their own, the 5-HT_3R-A subunit is believed to constitute an essential component of all serotonin-gated ion channels.

Importantly, peripheral injection of serotonin evokes acute pain that is attenuated by relatively selective 5-HT_3R antagonists (Richardson et al., 1985; Sufka et al., 1992). Few studies, however, have addressed the relative contribution of the 5-HT_3R to both acute and persistent pain, in part because it is difficult to sustain receptor antagonism *in vivo* using purely pharmacological methods. Moreover, some 5-HT_3R antagonists show unusual, non-linear dose-response relationships or exhibit actions at other serotonin receptor subtypes that are found in primary afferent nociceptors (most notably, blockade of 5-HT_4R by ICS 205-930; Bockaert et al., 1990). Obviously, these characteristics may confound the design or interpretation of *in vivo* pharmacological experiments. Indeed, such limitations may account for the controversy concerning the contribution of the spinal 5-HT_3R to nociceptive processing, where both pro- and anti-nociceptive effects of antagonists have been reported (Glaum et al., 1990; Alhaider et al., 1991; Ali et al., 1996).

To provide a more definitive assessment of the contribution of the 5-HT_3R to nociceptive processing, we generated mutant mice lacking the 5-HT_3R-A subunit and characterized their behavior in models of acute and persistent pain. Although these animals show normal acute pain responses, they exhibit significantly reduced persistent pain behavior in the setting of tissue injury, without a change in the magnitude of the associated inflammatory response. At the cellular level this may be explained by our observation that 5-HT_3R-As are not expressed in the substance P-containing afferents that are necessary for the plasma extravasation component of neurogenic inflammation

(Lembeck et al., 1982). Rather, our data suggest that serotonin, via an action at 5-HT_3Rs, contributes to specific features of tissue injury-induced pain by activating both myelinated Aδ -nociceptors and a previously undefined subset of C-fibers.

Material and Methods

Gene targeting: 5-HT_3R-A cDNA probes were used to identify genomic clones from a 129 mouse genomic DNA library. The targeting vector was generated from an 8kb EcoR1/Cla1 restriction fragment containing exons 5 - 9 of the 5-HT_3R-A gene. Exon 7 and 8 encoding transmembrane domains 1, 2, and 3 of the protein were deleted with BAL 31 and replaced by a PGKneo cassette for positive selection of recombinants. An HSV-TK cassette was subcloned downstream of the homologous region for negative selection. The targeting vector (50 µg) was linearized with Not1 and electroporated (240V/500 µF, Biorad Genepulser) into 129-derived embryonic stem (ES) cell line JM1 (generously provided by R. Pedersen and J. Meneses). After electroporation, transfected ES cells were allowed to recover for 48 hrs in non-selective medium, then grown for 8 days in medium containing 350 µg/ml G418 and 0.2 µM Ganciclovir. 180 clones surviving drug selection were screened for homologous recombination by Southern blot analysis. DNA was digested with Bbu1 (Sph1) or Pst1 and filters probed with an external 3' or 5' genomic probe, respectively. Two clones underwent correct targeting of the genetic locus and were also checked for random integration events using a PGKneo probe. Targeted clones were injected into C57BL/6 blastocysts to generate chimeras. Highly chimeric males were crossed to C57BL/6J or B6D2 females and germline transmission of the mutation was assessed by Southern blot analysis of tail DNA from agouti pups.

Heterozygotes were then intercrossed to generate wild type, heterozygous and null mutant mice used in this study.

Southern and Northern blotting: Genomic DNA was digested overnight with appropriate restriction enzymes, size fractionated on a 0.6% agarose gel and transferred onto a nylon membrane (Hybond N^+, Amersham) in 0.4 M NaOH. Hybridization with ^{32}P-labeled DNA probes was carried out overnight at 65°C in 1M NaCl, 1% SDS, 10% dextran sulphate (Pharmacia). Poly A^+ RNA was prepared from DRG of adult wild-type, heterozygous, or null mutant mice (35 ganglia per group) using a Micro-FastTrack kit (Invitrogen). RNAs were separated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane (Hybond N, Amersham) and hybridized overnight at 65°C in 1M NaCl, 1% SDS, 10% dextran sulphate with a ^{32}P-labeled full-length 5-HT_3R-A or $P2X_3$ cDNA probe.

Receptor autoradiography: Mice were killed by asphyxiation with CO_2, brains were removed, frozen in n-methylbutane that had been chilled with dry ice and stored at –70°C. Cryostat sections (10 µm) were thaw-mounted onto Superfrost Plus® (VWR) slides and stored at –70°C. Sections were rehydrated for 60 minutes with 5 mM HEPES buffer (pH 7.4) and covered with 150 µl of [^{3}H]BRL43696 (Wong et al., 1993; 10 nM in HEPES buffer pH 7.4). Nonspecific binding was defined on matched sections by the inclusion of zacopride (100 µM). After 60 minutes incubation at room temperature, sections were washed twice in ice cold HEPES buffer. Slides were dried, opposed to [^{3}H] Hyperfilm (Amersham), mounted in light tight cassettes and stored at room temperature

for 4 weeks. Films were developed with Kodak® D-19 (22°C) and analyzed with the MCID system (Image Research, Inc.).

Assessment of pain behavior: All experiments were approved by the UCSF IACUC. Pain responses to heat and mechanical stimuli, abdominal stretch response to acetic acid (0.6%) or serotonin (1.0mg/kg), and licking in the formalin test (2.0%/10 µl) were assessed as previously described (Hargreaves et al., 1988; Cao et al., 1998). Motor function was assessed on an accelerating rotarod treadmill. Licking behavior was measured after intraplantar injection of serotonin (10µg/10µl) or of the 5-HT_3R agonists 2-methylserotonin (10µg/10µl) or m-chlorophenyl-biguanide (1.0µg/10µl). Paw swelling was measured with a spring-loaded caliper (Mitutoyo). To establish a persistent tissue injury with inflammation, we made an intraplantar injection of Complete Freund's Adjuvant (CFA; 10µg/20 µl; Cao et al., 1998). To examine changes in a model of neuropathic pain, we studied animals after partial sciatic nerve section (Malmberg et al., 1998). Thermal and mechanical sensitivity were measured with von Frey filaments and a radiant heat stimulus at 1 and 3 days after the CFA and 3, 7, 10 and 14 days after the nerve injury, respectively.

Spinal cord electrophysiology: Single units were recorded as previously described (Martin et al., 2001). Neurons had mechanosensitive receptive fields on the plantar surface of the paw and were characterized by their responses to both brush and noxious heat (49 °C). Formalin (2%/20µl) was injected into the center of receptive field at time 0 and neuronal firing was recorded for 1 hr. We calculated the total number of spikes

evoked during the first (0-10 min) and the second phase (10-60 min) following formalin injection and used the Mann-Whitney U test to compare formalin-evoked neuronal activity between the groups of animals.

***In situ* hybridization and immunohistochemistry:** Cryostat sections (12 μm) from lumbar DRG were prepared from wild-type and mutant mice. Digoxigenin-labeled cRNA probes were transcribed from a linearized Bluescript vector (Stratagene) containing the entire 5-HT_3R cDNA, except for a small 5' fragment. Hybridization was conducted as previously described (Tecott et al., 1993). The hybridized probe was visualized by incubation with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Roche) followed by reaction with 4-nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Roche) according to the manufacturer's specifications. Sections were then incubated overnight in one of several antisera: rabbit anti-substance P (1:10,000; Peninsula), mouse anti-N-52 (1:000; Sigma), rabbit anti-CGRP (1:1000; Peninsula), guinea pig anti-VR1 (1:500), biotinylated IB4 (1:50; Vector). The primary antisera were localized by immunofluorescence with either Cy-3 or Cy-2 conjugated secondary antisera (1:600; Jackson Immunoresearch). To assess total number and sizes of cells in a given section, we used the FluroNissl Green stain (Molecular Probes).

Skin-nerve electrophysiology: The skin-saphenous nerve preparation was performed as previously described (Stucky and Lewin, 1999). Extracellular recordings of single fibers were made from the desheathed saphenous nerve of wild type and mutant mice (n = 20).

The conduction velocity of each fiber was determined by electrically stimulating its receptive field (Koltzenburg et al., 1997). Mechanical thresholds were assessed and fibers were considered to be nociceptors if their von Frey thresholds were ≥ 2.0 mN (range 2.0 to 22 mN) and if they exhibited slowly-adapting responses to a sustained mechanical stimulus. To examine chemical responsiveness we sealed off the receptive field with a metal ring and applied mCPBG (10 μM) for 3 min, washed for 2 min, and finally applied capsaicin (10 μM) for 3 min. We measured the number of chemical-induced action potentials during the first minute of application; a fiber was considered responsive if the drug elicited ≥ 3 action potentials.

Whole cell recordings from isolated DRG neurons: Lumbar ganglia (L1-L6) from adult null mutant and wild type mice (n=25) were removed, dissociated and cultured (Stucky and Lewin, 1999). Whole cell recordings were performed 18 to 30 hr after plating. We distinguished nociceptors from non-nociceptors by the presence of an inflection on the falling phase of the somal action potential as previously described (Stucky and Lewin, 1999) and only neurons with an inflection on the falling phase of the action potential were included in this study. In voltage clamp mode, neurons were superfused with mCPBG (3 μM) for 10 s followed by a 2 min wash and subsequently superfused with capsaicin (3 μM). We considered a neuron responsive if an inward current > 100 pA was induced. Directly after recording, IB_4-FITC (10 μg/ml) was added to the recording chamber for 10 min and the presence of IB_4 binding determined. For N-52 staining, neurons plated on Cell-Locate coverglasses (Eppendorf) were fixed with 4% paraformaldehyde for 10 min, washed in PBS and incubated overnight with mouse anti-

N-52 antibody (1:10,000; Sigma) followed by Cy3-conjugated anti-mouse secondary antisera (1:1000; Jackson Immuoresearch).

Results

5-HT_3R -A gene disruption

The 5-HT_3R-A gene was disrupted by deleting exons 7 and 8, which encode the first three putative transmembrane domains of the channel protein (Werner et al., 1994), and inserting a neomycin expression cassette (PGK-neo) at this position (Figure 1a). Homologous recombination of the targeting vector and germline transmission of the disrupted locus were verified by Southern blotting (Figure 1b). Northern blot analysis of message in dorsal root ganglia showed that null mutant mice were devoid of 5-HT_3R-A transcripts (Figure 1c). Heterozygotes expressed reduced transcript levels compared to wild-type siblings. Matings between heterozygous mice produced wild-type, heterozygous, and homozygous mutant offspring in normal Mendelian ratios. Homozygous mutant (knockout) animals were healthy, fertile, and did not differ in appearance from wild-type siblings. Gross motor ability, including walking on a rotating rod was comparable in wild-type and mutant mice.

Quantitative autoradiography using the selective 5-HT_3R radioligand, [^{3}H]-BRL 43694 (granisetron; Wong et al., 1993) revealed that null mutant mice lacked functional receptors in three areas that contain relatively high densities of 5-HT_3R ligand binding sites: the nucleus of the solitary tract (NTS), the area postrema (AP) and the trigeminal nucleus caudalis (TNC) (Gehlert et al., 1991). In agreement with the Northern blot

analysis, binding in the heterozygotes was approximately half that of wild-type mice (Figure 1d).

Acute pain responses are normal, but persistent pain responses are reduced in 5-HT_3R-A mutant mice

We compared wild-type and 5-HT_3R-A mutant mice in several acute pain models, including the hot-plate, tail-flick and Hargreave's/paw withdrawal (1988) tests of thermal nociception, the tail pinch test of mechanical nociception and the intraperitoneal (i.p.) acetic acid (i.e., low pH-induced) stretching test of visceral nociception. In each modality, we found no difference between the wild-type and mutant mice (Figure 2). On the other hand, the stretching response produced by i.p. serotonin was significantly reduced in the mutant mice (Figure 2). These results establish that the 5-HT_3R has functional relevance to the direct actions of serotonin, but that this receptor does not contribute to the acute pain produced by physiologically relevant stimuli.

We next examined the animals in a model of persistent pain using the formalin test. In this paradigm, a dilute formalin solution is injected into the plantar surface of the hindpaw and pain-related behavior (licking) is scored in two phases (for review, see Tjolsen et al, 1992). The first phase (~0-10 minutes) is produced by direct activation of nociceptors and therefore provides a measure of acute chemical pain. The second phase results in part from a delayed inflammatory response in the injected paw and thus serves as a model of persistent pain resulting from tissue injury. Consistent with acute pain being intact in null mutant animals, we found that first phase pain behavior did not differ in wild-type and mutant mice. In contrast, the second phase of pain behavior was

significantly reduced in the mutant animals, indicating that 5-HT_3Rs are essential for some component(s) of nociceptive processing that produces persistent pain (Figure 3a).

Because a decrease in the second phase pain behavior in the formalin test could result from changes at many levels of the neuroaxis (Wei et al., 2001), we examined electrophysiological responses of neurons in the deep dorsal horn (in the region of lamina V) following an intraplantar formalin injection. We first characterized the neurons as multireceptive based on their responses to both innocuous (brush) and noxious (49°C) stimuli. As for the behavioral profile, formalin evoked two phases of dorsal horn neuronal firing as previously shown in rats (Dickenson and Sullivan, 1987). The first phase consisted of an immediate increase in the firing rate that lasted for approximately two minutes; there was no difference in the total number of spikes evoked in the first phase (0-10 min) between wild-type (mean ± sem: 1236 ± 473) and mutant (1770 ± 617) mice (Figures 3b, c). After a quiescent period, the wild type mice exhibited a second phase of firing where there was a marked increase in total spikes (4082 ± 1726), which mirrored the second phase of pain behavior. Consistent with the observed deficit in second phase pain behavior in null mutant mice, the magnitude of neuronal firing during the second phase in the knockout animals was dramatically reduced (784 ± 410; $P = 0.0175$).

Finally, to identify the contribution of peripheral versus central 5-HT_3Rs to sustained formalin-evoked pain behaviors, we used a pharmacological approach to inhibit receptor function in specific sites. We found that peripheral (intraplantar) injection of the 5-HT_3R antagonist ondansetron reduced second, but not first phase pain behavior in wild-type mice (Figure 4a). Because 5-HT_3Rs are also found on the central (spinal) terminals of

primary afferents and on dorsal horn interneurons (Hamon et al., 1989; Kia et al., 1995) we also examined the effect of ondansetron administered directly into the cerebrospinal fluid (intrathecally). As observed after peripheral injection, intrathecal ondansetron dose-dependently suppressed the second phase paw-licking behavior in the formalin test (Figure 4b), but had no effect on the first (acute pain) phase (data not shown). These pharmacological results indicate that the reduced second phase formalin behavior in the knockout mice likely reflects loss of peripheral and central (spinal) 5-HT_3R activity (Oyama et al., 1996; also, see below).

The 5-HT_3R is not required for tissue or nerve-injury induce allodynia

In addition to producing persistent pain, tissue or nerve injury can induce a condition in which the response to noxious stimulation is exaggerated (hyperalgesia) and/or normally innocuous thermal or mechanical stimuli produce pain (allodynia) (Dubner and Basbaum, 1994). We tested the animals in a model of tissue inflammation produced by injection of CFA, as well as in a model of neuropathic pain produced by partial sciatic nerve injury (Malmberg and Basbuam, 1998). Injection of CFA produced a marked swelling of the paw and a decreased withdrawal threshold to thermal and mechanical stimuli, with similar magnitudes in 5-HT_3R mutant and wild-type mice (n=5 per group; data not shown). Partial nerve injury also produced a robust thermal and mechanical allodynia that did not differ between the two groups of animals (n=5 per group; data not shown). Importantly, we found that basal mechanical withdrawal thresholds, tested with calibrated von Frey hairs (Chaplan et al., 1994) did not differ in wild type and mutant mice in the absence of injury. These observations indicate that the persistence of pain in

the setting of injury, but not the concurrent sensitization process that contributes to allodynia, is dependent on 5-HT_3R activation.

Differential contribution of peripheral 5-HT_3Rs to pain and inflammation

Despite the reduction in second phase pain behavior in mutant animals, formalin-evoked paw swelling, a key indicator of inflammatory response, did not differ from that observed in wild-type mice (44±3% and 43±4% increase in paw diameter, respectively; Figure 5). These results suggest that formalin-induced release of serotonin in the setting of tissue injury induces pain behavior via activation of the 5-HT_3R, but that activity at this receptor does not contribute to concurrent edema. To directly test whether activation of 5-HT_3Rs is involved in edema, we assessed the magnitude of swelling produced by peripheral injection of serotonin or 5-HT_3R selective agonists. As predicted, in spite of the differences in pain behavior, the edema produced by intraplantar serotonin did not differ in mutant and wild-type mice (Figure 5). Paw injections of the 5-HT_3R agonists, m-chlorophenyl-biguanide (mCPBG; 1.0 µg/10 µl) or 2-methylserotonin (10 µg/10µl) also produced intense paw licking in the wild-type mice. Importantly, however, the 5-HT_3R agonists did not evoke significant paw swelling in either wild type or null mutant mice (Figure 5). Taken together, these results indicate that when serotonin is released in the setting of tissue injury, it induces pain and edema, but that the 5-HT_3R contributes only to the pain component of this response.

5-HT_3R-A is expressed by unique populations of primary afferent neurons

The presence of 5-HT_3Rs on primary afferent fibers has been inferred from the decreased binding of 5-HT_3R radioligands in the dorsal horn after denervating the spinal cord by transection of dorsal roots (Kidd et al., 1993) or by neonatal destruction of C-fibers with the neurotoxin, capsaicin (Hamon et al., 1989). In a previous study we demonstrated that DRG neurons express 5-HT_3R-A transcripts (Tecott et al., 1993). However, none of these studies identified the specific subset(s) of DRG neurons that express 5-HT_3Rs. To directly detect 5-HT_3R protein in DRG neurons, we first tested a number of commercial and non-commercial antibodies directed against the 5-HT_3R. Unfortunately, in every case the staining pattern revealed in null mutants was similar, if not identical, to that observed in wild-type littermates, suggesting that none of the antisera were specific for the 5-HT_3R. In contrast, *in situ* hybridization for 5-HT_3R-A message proved to be very specific, as indicated by the complete lack of 5-HT_3R-A mRNA in DRG sections from mutant animals (Figures 6a,b).

Figure 6i illustrates that 5-HT_3R-A mRNA is present not only in neurons with small cell bodies (i.e. presumptive C-fiber neurons), but is actually localized to many neurons with considerably larger diameters. In fact, approximately 80% of 5-HT_3R-A positive neurons (394/483) immunostained for N52, a neurofilament marker of myelinated primary afferents (Lawson et al., 1991) (Figures 6c,d). These data suggest that 5-HT_3Rs are expressed primarily by a subpopulation neurons with myelinated afferents as well as by some C-fiber nociceptors. Surprisingly, we found that at most 13% (61/483) of neurons that express the 5-HT_3R-A transcript co-stained with antisera directed against the

vanilloid/capsaicin receptor, VR1, indicating that a minority of 5-HT_3-responsive neurons are likely to be capsaicin-sensitive nociceptors (Figures 6e,f).

Interestingly, only 4% (31/739) of the total population of 5-HT_3R-A positive neurons were also immunoreactive for SP (Figures 6g,h) and an equivalently small proportion (4%; 25/656) bound the lectin IB_4 (data not shown). Because peripheral release of SP from nociceptors is critical to the swelling associated with neurogenic inflammation (Cao et al., 1998), the minimal overlap between 5-HT_3R and SP is consistent with the dissociation of 5-HT_3R contribution to pain and inflammation observed in the behavioral studies (Figure 5). Based on these results, we conclude that the 5-HT_3R-A is expressed by a subset of neurons with thinly myelinated fibers and by a population of C-fiber neurons, only some of which express traditional markers of nociceptors, notably VR1 and SP.

5-HT_3R agonists activate both Aδ- and C-fiber nociceptors

Because the majority of 5-HT_3R-A positive neurons do not immunostain for VR1, it is possible that the receptor is predominantly expressed by non-nociceptive primary afferents. To test the hypothesis that 5-HT_3Rs are in fact expressed on capsaicin-insensitive Aδ-and C-fiber *nociceptors*, we next examine the responses of physiologically characterized primary afferent nociceptors to the application of the 5-HT_3R agonist mCPBG in an *in vitro* skin-saphenous nerve preparation. In this system, cutaneous nociceptors can be examined *in situ* so as to measure the firing rate of single functionally-identified primary afferent neurons during chemical or natural stimulation of their receptive fields. Nociceptors were defined by their response to noxious mechanical stimuli. In wild-type mice, 16% (5/31) of Aδ–nociceptors responded to bath applied

mCPBG (10μM) (Figure 7a). The average number of spikes recorded during the first minute of agonist application was 35 ± 12.6 (mean ± SEM). By contrast, none of the mCPBG-responsive fibers responded to bath application of capsaicin.

Among primary afferent neurons with C-fiber conduction velocities 29% (9/31) responded to mCPBG application with an average of 13 ± 4.2 spikes in the first minute. The higher mCPBG-evoked activity in Aδ-fibers compared to C-fibers is consistent with our observation that the intensity of hybridization signal is greater in neurons with larger cell body diameters. Furthermore, of the 9 mCPBG–responsive C-fibers, 5 were also activated by capsaicin (10μM), an observation that agrees with the anatomical finding that approximately half of the 5-HT_3R C-fibers, as defined by the absence of N52 staining, are immunoreactive for VR1. Importantly, mCPBG never evoked responses in nociceptors from 5-HT_3R mutant mice (0/15 fibers), confirming the absence of functional receptors in the mutant animals. In other respect the mice lacking 5-HT_3Rs were identical to littermate controls. This was true for conduction velocities of Aδ-fibers (WT: 4.9 ± 0.5 m/s, mutant: 4.9 ± 1.2 m/s) and C-fibers (WT: 0.68 ± 0.03 m/s, mutant: 0.67 ± 0.04 m/s). and for von Frey mechanical thresholds of Aδ-fibers (median: 9.0 mN both groups) and C-fibers (median: 5.6 mN both groups).

Finally, we assessed responsiveness to mCPBG of cultured DRG neurons isolated from wild-type and mutant mice. The presence of an inflection on the falling phase of the somal action potential was used to distinguish nociceptors from non-nociceptors (Stucky and Lewin, 1999). Again, mCPBG was completely inactive on nociceptors from 5-HT_3R mutant mice (0/15). Of 70 wild-type neurons, 24 responded to bath application of 3.0 μM mCPBG and, consistent with the anatomical studies, *none* of the mCPBG responsive

neurons bound the lectin IB_4. The electrophysiological results confirm that the *in situ* hybridization analysis did not miss a population of small diameter afferents that bind IB_4. In a separate set of DRG cultures, in which 30 of 67 neurons responded to mCPBG, we found that 14 of the 30 mCPBG responsive nociceptors were N-52 positive (Figure 7b). Together, the electrophysiological data demonstrate that functional 5-HT_3Rs are found on unique subpopulations of myelinated and unmyelinated nociceptors.

Spinal 5-HT_3Rs facilitate the transmission of nociceptive messages

Several previous studies reported that spinal delivery of serotonin produces a caudally-directed scratching/biting behavior in mice (Fasmer and Post, 1983). This is consistent with a reported pronociceptive effect of 5-HT_3R agonists (Ali et al., 1996) and with our observation that intrathecal administration of 5-HT_3R antagonists reduced second phase behavior in the formalin test (Figure 4b). By contrast, some studies found an antinociceptive effect of intrathecal 5-HT_3R agonists and argued that this was mediated via activation of 5-HT_3Rs on GABAergic inhibitory interneurons (Glaum et al., 1990; Alhaider et al., 1991). Because the knockout mice provide a more specific assessment of the contribution of a particular receptor subtype we examined the effect of intrathecal injections of serotonin or selective 5-HT_3R agonists in wild-type and mutant mice. Consistent with the pronociceptive effects noted above, in the wild-type mice we observed a dose-dependent scratching behavior of the lower back after intrathecal administration of 0.2-20 μg serotonin (Figure 8a). In mutant mice the scratching response to intrathecal serotonin was significantly reduced. Surprisingly, the selective 5-HT_3R agonist m-chlorophenyl-biguanide did not produce scratching behavior (0.02-20 μg; data

not shown). This suggests that the 5-HT_3R is necessary, but not sufficient, for serotonin-mediated scratching (pain) behavior; other 5-HT receptors must act in concert to produce the behavior. Finally, although we found that the same dose of serotonin had an antinociceptive effect in the hot-plate test, there was no difference between the wild-type and mutant mice. At the highest dose (20 μg) intrathecal serotonin increased the hot-plate latency by 54±5 and 47±7% in the wild-type and mutant mice, respectively (Figure 8b). We conclude that the 5-HT_3R is not involved in the antinociceptive effect of serotonin.

Discussion

Based on the results of the present study, we can draw several important conclusions concerning the mechanisms through which activity at the 5-HT_3R contributes to nociceptive processing and pain. First, the 5-HT_3R is not required for acute pain responses to physiological stimuli. Second, the 5-HT_3R contributes to persistent pain without inducing a concomitant edema in the setting of injury. Third, the peripheral 5-HT_3R contribution to persistent pain is via an action on both myelinated and unmyelinated nociceptors. On the other hand, although these afferents are activated in the setting of tissue injury, we found no evidence to implicate the 5-HT_3R in their sensitization. This conclusion is based on the fact that profound allodynia developed in both wild-type and mutant mice after paw injections of CFA or following partial nerve injury. Thus activation, but not sensitization of primary afferents results from a serotonin action at the 5-HT_3R. Finally, we provide evidence that central serotonergic circuits modulate pain transmission via a facilitatory action at spinal 5-HT_3Rs.

Release of serotonin in the setting of injury thus has multiple consequences. Serotonin directly activates nociceptive afferents to increase the barrage of impulses transmitted to the spinal cord, resulting in an increase in pain behavior. It also contributes to peripheral neurogenic inflammation, via activation of small-diameter peripheral afferents and release of proinflammatory peptides, such as SP from peripheral terminals. The latter induces extravasation of proteins from postcapillary venules, which in turn contributes to peripheral edema (Lembeck et al., 1982). The contributions of serotonin to pain and swelling, however, are readily dissociable according to the receptors that are activated. We found that peripheral injection of serotonin produced both pain behavior and swelling of the hind paw in wild-type mice, but only the pain behavior was reduced in the 5-HT_3R null mutant mice. Consistent with these results, we found that 5-HT_3R agonists also induced pain, but without concomitant swelling of the paw. Finally, we found that despite a reduction of pain behavior in the null mutant mice when tissue injury was induced with formalin, swelling was not affected. This result agrees with a report showing that 5-HT_3R antagonists reduced the pain, but not the inflammation evoked by carrageenan (Eschalier et al., 1989). It is likely that serotonin-induced edema involves activation of 5-HT_{2a} receptors (Pierce et al., 1995; Germonpre et al., 1997). The fact that the 5-HT_3R appears not to contribute to the edema component of the neurogenic inflammatory response is, of course, consistent with our finding that 5-HT_3R-A message and substance P immunoreactivity did not colocalize in DRG neurons.

Previous studies reported that dorsal rhizotomy or neonatal capsaicin treatment significantly reduces the binding of ^{3}H-zacopride (a 5-HT_3R radioligand) in the superficial dorsal horn (Hamon et al., 1989; Kidd et al., 1993). Because the terminals of

unmyelinated primary afferents predominantly synapse in the superficial dorsal horn, it has been assumed that the 5-HT_3R is expressed primarily on capsaicin-sensitive C-fiber nociceptors. In fact, our anatomical and electrophysiological results indicate that the majority of nociceptors that express the 5-HT_3R are capsaicin-insensitive. Rather, the 5-HT_3R is predominantly expressed by myelinated (i.e., N52-positive) Aδ afferents and by small but unique population of C-fibers, which overlaps to some extent with both the traditional peptidergic (substance P-containing) and non-peptidergic subpopulations (Snider and McMahon, 1998). Importantly, our results are in accord with an earlier study of blood pressure regulation, which concluded that 5-HT_3R agonists and capsaicin stimulate different populations of vagal afferent fibers (Skofitsch et al., 1983).

Finally, we provide strong evidence that pain transmission is enhanced via an action at a spinal cord 5-HT_3R. Specifically, we found that the second phase pain behavior in the formalin test was significantly reduced after intrathecal administration of the 5-HT_3R antagonist, ondansetron (see also Oyama et al., 1996). Because the antagonist did not alter nociceptive thresholds when it was administered alone, i.e. in the absence of ongoing injury, there does not appear to be a tonic action of serotonin at the 5-HT_3R. Rather spinal cord levels of serotonin must be increased when pain is generated. Indeed pain-evoked release of serotonin into the spinal CSF has been described (Tyce and Yaksh, 1981). It is generally assumed that descending serotonergic pathways mediate an inhibition of pain transmission at the level of the spinal cord (Basbaum and Fields, 1984) in part via 5-HT_3R-mediated activation of GABAergic inhibitory interneurons (Alhaider et al., 1991). Our results suggest that spinal 5-HT_3Rs predominantly mediate a positive feedback that enhances nociceptive processing at the spinal cord level. Interestingly Zhuo

and Gebhart (1991) used electrical stimulation or glutamate microinjection into the rostroventral medulla to activate descending serotonergic axons and found a facilitation of nociceptive processing that could be blocked by intrathecal injection of 5-HT_1R antagonists. Our results indicate that parallel 5-HT_3R-mediated facilitation also occurs and they establish the conditions under which this facilitation comes into play, namely in the setting of tissue injury. Given that the 5-HT_3R is found on many spinal cord neurons, we cannot determine whether the positive feedback is exerted via an action on the central terminals of primary afferents that express the receptor or upon postsynaptic dorsal horn neurons.

In summary, using a combined genetic, pharmacological, electrophysiological and anatomical approach, we have provided new insights into the contribution of the 5-HT_3R to pain and inflammation. Our results are consistent with the fact that 5-HT_3R antagonists, which held initial promise as analgesic drugs, cannot by themselves provide satisfactory pain relief in patients (Greenshaw et al., 1997; Hamon et al., 1999). Because the great majority of C nociceptors do not express the 5-HT_3R, it follows that pain that arises from activation of these afferents will be refractory to 5-HT_3R antagonists. For this reason, we suggest that a combination of 5-HT_3R antagonists with drugs that interfere with the transmission of messages conveyed by unmyelinated afferents may provide a more effective approach to the relief of pain in the setting of injury.

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Figure 1: Functional disruption of the 5-HT_3R-A gene.

(a) The structure of the native and disrupted 5-HT_3R-A alleles are shown, together with the location of the 5' and 3' flanking probes used to characterize recombination products. The disrupted allele lacks three of the four putative transmembrane domains (white vertical bars) of the receptor and contains a neomycin selection cassette (PGK-Neo) in their place.

(b) Southern blot analysis of Pst1-digested tail DNA from wild type (+/+), heterozygous (+/-) or null mutant (-/-) 5-HT_3R-A mice using the 5' flanking fragment as probe. Wild type and mutant bands appear at 6.5 and 5.2 kb, respectively.

(c) Northern blot analysis of DRG mRNA from wild type, heterozygous and null mutant 5-HT_3R-A mice shows that knockout animals lack 5-HT_3R-A transcripts. Expression of ATP-gated ion channel ($P2X_3R$) mRNA (Chen et al., 1995; Lewis et al., 1995) served as a positive control for sample preparation and loading and liver mRNA served as a negative control for probe specificity.

(d) Null mutant mice lack specific binding sites for the 5-HT_3-selective radioligand [^{3}H]BRL 43694. In situ autoradiography was carried out for three brain regions known to contain 5-HT_3Rs, including the nucleus of the solitary tract (NTS), area postrema (AP) and trigeminal nucleus caudalis (TNC). Values represent average site densities as determined for 9 tissue sections prepared from each of 3 animals per genotype.

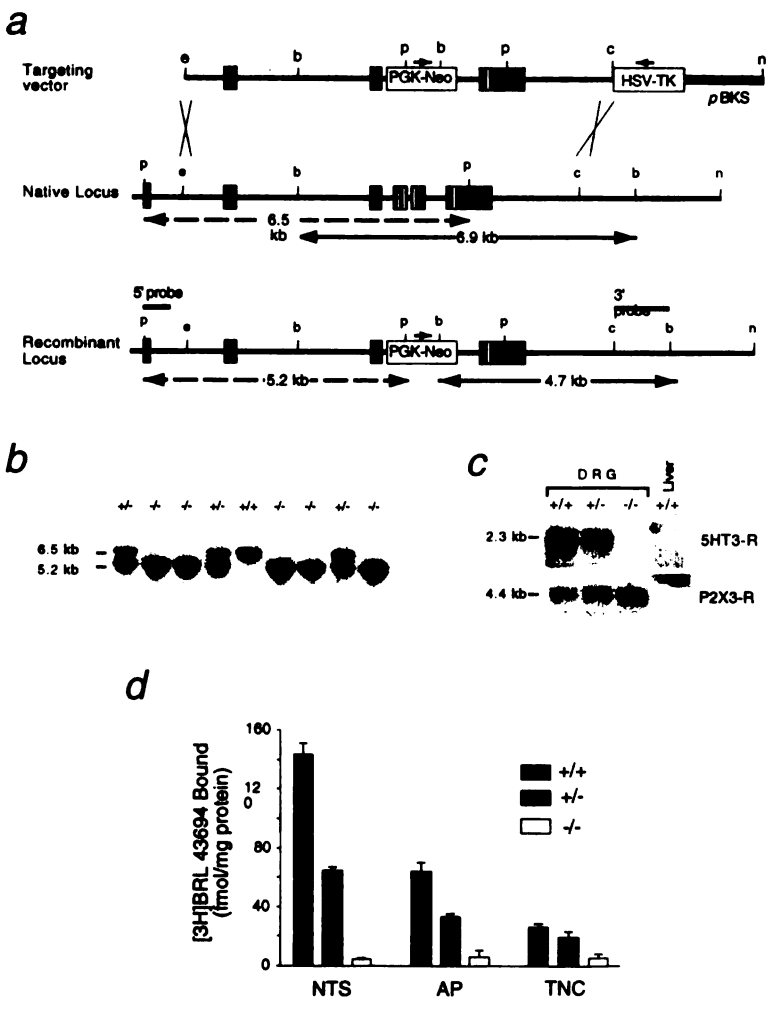

a
Targeting vector
PGK-Neo
HSV-TK
pBKS
Native Locus
6.5 kb
6.9 kb
5' probe
3' probe
Recombinant Locus
5.2 kb
4.7 kb
b
6.5 kb
5.2 kb
c
DRG
Liver
+/+ +/- -/- +/+
2.3 kb
5HT3-R
4.4 kb
P2X3-R
d
[3H]BRL 43694 Bound (fmol/mg protein)
160
120
80
40
0
+/+
+/-
-/-
NTS
AP
TNC

Figure 2: The 5-HT_3R is not required for acute pain responses to thermal, mechanical or visceral stimuli, but does contribute to behavioral indices of pain produced by serotonin. The response latencies in the 52.5°C hot-plate, Hargreave's thermal paw withdrawal and tail-flick tests of thermal nociception, in the tail pinch test of mechanical nociception and in the visceral pain response (stretching) to i.p. acetic acid were similar in 5-HT_3R mutant and wild-type mice ($p>0.05$, t-test, n=5 per group). By contrast, the visceral pain response to i.p. serotonin was significantly reduced in the mutant mice ($p < 0.01$, t-test, wt: n=8; ko: n=10 per group).

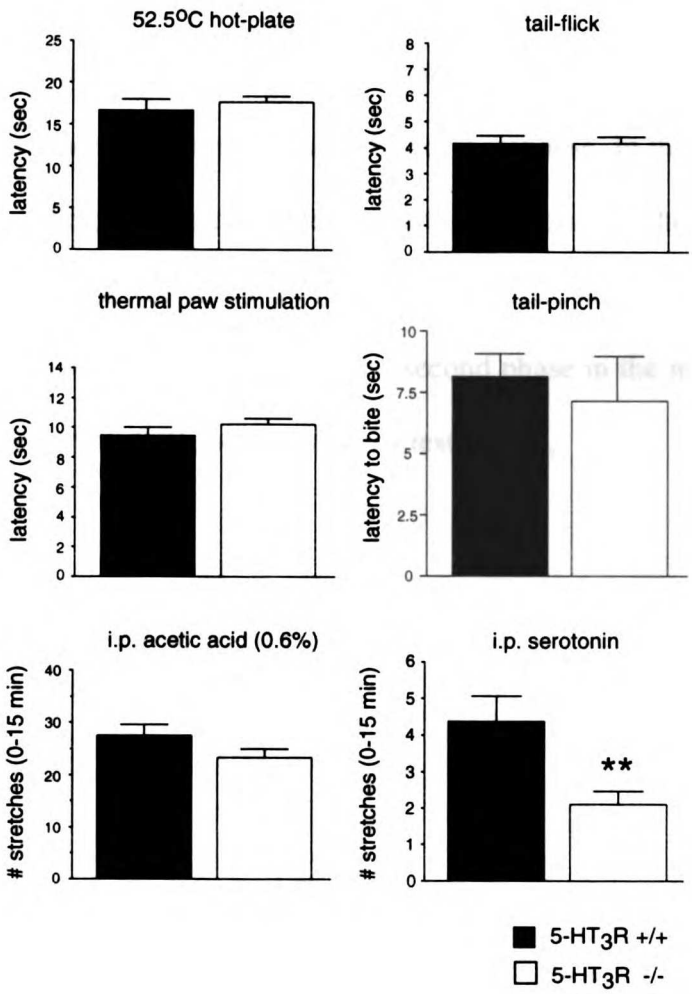
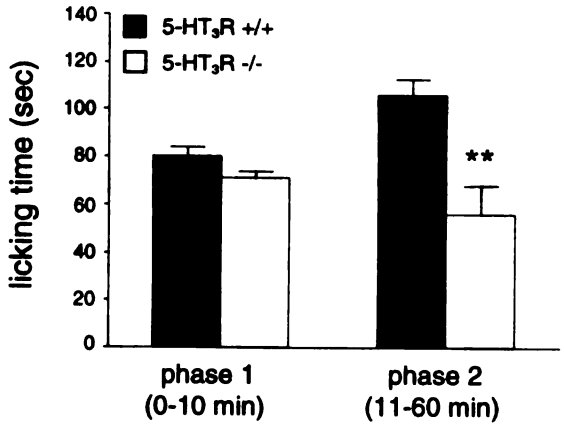
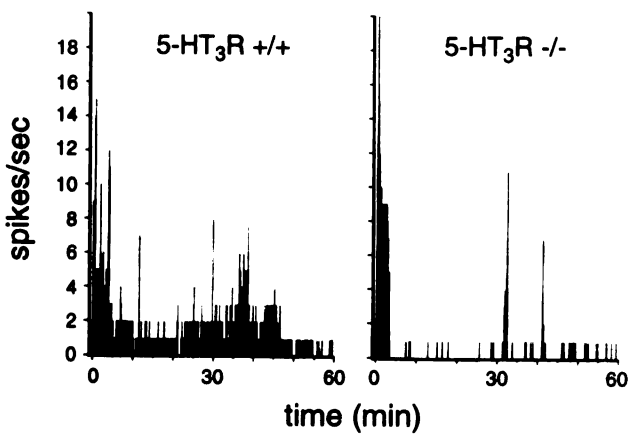

52.5°C hot-plate
latency (sec)
tail-flick
latency (sec)
thermal paw stimulation
latency (sec)
tail-pinch
latency to bite (sec)
i.p. acetic acid (0.6%)
stretches (0-15 min)
i.p. serotonin
stretches (0-15 min)
**
5-HT3R +/+
5-HT3R -/-

Figure 3: The 5-HT_3R is required for the second phase of pain behavior and of spinal cord neuronal firing in the formalin test. (a) Formalin induced paw licking during phases 1 (0-10 min) and 2 (11-60 min) in both wild-type and 5-HT_3R-A null mutant mice did not differ, but the magnitude of second phase pain behavior was significantly reduced in mutant animals (**: $p<0.01$; t-test, comparing the two groups, n=7-8 per group). (b) Response of a lamina V neuron in the spinal cord to the injection of formalin. In wild-type mice, formalin produced a characteristic biphasic increase in neuronal activity, which resembles the time course of the behavioral response. In the 5-HT_3R mutant mice, the first phase firing was comparable to wild-type mice, but there was only a modest second phase response. (c) Summary of formalin-evoked neuronal activity; significantly fewer total spikes were recorded during the second phase in the mutant mice, compared to wild-type mice (*: $p<0.01$; Mann-Whitney test; n = 8).

a


5-HT3R +/+
5-HT3R -/-
licking time (sec)
140
120
100
80
60
40
20
0
**
phase 1
(0-10 min)
phase 2
(11-60 min)

b


5-HT3R +/+
5-HT3R -/-
spikes/sec
18
16
14
12
10
8
6
4
2
0
0
30
60
0
30
60
time (min)

c

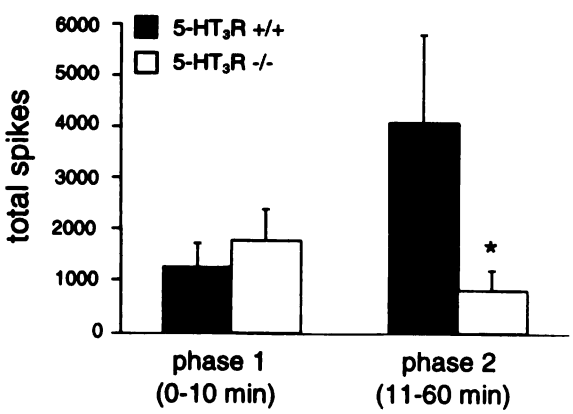

5-HT3R +/+
5-HT3R -/-
total spikes
6000
5000
4000
3000
2000
1000
0
*
phase 1
(0-10 min)
phase 2
(11-60 min)

Figure 4: Both peripheral and central (spinal) 5-HT_3Rs contribute to persistent pain behavior: (a) Intraplantar injection of the 5-HT_3R antagonist, ondansetron, dose dependently reduced the magnitude of pain (duration of licking) during the second phase of the formalin test. Contralateral injection was without effect, indicating that the locus of the drug action was in the injected paw. (b) Intrathecal (CSF) administration of ondansetron also produced a dose-dependent suppression of the paw-licking behavior in the second phase (**: $p<0.01$, ***: $p<0.001$; Student Newman-Keuls test, compared to saline injection, n=5 per group).

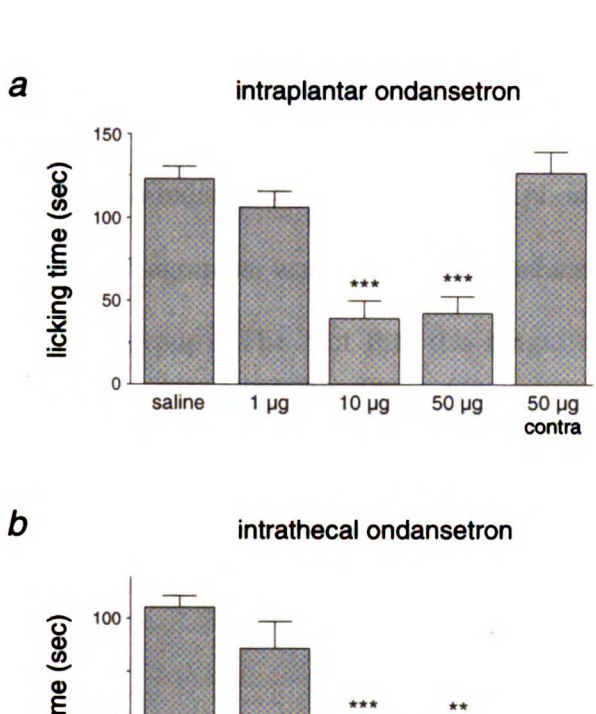

a
intraplantar ondansetron
licking time (sec)
150
100
50
0
***
***
saline
1 µg
10 µg
50 µg
50 µg
contra
b
intrathecal ondansetron
licking time (sec)
100
50
0
***
**
saline
1 µg
10 µg
25 µg

Figure 5: The peripheral 5-HT_3R contributes to pain, but not to paw edema induced by injury. Paw diameters prior to injection of algesic agents did not differ in wild type and null mutant mice. Intraplantar injection of 2.0% formalin or 10 μg serotonin (5-HT) produced a comparable increase in paw diameter, a measure of inflammation, in 5-HT_3R mutant and wild-type mice (***: $p<0.001$, t-test, n=7-8 per group). Consistent with these observations, i.pl. injection of the 5-HT_3R agonists 2-methyl 5-HT (10 μg), or m-chlorophenyl-biguanide (mCPBG; 1.0 μg) did not produce a significant change in paw diameter in either the mutant or wild-type mice ($p>0.05$, t-test, n=5-7 per group). In contrast, the licking behavior index of pain produced by i.pl. injection of either serotonin or the two selective 5-HT_3R agonists, was significantly reduced in the mutant mice (**: $p<0.01$; t-test, n=5-7 per group). The fact that the magnitude of the pain behavior produced by serotonin was greater than that produced by selective 5-HT_3R agonists indicates that multiple 5-HT receptors contribute to its pro-nociceptive action.

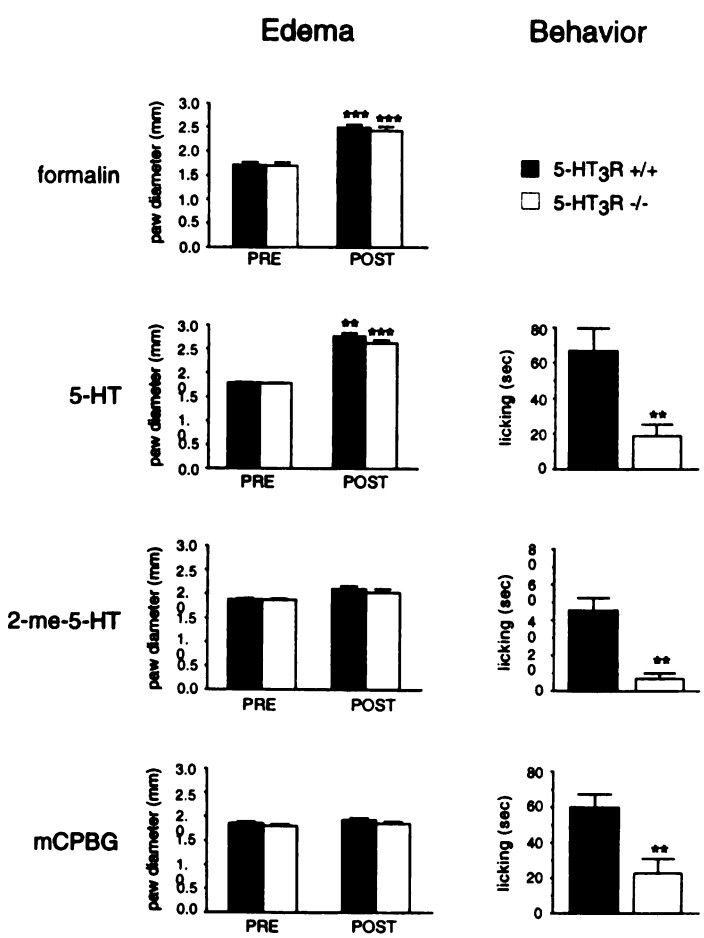
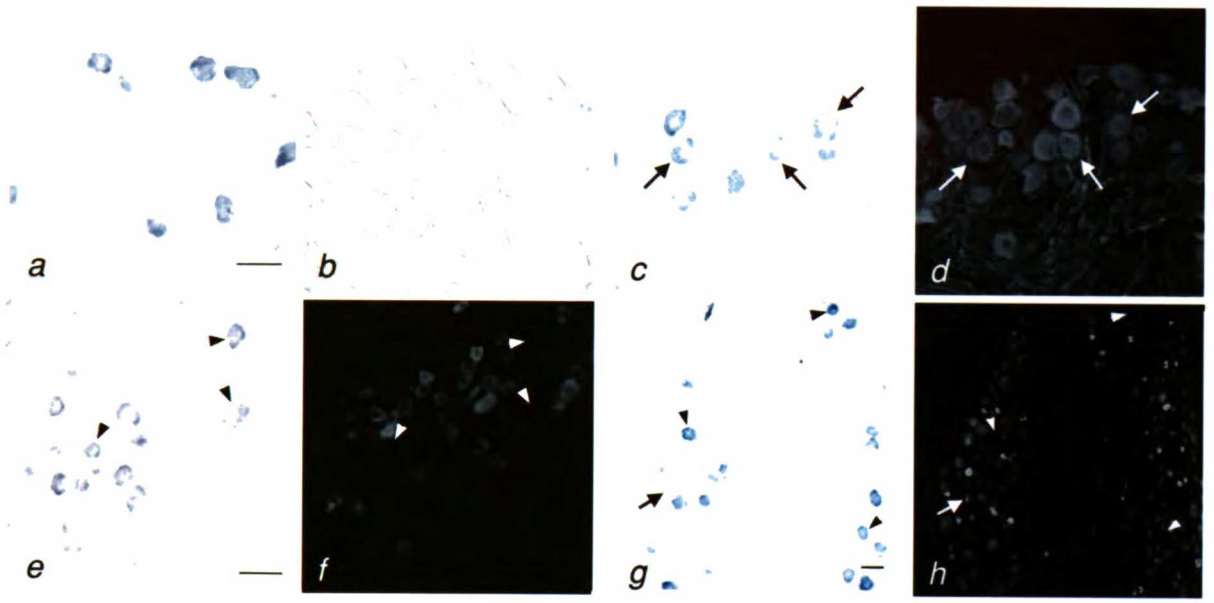

Edema
Behavior
formalin
5-HT
2-me-5-HT
mCPBG
paw diameter (mm)
licking (sec)
PRE
POST
5-HT3R +/+
5-HT3R -/-

Figure 6: Subsets of DRG neurons contain 5-HT_3R-A mRNA. *In situ* hybridization in (a) wild-type mice and (b) the absence of signal in 5-HT_3R-A null mutant mice confirms the probe specificity. The vast majority of 5-HT_3R mRNA-positive neurons (c) immunostain for N52 (d), a neurofilament marker of myelinated axons. Note that the double-labeled neurons (arrows) are of medium (~30 μm) diameter. There is minimal overlap between the 5-HT_3R-A neuronal population (e) and the subset of neurons that express VR1 (f). *Arrowheads* highlight examples of 5-HT_3R-A positive neurons that are not immunoreactive for VR1. Virtually none of the 5-HT_3R-A positive neurons (g) immunostain for SP (h). *Arrow* indicates a rare double-labeled cell and *arrowheads* highlight 5-HT_3R-A positive neurons that are not immunoreactive for SP. Size distribution of 5-HT_3R-A positive neurons in the adult mouse DRG (i). Only cells with visible nuclei were measured. Calibration bar equals 50 μm; calibration bar in (a) applies for (b-f), and calibration bar in (g) applies for (h).


a
b
c
d
e
f
g
h

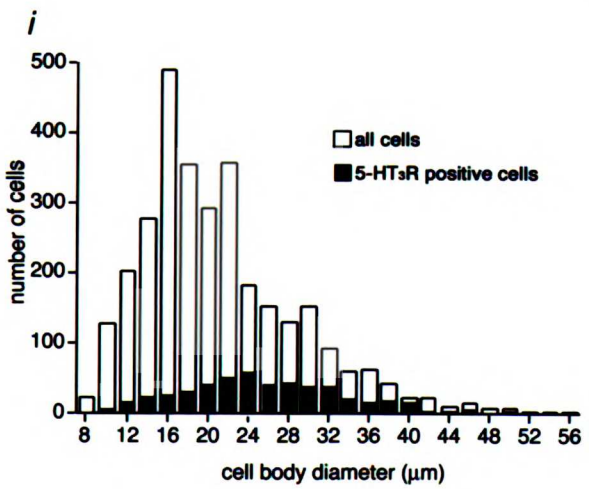

i
500
400
300
200
100
0
number of cells
all cells
5-HT3R positive cells
8
12
16
20
24
28
32
36
40
44
48
52
56
cell body diameter (μm)

Figure 7: Reponses of primary afferent neurons to the 5-HT_3R agonist mCPBG. (a) Recording from a single Aδ-fiber nociceptor (conduction velocity: 2.63 m/s) in a skin-nerve preparation from a wild type mouse. Fiber responded with action potentials only to high-intensity mechanical stimuli (≥40 mm indentation) and encoded the mechanical stimuli well, with slowly adapting responses to 10 s sustained indentation. The fiber responded vigorously during the first minute of mCPBG (10 μM) application but did not respond to subsequent application of capsaicin (10 μM; not shown). (b) Left: Fluorescent and phase images of wild type DRG neuron that was immunoreactive for neurofilament antibody N52 (closed arrow head). Open arrow heads indicate two smaller neurons that were negative for N52. Right: Patch clamp recordings from the N52 immunoreactive neuron show that the neuron responded to mCPBG (3μM) but not to capsaicin (3μM).

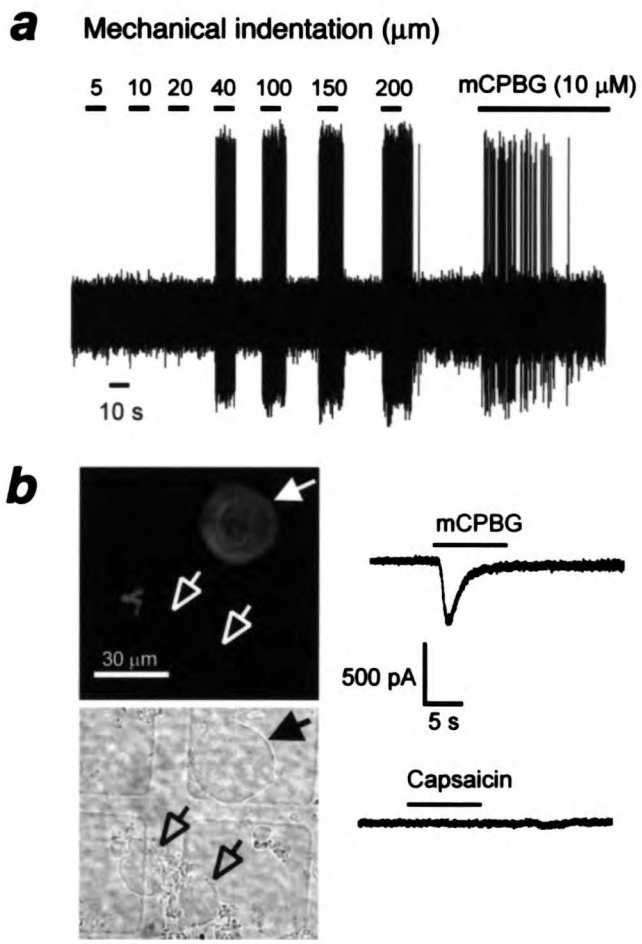
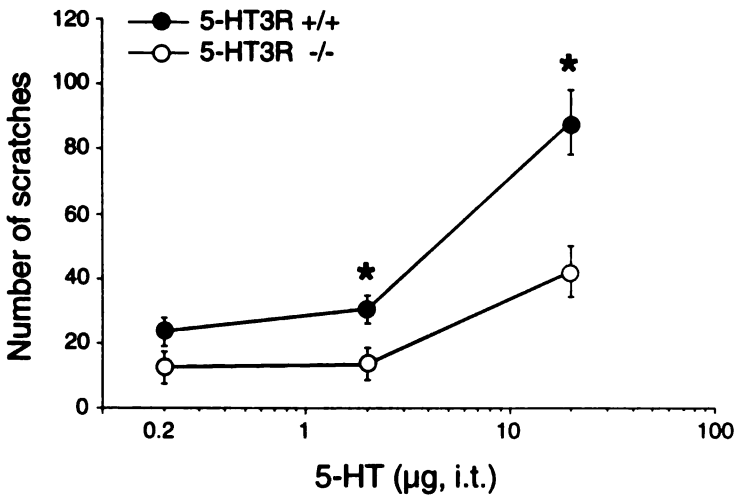

a
Mechanical indentation (μm)
5 10 20 40 100 150 200
mCPBG (10 μM)
10 s
b
30 μm
mCPBG
500 pA
5 s
Capsaicin

Figure 8: Spinal 5-HT_3Rs facilitate the transmission of nociceptive messages. Intrathecal injection of serotonin produced a dose-dependent caudally-directed scratching behavior (0-3 min) and (b) an increase in the response latency on the 52.5°C hot-plate test. Mutant mice showed significantly less scratching behavior (*: $p<0.01$; t-test, n=5 per group), but showed a similar antinociceptive effect compared to wild-type mice after spinal delivery of serotonin.


5-HT3R +/+
5-HT3R -/-
Number of scratches
120
100
80
60
40
20
0
0.2
1
10
100
5-HT (µg, i.t.)
*
*

Chapter 4

The contribution of autophosphorylated α-CaMKII to injury-induced persistent pain

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Departments of Neurobiology, Psychiatry, Psychology and the Brain Research Institute, University of California at Los Angeles, Los Angeles, CA.[2]

Abstract

Increases in neuronal activity in response to tissue or nerve injury can lead to prolonged functional changes in the spinal cord resulting in an enhancement/sensitization of nociceptive processing. To assess the contribution of α-CaMKII to injury-induced inflammation and pain, we evaluated nociceptive responses in mice that carry a point mutation in the α-CaMKII gene at position 286 (threonine to alanine). The mutated protein is unable to autophosphorylate and function independently of calcium and calmodulin. Responses to acute noxious stimuli did not differ between α-CaMKII T286A mutant and wild type mice. However, the ongoing pain produced by formalin injury was significantly reduced in the mutant mice, as was formalin-evoked spinal Fos-immunoreactivity. In contrast, the decreased mechanical and thermal thresholds associated with nerve injury, CFA-induced inflammation and formalin-evoked sensitization were manifest equally by wildtype and mutant mice. Double-labeling immunofluorescence studies revealed that α-CaMKII is expressed in the superficial dorsal horn as well as in a population of small diameter primary afferent neurons. In summary, our results suggest that α-CaMKII is a critical contributor, perhaps secondary to an NMDA-mediated calcium increase in postsynaptic dorsal horn nociresponsive neurons, to the spontaneous/ongoing component of tissue-injury evoked persistent pain.

Introduction

The activation of primary afferent nociceptors by tissue or nerve damage and the subsequent activity evoked within the central nervous system (CNS) gives rise to an acute sensation of pain. Injury can also result in persistent activation of peripheral nociceptors, which in turn initiates long-term functional changes in the dorsal horn of the spinal cord (Dubner and Basbaum, 1994). The behavioral manifestations of these changes include ongoing/spontaneous pain behaviors, exaggerated pain in response to a noxious stimulus (hyperalgesia), and pain in response to normally innocuous stimuli (allodynia). Importantly, in several paradigms, it has been demonstrated that allodynia and hyperalgesia can be prevented, or even reversed, by systemic or intrathecal administration of N-methyl-D-aspartate (NMDA) receptor antagonists (Davar et al., 1991; Coderre and Melzack, 1992; Mao et al., 1992; Yamamoto and Yaksh, 1992; Tal and Bennett, 1993; Chaplan et al., 1997). These results suggested that injury-induced hyperexcitablity of the spinal cord is mediated by the NMDA receptor and, presumably, events downstream of receptor activation.

Although there are many signaling pathways triggered by calcium influx through activated NMDA receptors, there is substantial evidence that the alpha isoform of calcium-calmodulin kinase type II (α-CaMKII) is critical to the development of activity-induced synaptic changes throughout CNS. For example, α-CaMKII contributes to long term potentiation (LTP) in the hippocampus and visual cortex as well as to experience-dependent plasticity in whisker barrel cortex (Silva et al., 1992; Stevens et al., 1994; Kirkwood et al., 1997; Glazewski et al., 1996). Furthermore, a specific property of α-CaMKII, namely autophosphorylation at position 286 (threonine), has been demonstrated

to be crucial for synaptic plasticity. Phosphorylation of the enzyme at this site enables α-CaMKII to switch into an 'on' state that retains its activity even after intracellular calcium levels return to baseline (Miller et al., 1988; Thiel et al., 1988; Lou and Schulman, 1989). Studies of a point mutant mouse in which T286 was changed to an alanine (T286A) revealed that autophosphorylation at this position is essential for the induction of NMDA-receptor-dependent LTP in the hippocampus (Giese et al., 1997).

Because α-CaMKII is necessary for long-term changes at synapses in the brain, it is possible that α-CaMKII also contributes to changes in nociceptive processing at the spinal cord level following injury. Importantly, α-CaMKII immunoreactivity has been localized to both spinal cord dorsal horn neurons and to the Lissauer tract, the axons of which are predominantly of primary afferent origin (Basbaum and Kennedy, 1986; Terashima et al., 1994; Bruggemann et al., 2000). Furthermore, non-selective kinase inhibitors reportedly decrease behavioral manifestations of persistent pain following injury (Coderre, 1992; Coderre and Yashpal, 1994). Discerning the specific signaling pathway that was critical in those studies, however, is impossible because the kinase inhibitor used in that work (namely H-7) is equally effective in the inhibition of PKA, PKC, and α-CaMKII (Malinow et al., 1989). In the present study, we tested the hypothesis α-CaMKII, specifically the calcium-independent component of its activity, contributes to nociceptive processing by assessing injury-induced behavioral changes in autophosphorylation (T286A) mutant mice. We found that autophosphorylation of α-CaMKII at position 286 is necessary for ongoing pain behaviors, but not the inflammation, associated with tissue injury. However, we found that α-CaMKII autophosphorylation is not required for injury-induced allodynia and hyperalgesia.

Taken together, these observations highlight a dissociation between the molecular mechanisms underlying spontaneous/ongoing pain and the stimulus evoked abnormal pain behaviors that occur in the setting of injury.

Materials and Methods

Generation of mutant mice: α-CaMKII T286A mutant mice were generated as described previously (Giese et al., 1998). The mice were backcrossed 2 to 3 times into a C57Bl6 background from the original 129sv source. Mice were age- and weight-matched for all experiments. All experiments were performed blind to the genotype of the mice.

Assessment of nociceptive behavior: Experiments were approved by the UCSF Institutional Animal Care and Use Committee. Acute pain responses were assessed in the 52.5°C hot plate test, tail flick test, and paw withdrawal to a radiant heat stimulus as previously described (Cao et al., 1998). Mechanical thresholds were determined with calibrated von Frey hairs using the up-down paradigm of Chaplan et al (1994). To assess the sensitivity of mice to chemical stimuli, mice were given a 5 μg intraplantar injection of capsaicin and the amount of time spent licking the injected paw was monitored for 5 minutes. To quantify the magnitude of the inflammatory response, we measured the paw diameter with a spring-loaded caliper (Mitutoyo) 30 minutes after the capsaicin injection.

For the formalin test, the mice received a 10 μl i.pl. injection of 2% formalin and the licking behavior of the injected paw was observed for 60 min in 2 min periods at 5 min intervals. We also determined the magnitude of the formalin-evoked inflammatory response by measuring paw diameter at 90 min postinjection, In a separate group of mice,

formalin-evoked thermal hyperalgesia was assessed by measuring paw withdrawal latencies to a radiant heat stimulus following an intraplantar formalin injection.

To establish a persistent tissue injury with inflammation, we made an intraplantar injection of complete Freund's adjuvant (CFA; 10μg/20 μl). On days 1 and 3 after the injection, paw diameter was measured as described above. The nerve injury model was produced by tightly ligating 1/3 to 1/2 of the sciatic nerve (Malmberg and Basbaum, 1998). Thermal and mechanical sensitivity were measured with a radiant heat stimulus and von Frey filaments at 1 and 3 days after the CFA and 3, 7, 10 and 14 days after the nerve injury, respectively.

Immunocytochemistry: For spinal cord immunocytochemistry, mice were deeply anesthetized with pentobarbital (100mg/kg) and perfused with a 10% phosphate-buffered formalin fixative. The spinal cords were removed and post-fixed for 4 hr in the same fixative and then cryoprotected in phosphate-buffered 30% sucrose overnight. The spinal cords were then sectioned transversely at 30μm on a freezing microtome. For trigeminal ganglia immunocytochemistry, mice were deeply anesthetized and decapitated and the trigeminal ganglia were quickly removed and immediately frozen. Twelve μm sections of the ganglia were then cut on a cryostat. We used the following antisera for immunofluorescence double-labeling studies: mouse anti-α-CaMKII (1:500; Chemicon); rabbit anti-PKCγ (1:5000; Santa Cruz); rabbit anti-substance P (1:10,000; Peninsula); biotinylated-IB4 (1:50; Vector); and goat anti-peripherin (1:2000; Santa Cruz). Secondary antibodies were conjugated with either Cy-3 (1:600; Jackson Immunoresearch) or Cy-2 (1:200; Jackson Immunoresearch).

Fos protein immunoreactivity was examined on spinal cord sections from mice that were studied in the formalin test and perfused 90 minutes following the formalin injection. Fos antisera (kindly provided by Dr. Dennis Slamon, UCLA) was diluted 1:30,000. Immunostaining was performed according to the avidin-biotin-peroxidase method (Hsu et al., 1981). We used a nickel-intensified diaminobenzidine protocol with glucose oxidase to localize the horseradish peroxidase immunoreaction product. To quantitate the number of Fos-like immunoreactive neurons, we first examined tissue sections using darkfield microscopy to determine the segmental level and outline gray matter landmarks. We counted the number of Fos-like immunoreactive neurons in five sections from the L4-5 level. All counts were made with brightfield microscopy using a 10 X objective. Labeled nuclei were plotted onto the outlines with a camera lucida attachment. The investigator responsible for plotting and counting the Fos-like immunoreactive neurons was blind to genotype of the sections.

Data and statistical analysis: All graphs are presented as mean ± SEM. For statistical analyses of parametric data, we used Student's t test or ANOVA followed by the Fisher's PLSD test for multiple comparisons when appropriate. Nonparametric data (mechanical withdrawal thresholds; see Chaplan et al., 1994) were analysed using the Friedman test for within-group comparisons and the Mann-Whitney test for comparisons between groups. Critical values that reached the $p < 0.05$ level were considered statistically significant.

Results

General behavior and nociceptive pain responses

The generation and general behavior of α-CaMKII T286A mutant mice have been described previously (Giese et al., 1998). Briefly, the point mutation results in viable mice that are indistinguishable from wild-type littermates. Immunocytochemical analysis demonstrated that the mutation does not alter expression of the α-CaMKII gene in either the forebrain (Giese et al., 1998) or the spinal cord (data not shown).

We first compared wild-type and α-CaMKIIT286A mutant mice in several acute pain models, including the hot-plate and tail-flick tests of thermal nociception, the von Frey test of mechanical nociceptive thresholds, and the capsaicin test of chemical nociception. In each test, we found no difference between wild-type and mutant mice (Figure 1; tail flick data not shown). Additionally, the wild-type and mutant mice did not differ with respect to capsaicin-evoked edema. These results suggest, not surprisingly, that the autophosphorylation of α-CaMKII does not contribute to the processing of acute noxious stimuli. Furthermore, these data indicate that the nociceptive afferents are intact despite the presence of the mutant protein.

Persistent pain behavior

We next examined the mice in the formalin test, a model of persistent pain. In this paradigm, a dilute formalin solution is injected into the plantar surface of the hindpaw and pain-related behavior (licking) is scored in two phases (for review, see Tjolsen et al., 1992). The first phase behavior is presumed to be driven by direct activation of primary afferent nociceptors and therefore provides a measure of acute

chemical pain. The second or persistent phase likely results from an interaction of ongoing peripheral input produced by the tissue injury and a central sensitization of dorsal horn neurons subsequent to activity generated during the first phase. Consistent with our finding that acute nociception in the mutant mice is intact, we found no difference in first phase behavior between wild-type and mutant mice. However, second phase behavior was dramatically reduced in the α-CaMKIIT286A mutant mice (Figure 2a). Importantly, this phenotype does not appear to be dependent on background strain as mutant mice produced by backcrossing both 2 and 3 times onto a C57Bl6 background exhibited the same decrease in second phase formalin behavior.

Because it has been proposed that second phase behavior is dependent on the formalin-evoked inflammatory process (Taylor et al., 2000), we also examined the magnitude of swelling produced by the formalin as an index of the inflammation that is produced. In fact, the edema component of formalin-evoked inflammation was equivalent in the two groups of mice (Figure 2b). These data suggest that phosphorylation of α-CaMKII at position 286 is a necessary step in generating the ongoing/spontaneous pain behavior of the second phase of the formalin test but that this phosphorylation contributes neither to acute pain behavior, nor to the inflammatory response that occurs in the injured hindpaw.

Activity of populations of spinal cord nociceptive neurons can be determined by assessing expression of the *c-fos* protein (Hunt et al., 1987; Menetrey et al., 1989). Importantly, injection of formalin produces highly reproducible patters of Fos-immunoreactivity (Fos-IR) in the lumbar spinal cord (Presley et al., 1990; Abbadie et al., 1992; Abbadie et al., 1997). Consistent with the reduced second phase behavior, we

found significantly fewer formalin-evoked Fos-IR neurons in laminae V-VI of L4/5 spinal cord sections ipsilateral to the formalin injection in mutant mice than in their wild-type littermates (Figure 3).

Injury-evoked changes in thermal and mechanical nociceptive thresholds

In addition to producing an ongoing persistent pain (manifest, for example, in second phase behavior of the formalin test), tissue or nerve injury can result in allodynia and hyperalgesia. As noted above, in the absence of injury we found that basal mechanical and thermal withdrawal thresholds did not differ in wild type and mutant mice. Because central sensitization has been implicated in the development of allodynia and hyperalgesia, we next tested the hypothesis that autophosphorylation of α-CaMKII contributes to threshold changes associated with injury. Specifically, we tested the animals in a model of tissue inflammation produced by injection of Complete Freund's Adjuvant (CFA), as well as in a model of neuropathic pain that is produced by partial sciatic nerve injury (Malmberg et al., 1997). Surprisingly, we found that in both wild-type and mutant mice, CFA injection (Figure 4) and nerve injury (data not shown) produced significant increases in thermal and mechanical sensitivity of the ipsilateral hindpaw at each time point assessed following the injury. Specifically, the latency to respond to the thermal (heat) stimulus as well as the force required to evoke paw withdrawal were significantly and equivalently reduced in both wild-type and α-CaMKIIT286A mutant mice. These data suggest that autophosphorylation of α-CaMKII is not necessary for either the induction or maintenance of injury-induced changes in stimulus-evoked nociceptive processing.

Because the autophosphorylation of α-CaMKII at position 286 has been demonstrated to be dependent on the frequency of calcium transients (DeKoninck and Schulman, 1998), it is possible the stimuli associated with either CFA injection or nerve injury are not sufficient to drive T286 phosphorylation and the subsequent calcium-independent activity of α-CaMKII. On the other hand, because the results from the formalin studies suggested that the formalin stimulus is sufficient to cause autophosphorylation of α-CaMKII and that this event is important in maintaining ongoing/spontaneous pain behavior associated with the injury, we reassessed the hypothesis that autophosphorylation of α-CaMKII can contribute to injury-evoked threshold changes. We measured the thermal nociceptive thresholds in wild-type and mutant mice following an intraplantar formalin injection. Despite the decrease in second phase licking behavior in the mutant mice, formalin nevertheless produced a significant decrease in latency to respond to the heat stimulus in both wild-type and mutant mice (Figure 5). Importantly, the difference in magnitude of threshold change did not differ in the two groups of mice.

Expression of α-CaMKII in nociceptive pathways

Previous studies in the rat indicated that α-CaMKII is expressed in nociceptive processing regions of the dorsal horn (Basbaum and Kennedy, 1986; Terashima et al., 1994; Bruggemann et al., 2000). In the mouse, we found intense α-CaMKII immunoreactivity in dorsal horn neurons localized to laminae I and II, as well as in some neurons of the deeper dorsal horn, specifically in laminae V and VI. Double-labeling with antisera directed against substance P (SP) as well as the lectin IB_4 highlight the

presence of α-CaMKII in regions of the spinal cord implicated in nociception (Figure 6a,b). In light of the evidence for a contribution of PKCγ, a calcium-dependent serine-threonine kinase, to injury-induced persistent pain behaviors, it was of particular interest that we found extensive colocalization of α-CaMKII-IR with PKCγ-IR in interneurons of inner lamina II (Figure 6c).

Because previous work localized α-CaMKII-IR to axons of the Lissauer's tract (Basbaum and Kennedy, 1986), in which as many as 80% of the fibers are believed to be of primary afferent origin (Coggeshall et al., 1981), we evaluated expression of the enzyme in primary afferent ganglia. Perfusion fixation reduced α-CaMKII-IR in trigeminal and dorsal root ganglia to levels below our detection ability, presumably reflecting low levels of antigen presence in these tissues. α-CaMKII-IR was however readily detectable in the primary afferent neurons of fresh, frozen trigeminal ganglion tissue. Consistent with previous studies of α-CaMKII-IR in the rat (Bruggemann et al., 2000), α-CaMKII-IR was restricted to a subset neurons that also express the neurofilament peripherin, a marker of small diameter afferents (i.e. presumed nociceptors) (Figure 6d).

Discussion

In the present study, we assessed the nociceptive phenotype of mice in which calcium-independent activity of α-CaMKII cannot be generated. Our results suggest that sustained α-CaMKII activity is necessary for the persistence of ongoing/spontaneous pain behaviors in the setting of tissue injury, but that it is not required for stimulus-evoked threshold changes, namely thermal or mechanical allodynia that also occur when

there is peripheral tissue or nerve injury. Because we observed a reduction in formalin-evoked Fos expression, we suggest that the contribution of α-CaMKII is at the level of the spinal cord dorsal horn, where it is important in the generation and maintenance of **certain forms** of central sensitization.

Although studies of α-CaMKII null mutant mice suggested that the enzyme may contribute to acute nociception (Chen et al., 1994), we did not observe any differences between wild-type and α-CaMKII T286A mice with respect to either thermal or mechanical thresholds. Similarly, the behavioral and inflammatory responses to acute, noxious chemical stimuli were not affected by the mutation. These results indicate that activation of the primary afferent nociceptors as well as the central neurons that contribute to acute pain processing does not require the autophosphorylation and subsequent calcium-independent activity of α-CaMKII. Given that acute pain is likely to result from glutamate induced activity of second order neurons, via interaction with AMPA-type glutamate receptors, this result is not entirely unexpected (Coderre and Melzack, 1992).

By contrast, autophosphorylation of α-CaMKII at position 286 is clearly important in generating ongoing/spontaneous pain behavior in the setting of tissue injury produced by formalin injection. Importantly, the attenuation of nociceptive scores is not due to decreased inflammation as the formalin-evoked edema did not differ between wild-type and mutant mice. Interestingly, a similar phenotype was observed in mice deficient for the 5-HT_3 receptor, an ionotropic serotonin receptor (Chapter 3). Specifically, second phase formalin-evoked behavior was reduced, though not to the same extent as in the present study, in the 5-HT_3 receptor null mice without a

concomitant change in edema. These results as well as those of the present study suggest that formalin-evoked edema is not sufficient to produce the persistent pain behaviors associated with the second phase of the formalin test. (Hunter and Singh, 1994).

Additionally, we observed a significant decrease in the number of formalin-evoked Fos-IR neurons in lamina V/VI in mutant mice suggesting that least one locus of difference between wild-type and CaMKIIT286A mice is at the level neuronal activity in the deep dorsal horn. The selective decrease in formalin-evoked Fos-IR in lamina V/VI is consistent with work by Bon et al. (2001) demonstrating that late phase formalin licking behavior in mice is correlated with Fos-IR in lamina V/VI. By contrast, early phase licking behavior was shown to correlate with Fos-IR in spinal cord neurons located in lamina I/II and in the present study, we found no difference between the wild-type and mutant mice in either first-phase licking behavior or superficial Fos-IR. Similar regional differences in the central patterns of activity generated during the two phases of formalin behavior have been identified using Fos-IR in combination with pharmacological tools that independently disrupted first and second phase formalin behaviors in the rat (Abbadie et al., 1997).

Interestingly, although this seemingly subtle mutation in α-CaMKII produces a robust plasticity deficit in hippocampal and neocortical regions, the effects of the mutation on injury-evoked spinal cord plasticity are quite limited. No differences in mechanical or thermal sensitivity were observed in any injury model, even when the injury stimulus was a formalin injection, a stimulus that appears to be sufficient to drive α-CaMKII autophosphorylation. This observation suggests that despite the NMDA receptor dependence of both injury-induced spinal cord plasticity and experience

dependent cortical plasticity, the molecular changes that underlie these processes are indeed quite different. The results of the present study indicate that calcium-independent CaMKII activity is necessary neither for the induction nor the maintenance of spinal cord plasticity following a slow-onset inflammation (CFA) or partial nerve injury.

The absence of change in stimulus-evoked response is perhaps even more surprising given the extensive colocalization of α-CaMKII with PKCγ in the spinal cord. PKCγ is a calcium-dependent enzyme that has been implicated in injury-induced changes in spinal cord processing. Specifically, studies of PKCγ null mutant mice revealed that these mice not only exhibit decreased spontaneous behavior during the second phase of the formalin test but that they also show reduced nerve injury-induced stimulus-evoked threshold changes relative to wild-type mice (Malmberg et al., 1997). If one site of formalin-evoked α-CaMKII activity is the PKCγ -containing interneurons, the phenotypic differences between α-CaMKIIT286A mutant mice and PKCg mutant mice could be due to the phosphorylation of distinct target proteins by these two serine-threonine kinases.

Because α-CaMKII-IR is localized to both peripheral and central neurons, it is not clear whether the behavioral consequences of the mutated enzyme are due to changes in pre- or post- synaptic activity (or to both). Importantly, α-CaMKII substrate proteins in both axon terminals and dendritic spines have been proposed to contribute to activity-induced plasticity. For example, synapsin I, a protein implicated in regulating the release of neurotransmitter from presynaptic terminals, can be phosphorylated by α-CaMKII (Llinas et al., 1985; Benefenati et al., 1992). Thus, phosphorylation of synapsin I by α-CaMKII has been suggested to increase the availability of synaptic vesicles for release. Thus, one mechanism by which α-CaMKII may contribute to persistent pain behavior is

by increasing transmitter release from primary afferent nociceptors. Studies of formalin-evoked spinal glutamate release in the rat indicate that glutamate release is substantially increased during the first phase (Malmberg and Yaksh, 1995a,b). Importantly, glutamate release was not reported to be significantly greater than baseline during the second phase of the formalin test. Therefore, if a decrease in presynaptic α-CaMKII activity contributes to the diminished second phase formalin behavior in the α-CaMKIIT286A mutant mice, then it may be attributable to changes in vesicle fusion and subsequent glutamate release during the acute phase but not during the persistent phase.

The contribution of α-CaMKII to synaptic plasticity in other regions of the nervous system, especially the hippocampus, has focussed largely on the effects of α-CaMKII in postsynaptic neurons. α-CaMKII is concentrated in postsynaptic densities where its interaction with a number of proteins, including AMPA- and NMDA-type glutamate receptors, has been demonstrated to contribute to activity-induced synaptic plasticity (McGlade-McCulloh et al., 1993; Kitamura et al., 1993; Omkumar et al., 1996). For example, α-CaMKII has been demonstrated to enhance synaptic efficacy by inducing synaptic insertion (Rongo and Kaplan, 1999; Hayashi et al., 2000) and increasing the single-channel conductance of AMPA receptors (Barria et al., 1997). Additionally, α-CaMKII directly interacts with NMDA receptors, specifically the NR1, NR2A, and NR2B subunits and this interaction is enhanced when α-CaMKII is phosphorylated at position T286 (Gardoni et al., 1999; Leonard et al., 1999; however, see Strack and Colburn, 1998). Furthermore, there is evidence to suggest that α-CaMKII/NMDA receptor interactions are associated with synaptic plasticity in the hippocampus (Leonard et al., 1999; Gardoni et al., 2001).

Importantly, the interactions of α-CaMKII with glutamate receptors does not appear to be restricted to cortical centers as recordings from substantia gelatinosa neurons in spinal cord slices revealed that AMPA, kainate, and NMDA-mediated currents are enhanced following intracellular injection of α-CaMKII (Kolaj et al., 1994). Differences in glutamatergic receptor activity between the α-CaMKIIT286A wild-type and mutant mice may contribute to the reduced second phase behavior observed in the mutant mice. Indeed, glutamate receptors have been implicated in persistent pain behaviors associated with formalin-induced tissue injury (Coderre and Melzack, 1992; Hunter and Singh, 1994; Chaplan et al., 1997; Nishiyama et al., 1999).

It has also been hypothesized that α-CaMKII can activate the mitogen-activated protein kinase (MAPK) cascade (Kim et al., 1998; Chen et al., 1998) through the phosphorylation of SynGAP, a synaptic RasGAP that associates with post-synaptic density proteins. Recently, activation of the MAPKs ERK1 and ERK2 in spinal cord neurons by noxious inputs was demonstrated to contribute to second phase formalin behavior (Ji et al., 1999; Karim et al., 2001). A decreased activation of this pathway the α-CaMKIIT286A mutant mice may also in part mediate the decreased persistent pain behavior associated with formalin injection. Finally, α-CaMKII expression is restricted to excitatory neurons of the spinal cord, suggesting that any central contribution of autophosphorylated CaMKII to second phase behavior is the result of CaMKII activity in non-GABAergic neurons (Benson and Jones, 1992).

In summary, the results of the present study indicate that the autophosphorylation of α-CaMKII contributes to the persistent pain, but neither the acute pain nor the edema, associated with the formalin test, model of persistent pain. Furthermore, this

autophosphorylation and subsequent calcium-independent activity of α-CaMKII does not mediate changes in threshold associated with either formalin injection or other models of persistent pain. Taken together, these observations highlight a dissociation between molecular mechanisms underlying the spontaneous and stimulus-evoked pain behaviors associated with tissue injury.

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Figure 1: Responses to acute, noxious thermal, mechanical, and chemical do not differ between wild-type and mutant mice. (a) Wild-type and mutant mice did not differ in latency to lick the hind paw when placed on at 52.5°C hot plate. Data are presented as mean latency to respond ± SEM; n = 3 per group. (b) Mechanical sensitivity was determined using the up-down paradigm of Chaplan et al. (1994). Data are presented as the mean von Frey hair threshold in grams ± SEM; n = 3 per group. (c) No difference in capsaicin-evoked licking of the injected hindpaw (5 ug, i.pl.) was observed between wild-type and mutant mice. Data are presented as mean licking time ± SEM; n = 7 wild-type and 5 mutant mice. (d) Thirty minutes after the capsaicin injection, the paw diameters of the wild-type and mutant mice did not differ.

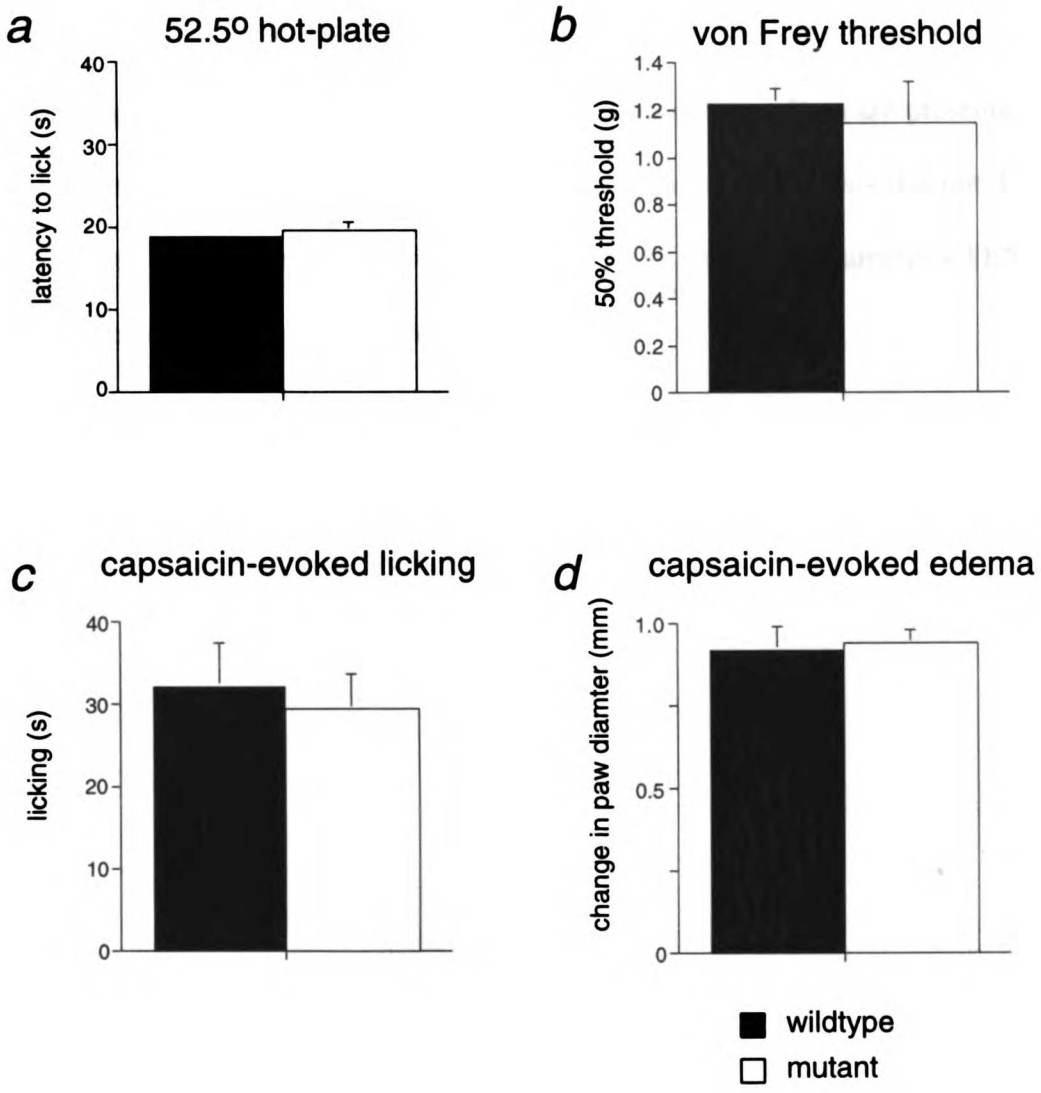

a
52.5º hot-plate
latency to lick (s)
40
30
20
10
0
b
von Frey threshold
50% threshold (g)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
c
capsaicin-evoked licking
licking (s)
40
30
20
10
0
d
capsaicin-evoked edema
change in paw diamter (mm)
1.0
0.5
0
wildtype
mutant

Figure 2: Formalin-evoked behavior and edema in wild-type and mutant mice. (a) Hindpaw licking responses were measured following an intraplantar injection of 2% formalin in a total volume of 10 μl. No behavioral difference between wild-type and mutant mice was observed during the first phase (0-10 min). Second phase (10-40 min) behavior was significantly ($p<0.01$; t test) reduced in mutant mice. Data are presented as mean licking time ± SEM; $n = 5$ per group. (b) Formalin-evoked edema did not differ between wild-type and mutant mice. Data are presented as mean paw diameter ± SEM of the formalin injected and noninjected paws.

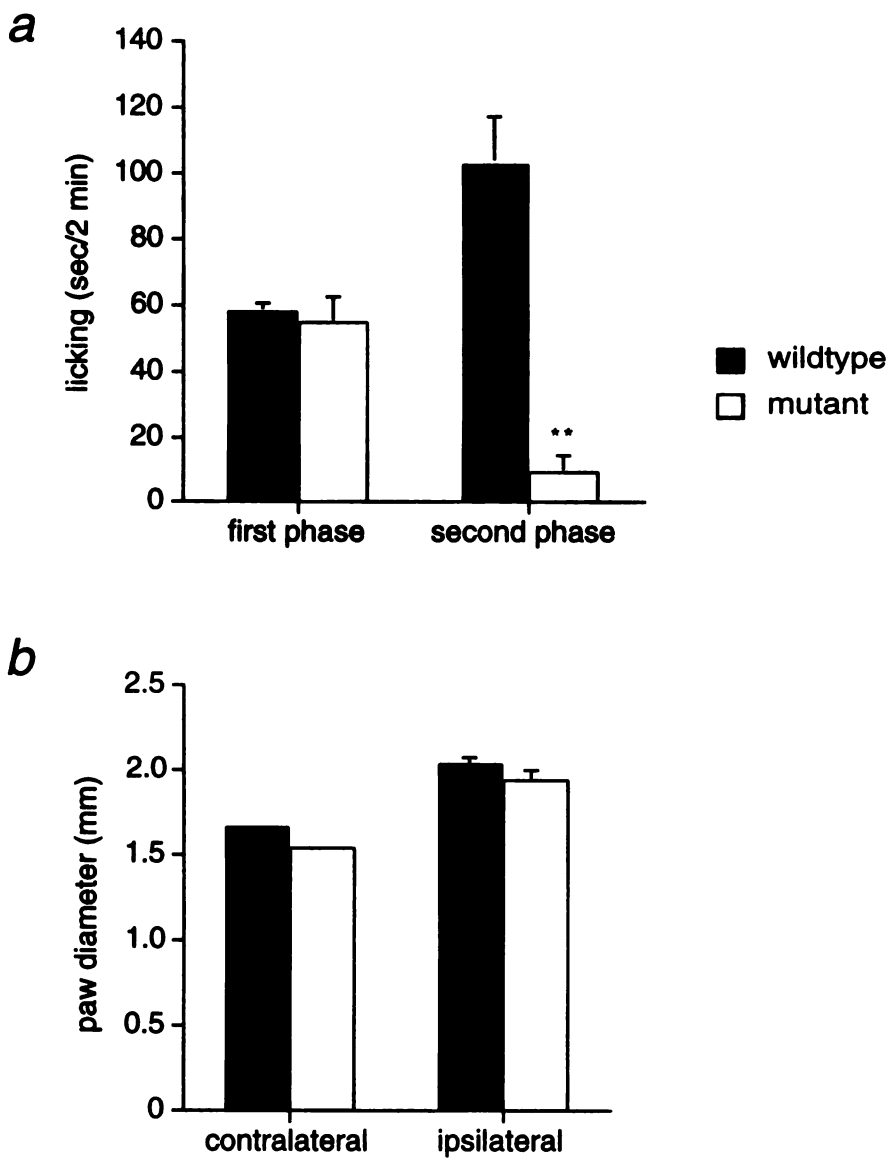

a
140
120
100
80
60
40
20
0
licking (sec/2 min)
**
first phase
second phase
wildtype
mutant
b
2.5
2.0
1.5
1.0
0.5
0
paw diameter (mm)
contralateral
ipsilateral

Figure 3: Formalin-evoked Fos-immunoreactivity. Number of Fos-like immunoreactive neurons in the spinal cord following formalin ± SEM on the formalin-injected side at the L4-L5 level; n = 5 mice per group. There was a significant difference in Fos expression between the two groups ($p<0.05$, two way ANOVA comparing group and laminae. Specifically, the mutant mice showed a significant reduction ($p<0.05$, PLSD Fisher's test) of Fos positive cells in laminae V/VI compared with wild-type mice.

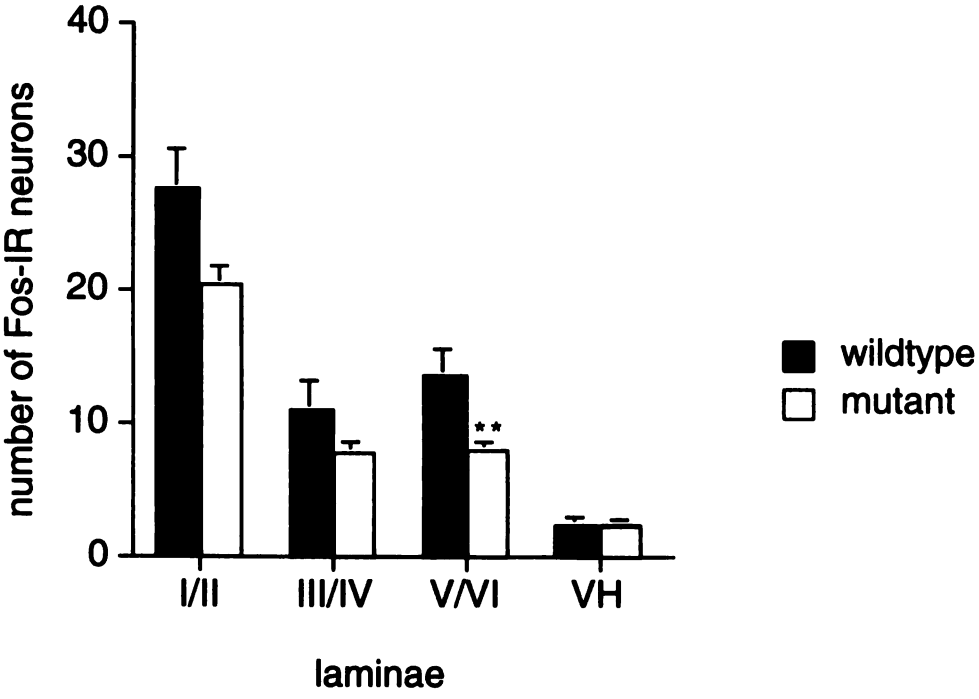

40
30
20
10
0
number of Fos-IR neurons
I/II
III/IV
V/VI
VH
laminae
**
wildtype
mutant

Figure 4: CFA-induced changes in thermal and mechanical thresholds. An injection of CFA (5 μg in 10 μl) into the intraplantar surface of the hindpaw produced comparable reductions in (a) mechanical and (b) thermal thresholds in wild-type and mutant mice. Data are presented as mean latency/threshold ± SEM; n = 3 mice per group. There was no difference in edema between the two groups (data not shown).

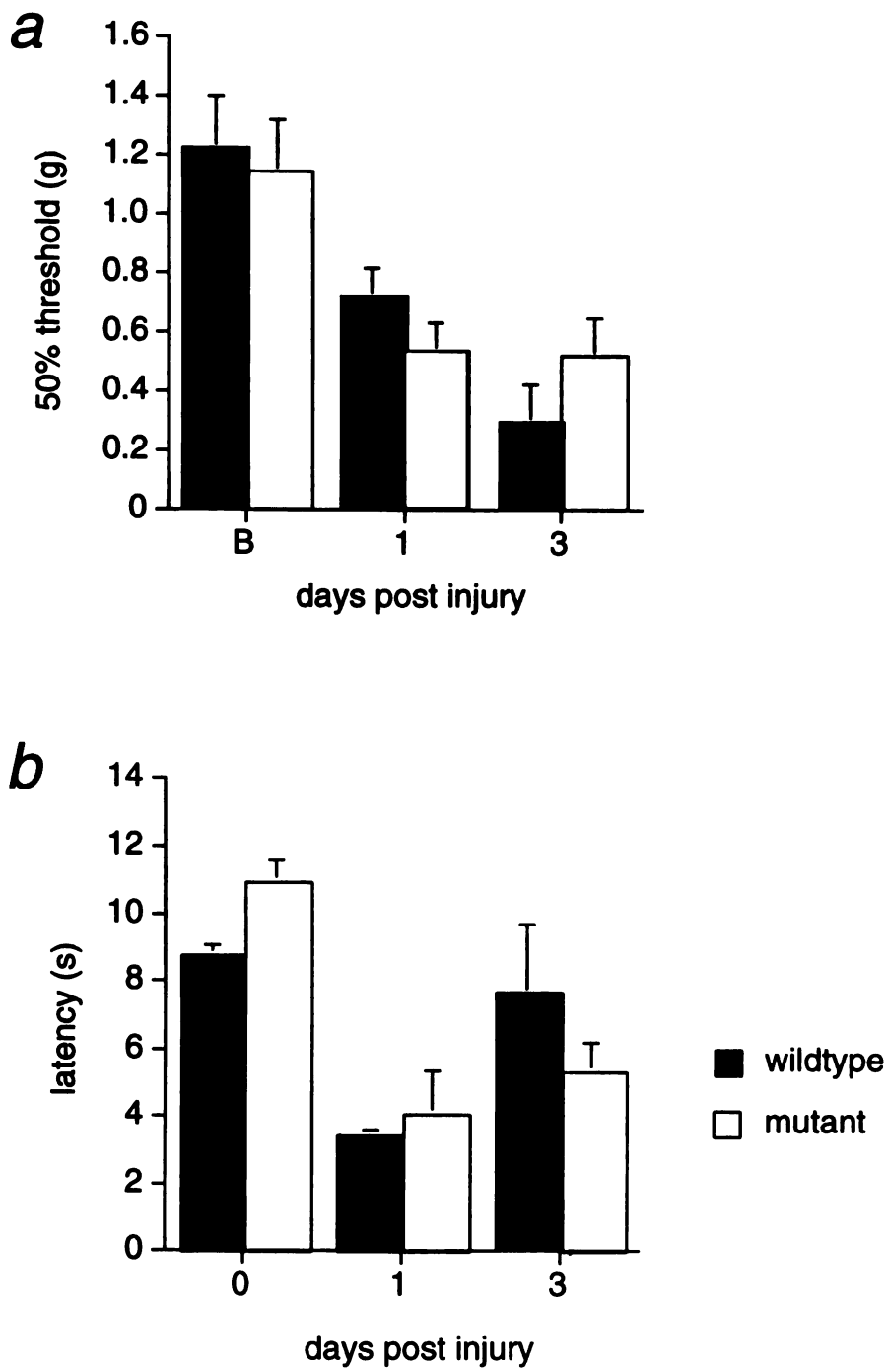

a
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
50% threshold (g)
B
1
3
days post injury
b
14
12
10
8
6
4
2
0
latency (s)
0
1
3
days post injury
wildtype
mutant

Figure 5: Formalin-evoked changes in thermal latencies. Paw withdrawal latencies were measured before and at time points after intraplantar injection of 10µl of 2% formalin. The injection produced a significant reduction in paw withdrawal latency in both groups of mice, however, there was no difference between the groups. Data are presented as mean latency ± SEM; $n = 4$ wild-type and 5 mutant mice per group.

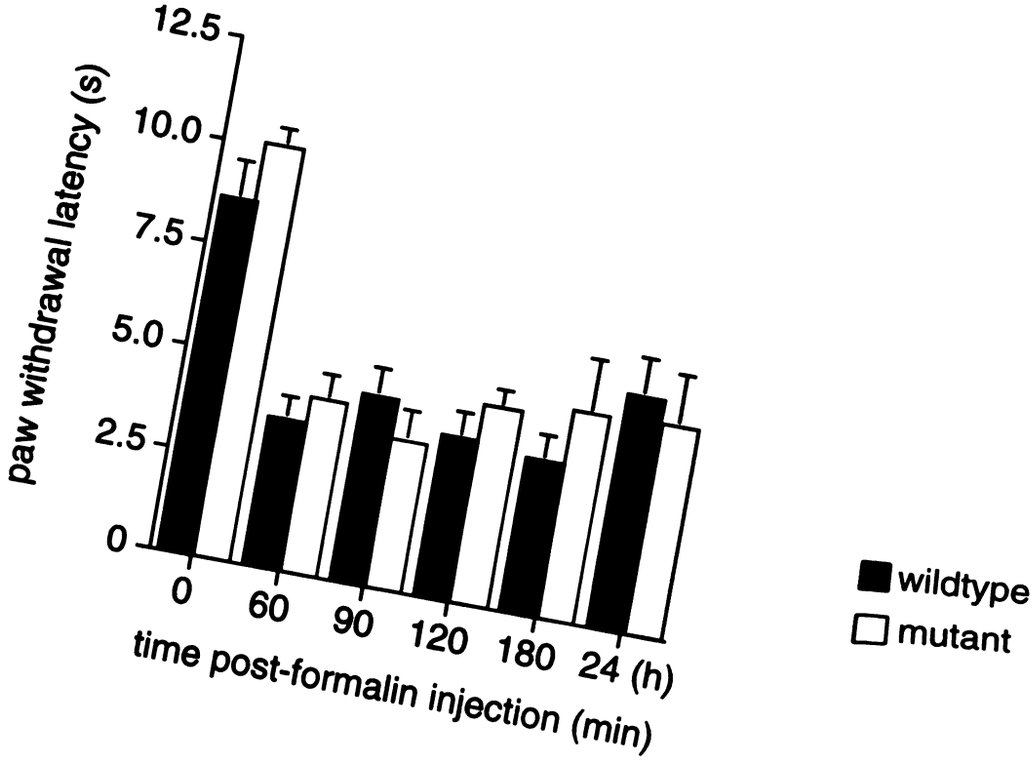
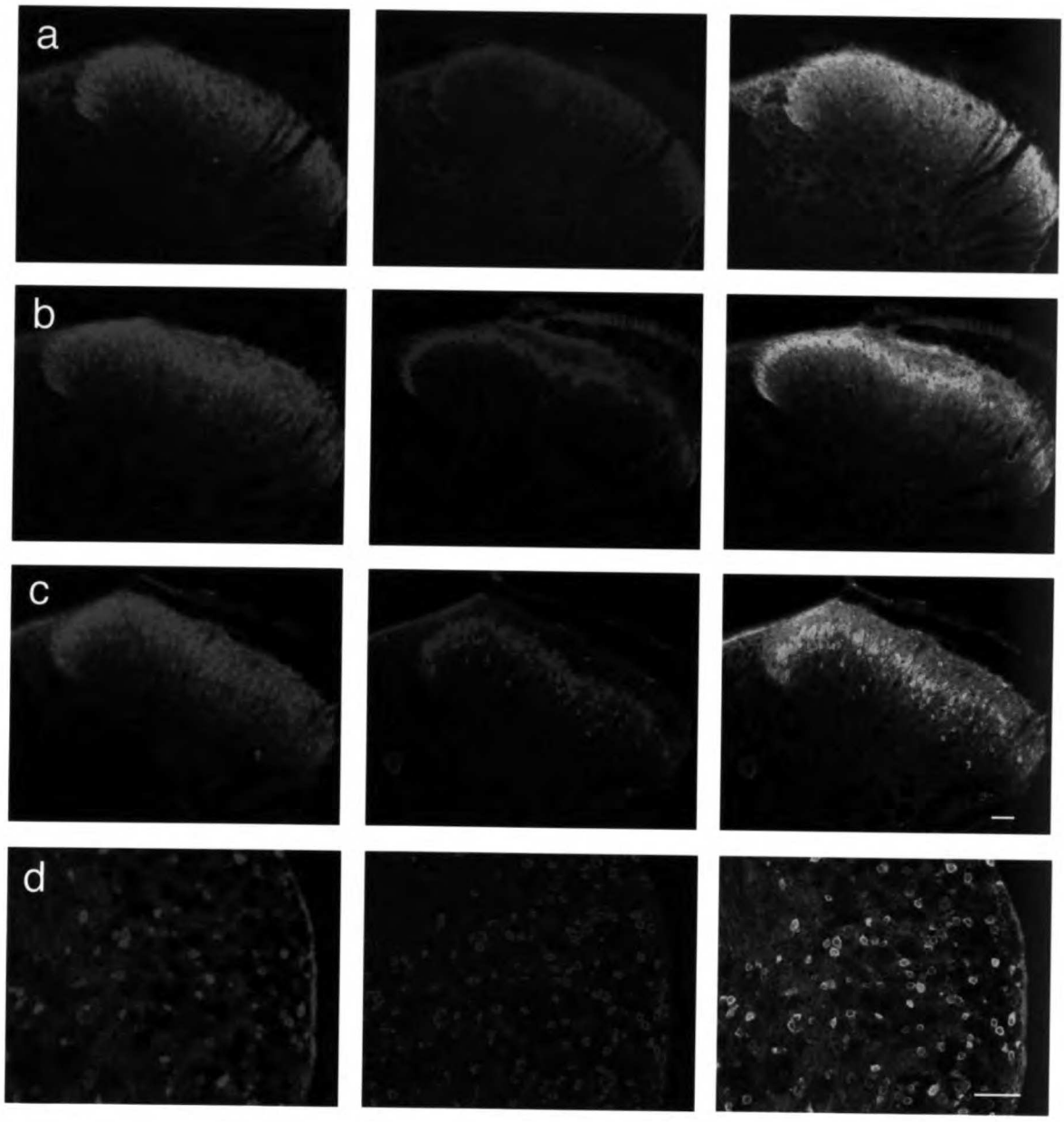

12.5
10.0
7.5
5.0
2.5
0
paw withdrawal latency (s)
0
60
90
120
180
24 (h)
time post-formalin injection (min)
wildtype
mutant

Figure 6: Expression of α-CaMKII in the dorsal horn and trigeminal ganglia. (a,b) α-CaMKII-IR is localized to lamina I and II of the spinal cord as evidenced by the double-labeling of -α-CaMKII with SP (a, green) and IB4 (b, green). (c) α-CaMKII (red) is expressed by the PKCg-containing (green) interneurons of lamina IIi. (d) Double labeling for α-CaMKII (red) and peripherin (green) revealed that α-CaMKII expression in primary afferent neurons is restricted to those with unmyelinated afferents.


a
b
c
d

Chapter 5

Reduced Development of Tolerance to the Analgesic Effects of Morphine and Clonidine in PKCγ Mutant Mice

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Abstract

A variety of second messenger systems have been implicated in the intracellular mechanisms of tolerance development to the analgesic actions of morphine, a mu opioid, and clonidine, an alpha-2 adrenergic receptor agonist. Here, we studied mice that carry a null mutation in the gene encoding a neuronal specific isoform of PKC, namely, PKCγ. We used the tail flick test to construct dose-response curves before and 4 days after chronic morphine (75 mg pellets, sc) or clonidine treatment (0.3 mg/kg, sc, twice daily). Baseline tail flick latencies did not differ in PKCγ mutant and wild-type mice (3-4 sec). Both morphine and clonidine produced a dose-dependent suppression of the tail flick response with an ED_{50} value (2.0 mg/kg for morphine and 0.1 mg/kg for clonidine) that was similar for naive mutant and wild-type mice. In contrast, after 4 days of drug delivery, mutant mice showed significantly less rightward shift in the dose-response curve to morphine (6-fold for wild-type and 3-fold for mutant mice) and to clonidine (5-fold for wild-type and no shift for the mutant mice). These results indicate that PKCγ contributes to the development of tolerance to the analgesic effects of both morphine and clonidine.

Chronic morphine treatment can also result in sensitization of spinal cord neurons and increased pain behaviors following a noxious insult. To assess the contribution of PKCγ to this process, we studied the responses of wild-type and mutant mice to an intraplantar injection of formalin (a model of persistent pain) following chronic morphine treatment. Although morphine tolerance increased formalin-evoked persistent pain behavior and Fos-LI in wild-type mice, there was no difference between placebo- and morphine-treated mutant mice suggesting that PKCγ also contributes to chronic morphine-induced changes in nociceptive processing.

Introduction

Several hypotheses have been proposed to explain the development of tolerance and dependence that results from chronic morphine administration (Cox, 1991). The possibility that opioid receptor downregulation underlies tolerance has largely been ruled out (Smith,1988). A more accepted view holds that tolerance results from decreased coupling of the opioid receptor to an inhibitory G protein (Christie et al., 1987; Smith, 1988; Cox, 1991) which in turn, regulates many downstream effects, including inhibition of adenyl cyclase (Sharma et al., 1975) and increased potassium conductance (North et al., 1987). Based on this hypothesis, many studies of opioid tolerance and dependence have focussed on cAMP-dependent mechanisms, including protein kinase A (PKA)-mediated phosphorylation of specific cellular proteins. Interestingly, PKA has been implicated in other forms of neuronal plasticity including long-term potentiation in the hippocampus and injury-induced changes in nociceptive processing at the level of the spinal cord (Frey et al., 1993; Huang et al., 1995; Malmberg et al., 1997a).

A limitation of the decreased coupling hypothesis is that it cannot account for the induction of the acute withdrawal syndrome that is produced by injection of an opiate antagonist, such as naloxone, into morphine tolerant animals. The fact that opiate antagonists induce withdrawal in morphine tolerant animals suggests that in the tolerant state, morphine remains functionally bound to opioid receptors and, in doing so, prevents the induction of a withdrawal syndrome. This observation has given rise to another hypothesis to explain tolerance and dependence, namely that tolerance to morphine results from a compensatory response in neurons that express the opioid receptor or of neural circuits in which such neurons participate (Nestler and Tallman, 1988; Nestler et al., 1993; Nestler et al., 1994). In this model of the tolerant state, the opioid receptor is functionally coupled to inhibitory G proteins and the compensatory responses balance the inhibitory effects of morphine. Naloxone induces withdrawal because it blocks the effect of the opioid, leaving an unopposed action of the compensatory response system. Thus, the

tolerant state has been referred to as one of latent sensitization; the sensitization is only manifested when the opiate antagonist is injected (Fry et al., 1980)

The hypothesized latent sensitization in the tolerant animal has features in common with the sensitization of spinal cord neurons that occurs after prolonged or intense noxious stimulation. For example, we have reported that chronic morphine results in enhanced noxious stimulus-evoked Fos expression in spinal cord neurons (Rohde et al., 1997). This result could be explained if spinal cord neurons were made hyperexcitable/sensitized by chronic opiate administration. Furthermore, both the behavioral manifestations of injury-induced sensitization of spinal cord neurons, namely allodynia and hyperalgesia, as well as morphine tolerance and dependence can be reduced by NMDA antagonists (Davar et al., 1991; Tanganelli et al., 1991; Trujillo and Akil, 1991; Coderre and Melzack, 1992; Mao et al., 1995a). A similar parallel between the tolerant and the injured state is observed downstream of the NMDA receptors. Specifically, NMDA receptor activation contributes to neuronal plasticity by a calcium-mediated activation of a variety of second messenger systems, including protein kinase C (PKC) and nitric oxide. PKC has been strongly implicated in spinal cord mechanisms of allodynia and hyperalgesia and in the development of tolerance to the analgesic effects of morphine (Mao et al., 1992; Mao et al., 1995a,b,c; Malmberg et al., 1997b).

Importantly, studies to date implicating PKC in tolerance development have relied on the use of relatively non-selective antagonists (Mayer et al., 1995; Granados-Soto et al., 2000). Furthermore, despite the discovery of multiple PKC isozymes with distinct distributions and functions, the contribution of specific PKC isozymes to tolerance development has not been investigated. Here we studied mice with a null mutation in the gene that encodes the gamma isozyme of PKC (PKCγ). We chose to study the development of morphine tolerance and dependence in these mice because previous work in our laboratory demonstrated that these mice do not develop nerve-injury induced persistent pain, a condition generally attributed to central sensitization mechanisms (Malmberg et al.,

1997b). We also assessed the development of tolerance to clonidine, an alpha-2 adrenergic receptor agonist, which, like morphine, exerts its effects via an inhibitory G protein. Consistent with a contribution of PKCγ to the sensitization of spinal cord neurons, we found that PKCγ mutant mice did not become as tolerant to the effects of morphine as did their wild type littermates. Similar results were obtained with clonidine. Finally, we investigated the contribution of PKCγ to morphine tolerance-induced sensitization of spinal cord neurons by examining formalin-evoked behavior and Fos-LI in wild-type and mutant mice treated with chronic morphine.

Material and Methods

Generation of mutant mice: The PKCγ mutant mice were produced as described previously (Abeliovich et al., 1993a) and maintained on a hybrid 129/OLA x C57BL/6 genetic background. Importantly, the 129 strain used here (129/OLA) is distinct from that reported to have reduced morphine tolerance development (129/Sv/Ev; Kolesnikov et al., 1998). Mutant and wild-type male mice were age-matched (10-14 weeks) for experiments. The experimenter was blind to the genotype of the mice in all studies. All animal experiments were reviewed and approved by the Institutional Animal Care and use Committee of the University of California, San Francisco.

Tail flick test: A tail-flick apparatus with a radiant heat source connected to an automatic timer was used to assess the analgesic response. Baseline latencies were determined by averaging three measurements, and a maximum latency of 10 sec was used to minimize damage to the tail. Tail-flick latencies were determined prior to and 30 min after drug administration.

Tolerance studies: Mice were made tolerant to morphine by s.c. implantation of 75 mg morphine pellets (provided by NIDA). Tolerance to the analgesic effect of clonidine was induced by twice daily s.c injections of 0.3 mg/kg clonidine (Paul and Tran, 1995). Dose-response curves to the analgesic effect of morphine or clonidine were established before and on the 5th day after the start of the chronic morphine or clonidine treatment.

Naloxone-precipitated withdrawal: Six days after the implantation of morphine pellets (i.e., one day after the testing of the analgesic effect of morphine), mice were challenged with naloxone, 1.0 mg/kg, s.c.. The mice were placed in Plexiglas cylinders and naloxone-induced jumping behavior was observed for 15 min. At several points during the observation period, the presence or absence of ptosis, salivation and diarrhea were noted. Body weights of the mice were recorded before the injection of naloxone and then again at 60 min post injection. The mice were subsequently (at 90 min) perfusion fixed (see below) and the spinal cords were removed and prepared for Fos immunocytochemistry.

Formalin test: To determine if chronic morphine treatment resulted in an increased pain responsiveness (hyperalgesia) to a noxious stimulus, we used the paw formalin test. Placebo- or morphine-pelleted mice (distinct from those used in tail-flick and withdrawal studies) received a 10 µl intraplantar injection of 1% formalin, a concentration that produces a sub-maximal response in naïve animals. After the formalin injection, the duration of paw licking was measured in 2-min periods at 5-min intervals for 40 min. We defined the first phase response as the total time spent licking at the 1 and 5 min time points and the second phase response as the duration of licking that occurred 10 to 40 min after the injection of formalin. To quantify the magnitude of the inflammatory response we measured the paw diameter with a spring-loaded caliper (Mitutoyo) 90 min after the formalin injection. Ninety minutes after the formalin injection, the mice underwent perfusion fixation and the spinal cords were removed and prepared for Fos immunocytochemistry (see below).

Immunocytochemistry: For immunocytochemistry, the mice were deeply anesthetized with pentobarbital (100 mg/kg) and perfused with a 10 % phosphate-buffered formalin fixative. Following perfusion, the spinal cords were removed, postfixed for 4 hours in the same fixative and then cryoprotected in 30% sucrose overnight. The spinal cords were sectioned transversely (30 μm) on a freezing microtome. The sections were incubated overnight at 4°C in a 1:30,000 dilution of Fos protein antiserum, kindly provided by Dr. Dennis Slamon, UCLA. Immunostaining was performed according to the avidin-biotin peroxidase method (Hsu et al., 1981) using a nickel-intensified diaminobenzidine protocol with glucose oxidase to localize the horseradish peroxidase immunoreaction product. Reacted sections were mounted on gelatin-coated slides, dried, dehydrated and then coverslipped with Eukitt (Calibrated Instruments Inc.).

Double-immunofluorescence staining was performed to determine the localization of PKCγ and mu-opioid receptors (MOR). Thirty μm transverse and sagittal sections of wild-type mouse spinal cords were incubated overnight in guinea pig anti-MOR antibody (kindly provided by Dr. Robert Elde, University of Minnesota) and rabbit anti-PKCγ antibody (Santa Cruz Biotechnology Laboratory). Sections were then incubated in a Cy-2 conjugated goat anti-guinea pig antibody and a Cy-3 conjugated goat anti-rabbit antibody. Images were collected on a BioRad MRC-600 confocal microscope and processed in Adobe Photoshop 5.5.

Data and statistical analysis: All graphical presentations are expressed as mean ± SEM. ED_{50} (effective dose resulting in a 50% reduction of the control response) and 95% confidence intervals (CI) were calculated using a least square linear regression method according to the formulae given by Tallarida and Murray (1987). Statistical significance was determined by factorial analysis of variance (ANOVA) or one-way ANOVA, where appropriate, followed by Student Newman-Keuls post hoc test provided that the F ratio gave $P < 0.05$. To quantitate the number of Fos-like immunoreactive neurons, we took

photographs of 5-10 sections from L4/L5 at low (4x) power on a Nikon Microphot-FXA microscope. The photographs were divided into four segments: laminae I-II, III-VI, V-VI and the ventral horn. A person blinded to the groups counted the number of Fos-immunoreactive neurons. Five to 10 spinal cord sections were counted per mouse and averaged so that each mouse had a mean value for the regional Fos immunoreactivity.

Results

General behavior

The generation and general behavior of the PKCγ mutant mice have been described previously. Briefly, the deletion of PKCγ produces viable mice with a normal appearance. The mutant mice have modest impairments in tests of learning and memory (Abeliovich et al., 1993a,b) and some motor incoordination (Chen et al., 1995), which may be related to the elimination of multiple climbing fiber innervation of Purkinje cells (Kano et al., 1995). We have demonstrated that "acute" nociceptive responses are normal in the mutant mice, but thermal and mechanical allodynia associated with nerve injury are significantly reduced (Malmberg et al., 1997b). As there is no difference between mutant and wild-type mice in the tail-flick test or the first phase of the formalin test, we do not believe that the reported mild ataxia and motor incoordination influenced the results in the present study. Importantly, the gamma isozyme of PKC is not expressed until after birth so developmental abnormalities and/or compensatory responses to its loss are less likely to have occurred.

Effect of acute and chronic morphine treatment

Baseline tail flick latencies were similar in PKCγ mutant and wild-type mice (3-4 sec). Systemic delivery of morphine produced a similar dose-dependent suppression of the tail-flick response in both wild-type and PKCγ mutant mice (Fig. 1A). The morphine ED_{50} value and 95% CI was 2.4 (2.1-2.8) mg/kg in the wild-type mice and 2.1 (1.7-2.6) mg/kg in the PKCγ mutant mice. Chronic delivery of morphine (75 mg morphine pellet, s.c.)

produced a rightward shift in the morphine dose-response curve in both groups of mice (Fig. 1B). However, after 4 days of drug delivery, the morphine dose-response curve for the mutant mice showed a significantly smaller shift as compared to wild-type (6-fold for wild-type and 3-fold for mutant mice, Fig. 1B). The ED_{50} value for morphine in the pelleted mice was 14 (11-18) mg/kg in wild-type mice and 6.4 (4.8-8.4) mg/kg in the PKCγ mutant mice.

Effect of acute and chronic clonidine treatment

Subcutaneous injections of clonidine also produced a dose-dependent suppression of the tail-flick response with similar ED_{50} values in the naive wild-type [95 (79-115) μg/kg] and the PKCγ mutant [115 (97-135) μg/kg] mice (Fig. 2A). In contrast, after 4 days of clonidine treatment (twice daily injections of 0.3 mg/kg), the clonidine dose-response curve shifted 5-fold in wild-type mice, but there was no shift in the mutant mice (Fig. 2B). To determine if the development of antinociceptive tolerance to morphine and clonidine had the same underlying mechanism, we made wild-type mice tolerant to morphine (as described above) and generated clonidine dose-response curves before and after the induction of morphine tolerance. In these studies, we saw no shift in the dose-response curve to clonidine in morphine tolerant mice, indicating that there was no cross-tolerance between the two drugs in wild-type mice (data not shown). Although cross-tolerance between opioid and alpha-2 agonists has been demonstrated following repeated s.c. morphine injections (Post et al., 1988; Paul and Tran, 1995), Li and Roerig (1999) were also unable to demonstrate cross-tolerance using a morphine pellet paradigm to produce tolerance in mice. These authors suggest that the development of cross-tolerance between opioid and alpha-2 agonist depends, at least to some extent, on the route of chronic drug administration.

Naloxone-induced withdrawal behavior

In other groups of mice, 1.0 mg/kg naloxone was injected s.c. on the sixth day after morphine pellet implantation. This injection produced withdrawal responses in all mice (13 wild-type and 18 mutant mice). Withdrawal behaviors included vertical jumping, diarrhea, salivation, ptosis and serpentine movement of the tail. The jumping behavior was observed within 1 min of the naloxone injection and was no longer present 15 min after the injection. Interestingly, although tolerance to the analgesic effect of morphine was reduced in the PKCγ null mice, we detected significantly *more* jumping in the mutant mice (64±6 jumps) as compared to wild-type mice (38± 8 jumps) during the first 15 minutes following naloxone injection (Fig. 3A). In contrast, we noted less diarrhea in the mutants than in wild-type mice and this was reflected by the difference in loss of body weight 60 min after the naloxone injection (Fig. 3B). Specifically, wild-type mice showed a 7.7±0.2% reduction in body weight one hour after naloxone injection whereas the body weight of mutant mice had decreased only 4.6±0.1% at the same time point. Furthermore, the diarrhea started later in the mutant (10±0.6 min) compared to the wild-type group (5.3±0.5 min). The other signs of withdrawal, such as salivation, ptosis and tail movements were similar in the wild-type and mutant mice.

The spinal cords of 6 wild-type and 6 mutant mice (randomly selected from the above groups) were also processed for Fos immunoreactivity, which increases after naloxone-induced withdrawal (Rohde et al., 1996). We observed no difference in Fos immunoreactivity in any of the laminae at either lumbar (total Fos: 97±16 in the wild-type and 108±14 in the mutant mice) or sacral (total Fos: 278±18 in the wild-type and 282±36 in the mutant mice) segments of the spinal cord.

Formalin test in morphine tolerant mice

During the first 1-3 days following morphine pellet implantation, the mice appeared hyperactive and had Straub tails. On the fifth day after pellet implantation, when the formalin test was performed, these altered behaviors were absent and there was no

difference in body weight between the groups (20-22 grams). An i.pl. injection of 1% formalin produced a characteristic biphasic licking behavior in the mice. We used a low concentration of formalin to avoid a ceiling effect that would have made it impossible to detect increased behaviors in tolerant animals. The first phase behavioral response of the formalin test was similar in all groups (Fig. 4). Consistent with there being no change in first phase behavior, the tail flick latency was not altered in morphine-tolerant animals. In contrast to the acute behavior, the second phase response was greatly enhanced in morphine-treated wild-type mice compared to the placebo-pelleted wild-type mice (Fig. 4). On the other hand, in the mutant mice there was no difference in the second phase between morphine- and placebo-pelleted mice and the morphine-treated mutant mice showed significantly less second phase licking compared to the morphine-treated wild-type mice (Fig. 4).

Despite the behavioral differences, chronic morphine treatment had no effect on the peripheral inflammatory response produced by the formalin injection. There was a similar paw swelling in placebo-treated (35±3% increase in paw diameter) and morphine-pelleted (36±5%) wild-type mice. Similarly, in the mutant mice, the paw swelling in the placebo group (18±2%) did not differ from that of the morphine-treated mice (18±6%). However, consistent with our previous finding (Malmberg et al., 1997b), the mutant mice showed significantly less inflammatory response when compared to wild-type mice ($p<0.001$; t-test comparing wild-type with mutant mice pooled from the placebo and morphine groups).

As previously described, the formalin injection evoked Fos expression, a marker of neuronal activity, in the superficial laminae in the spinal segments L4-L5 ipsilateral to the injection. In laminae I-II, the area of the dorsal horn where primary afferent nociceptors terminate, we observed a significant increase ($p<0.05$; ANOVA followed by Student Newman-Keuls test) in Fos-IR in morphine-pelleted wild-type mice relative to placebo-treated wild-type mice and morphine-treated mutant mice (Fig. 5). There was no significant difference in Fos-IR between wild-type and mutant placebo-pelleted mice. Furthermore,

there was no difference between the groups in the number of Fos-IR neurons in deeper laminae (III-VI and the ventral horn).

PKCγ and mu opioid receptor immunoreactivity

An important insight into the possible mechanism through which PKCγ may influence chronic morphine evoked changes was revealed in double-immunofluorescence studies. As seen in our previous work (Malmberg et al., 1997b), we found that PKCγ immunoreactivity is largely restricted to a population of interneurons in the inner portion of lamina II (Figure 6). In contrast, immunoreactivity for the mu opioid receptor (MOR) was localized to laminae I, IIo, and the dorsal portion of IIi. Consistent with the work of Polgár et al. (1999), we found virtually no overlap of PKCγ and MOR expressing cells.

Discussion

The present study provides evidence to support the hypothesis that PKCγ contributes to the neuronal changes associated with the chronic delivery of the analgesic agents morphine and clonidine. Our results suggest that PKCγ contributes to the compensatory neuronal responses that give rise to analgesic tolerance following chronic drug administration. Furthermore, the distinctions that we observed in withdrawal-associated behaviors as well as nociceptive processing between morphine-treated wild-type and mutant mice suggests that PKCγ also contributes to the latent sensitization of neurons/neuronal circuits that arises as a result of chronic morphine treatment.

PKCγ and analgesic tolerance

Our results are consistent with results from other laboratories that used pharmacological approaches to investigate the contribution of PKC to tolerance development. For example, Mayer and colleagues (1995) demonstrated that intrathecal coadministration of GM1 ganglioside and morphine reduced the development of tolerance

to the analgesic effects of morphine in rats. GM1 ganglioside purportedly inhibits PKC activity by preventing translocation of the enzyme to the plasma membrane. The authors also reported that the increase in PKC translocation associated with chronic morphine treatment could be decreased by GM1 ganglioside. A caveat in these studies is GM1 ganglioside has been shown to inhibit phospholipase A (Hungund et al., 1994) and modulate Ca^{2+} channel activity (Carlson et al., 1994) As a result, it is not certain that the behavioral effects of the drug reflected blockade of PKC function. Recently, Granados-Soto et al. (2000) reported that i.t. administration of chelerythrine, a more selective PKC inhibitor, also prevents the development of analgesic tolerance to spinally-administered morphine. Although more selective for PKC than GM1 ganglioside, chelerythrine does not permit evaluation of the contribution of particular PKC isozymes to tolerance development.

In the present study we used a mouse in which one isozyme, namely gamma, has been deleted and we demonstrated a significant contribution of this specific PKC isozyme to morphine tolerance. Although a previous immunohistochemical study implicated PKCγ in tolerance development (Mao et al., 1995c), the general PKCγ -IR pattern revealed in those studies (namely extensive PKCγ -IR in laminae I and II) was markedly different from that observed in our work and in other laboratories (Polgár et al., 1999). Because we were able to perform control immunocytochemical experiments in the PKCγ null mouse, we were able to identify antibodies selective for PKCγ. These studies reveal that PKCγ expression is indeed restricted to a population of interneurons in inner lamina II of the spinal cord (Malmberg et al., 1997b) and not the broader distribution previously suggested (Mao et al., 1995c).

Because tolerance to the analgesic effects of morphine was not completely abolished in the PKCγ mutant mice, it is likely that other isozymes or second messenger systems also contribute to tolerance development. For example, other isozymes of PKC, including alpha, betaI, beta II, are expressed in the spinal cord (Malmberg et al., 1997b) and chronic i.t. morphine has been shown to upregulate both PKCα and PKCγ expression in the spinal

cord (Granados-Soto et al., 2000). By contrast, using morphine pellets to establish tolerance, Li and Roerig (1999) found a *decrease* in PKCγ and PKCα protein levels in the spinal cord even though overall PKC activity was enhanced. Important distinctions in the studies assessing changes in PKC activity and expression with tolerance include the use of different species (rat vs. mouse) and different paradigms to produce tolerance (i.t. injections vs. pellets). Furthermore, in the case of PKCγ, different antibodies were used to determine changes in protein level. The anti- PKCγ antibody used by Li and Roering (1999; Santa Cruz) does not reveal any immunostaining in the PKCγ null mouse. Thus, antibody specificity is not an issue in that study.

Tolerance to agents that activate G-protein coupled receptors has also been observed with chronic agonist exposure. In fact, although many studies have focussed on the compensatory changes associated with opiates, it has been shown that alpha-2 adrenergic and opioid receptor stimulation produce similar effects on signal transduction pathways (Law and Loh, 1992). With this in mind, we hypothesized that the PKCγ mutant mice would also show decreased analgesic tolerance in the setting of chronic clonidine, an alpha-2 adrenergic receptor agonist. In fact, the effect of the mutation was, if anything, more significant in the case of clonidine than morphine. There was no shift in the clonidine dose-response curve in the PKCγ mutant mice following 4 days of clonidine treatment suggesting that analgesic tolerance to this treatment regime was completely prevented.

Contribution of PKCγ to morphine dependence

Although the PKCγ mutant mice did not develop tolerance to the same extent as the wild-type mice, the consequences of the PKCγ mutation on morphine dependence (as assessed by precipitated withdrawal) were somewhat surprising. Because the mutant mice were less tolerant, one might have predicted that the signs of withdrawal would also be decreased. This was, in fact, true for the gastrointestinal signs of withdrawal (namely diarrhea and weight loss) but the mutant mice, despite a slight motor incoordination

(Abeliovich et al., 1993b), showed significantly *greater* jumping behavior following an injection of naloxone compared to wild-type mice. The presence of decreased tolerance with increased withdrawal behavior points to a dissociation not only of tolerance and dependence but also of different symptoms of dependence. Interestingly, recent work by Murtra and colleagues (2000) reveals a similar dissociation in mice lacking the NKI receptor. Following an injection of naloxone, morphine-treated NK1 receptor mutant mice displayed all of the classical withdrawal signs with the exception of jumping. Furthermore, although B-arrestin-2 mutant mice fail to develop antinociceptive tolerance to morphine, they still become physically dependent (Bohn et al., 2000).

Of course, tolerance to the analgesic effect of morphine in the tail flick test, a spinal reflex, involves opioid receptor changes far more localized than the broad spectrum of behaviors seen when withdrawal is precipitated by systemic naloxone injection. For example, i.c.v. administration of a quaternary derivative of naloxone in morphine tolerant rats induces some withdrawal signs, such as jumping and wet dog shakes, but not others, including diarrhea (Maldonado et al., 1992). Thus, the seemingly opposing effects of the PKCγ mutation on naloxone precipitated withdrawal (i.e. an exacerbation of withdrawal behavior despite reduced analgesic tolerance) may be due to a contribution of PKCγ to subsets of the circuits that underlie different withdrawal symptoms. Consistent with this hypothesis, Fundytus and Coderre (1996) showed that a single i.c.v. chelerythrine injection just prior to naloxone administration in morphine tolerant rats increases the frequency of jumping and wet-dog shakes. This suggests that supraspinal PKC contributes to this component of the withdrawal syndrome. On the other hand, because we did not observe any difference in withdrawal-evoked Fos-LI at the level of the spinal cord between mutant and wild-type mice, it appears that PKCγ does not contribute to dependence-associated changes in spinal cord processing, despite the clear contribution to tolerance development. This further highlights a dissociation between the development of morphine tolerance and dependence.

Tolerance and hyperalgesia

As described above, it has been argued that opiates are not inactive in the tolerant state, but rather a compensatory response counteracts their inhibitory effects. The compensatory response, which corresponds to latent sensitization, is only manifest when an opiate antagonist is administered or when a noxious stimulus is applied, resulting in withdrawal or hyperalgesia, respectively. In the present study, we provide evidence that PKCγ contributes to the latent sensitization associated with hyperalgesia. Thus, morphine-tolerant wild-type mice showed significantly greater second phase behavior and formalin-evoked Fos-LI than did placebo-pelleted wild-type mice. In contrast, there was no difference in second phase behavior or Fos-LI between morphine- and placebo-pelleted mutant mice. Importantly, we found no difference in the mutant mice despite the fact that tolerance to morphine was not completely lost. The increased behavior and Fos-LI suggest the lumbar spinal cord neurons were indeed sensitized during the development of morphine tolerance. The absence of such changes in the mutant mice argues that, as in the case of nerve injury (Malmberg et al., 1997b), PKCγ contributes to the tolerance-induced sensitization of spinal cord circuits.

Previously, we compared the patterns of Fos expression produced by formalin injection in naive and morphine-tolerant rats (Rohde et al., 1997). These studies found that although there was no difference in flinching (a measure of pain) between morphine-tolerant and control groups, formalin-evoked Fos expression was significantly increased in morphine-tolerant rats. The fact that the increased Fos expression occurred without increased pain behavior suggested either that we were already at a ceiling for pain behavior, or that the cellular manifestation of the compensatory response (i.e. Fos expression) may not have occurred in the circuits that generate pain behavior. In the present study, we used a low concentration of formalin and we were able to demonstrate both an increase in Fos-LI and an increase in persistent pain behavior in morphine-tolerant. These observations

indicate that the increase in formalin-evoked Fos-LI in morphine tolerant mice is indeed in neurons that contribute to the "pain" behaviors. Notably, in a previous report (Malmberg et al., 1997b), we found decreased second phase behavior in PKCγ mutant mice, however, in the present study, we found that second phase behavior did not differ between placebo-pelleted wild-type and mutant mice. A possible explanation for this discrepancy is that the lower formalin concentration used in the present study (1.0% vs. 2.0%) was not sufficient to produce observable differences in second phase behaviors between genotypes.

Spinal cord circuitry

The molecular mechanisms by which PKCγ contributes to the development of tolerance is not clear. Activation of the mu opioid receptor can enhance NMDA receptor currents in part via its phosphorylation by PKC (Chen and Huang, 1991). Importantly, Polgár et al (1999) as well as our own observations (see Fig 6) indicate that the gamma isozyme of PKC is expressed in very few of the mu opioid receptor positive cells in the spinal cord. In fact, the distinction between MOR-IR and PKCγ -IR in the spinal cord is striking, with MOR-IR being restricted to laminae I, IIo, and the dorsal portion of IIi and PKCγ to the ventral aspect of inner lamina II. Because of this segregation, PKCγ cannot be the isoform that underlies the interaction between mu opioid and NMDA receptors. Rather, we propose that PKCγ contributes to the development of tolerance to the analgesic effects of morphine through its participation in a neuronal circuit that involves PKCγ–containing neurons and mu opioid-receptor expressing neurons. Because the distribution of alpha-adrenergic receptors does not appear to overlap with PKCγ, this is also likely for the development of analgesic tolerance to clonidine (Stone et al., 1998).

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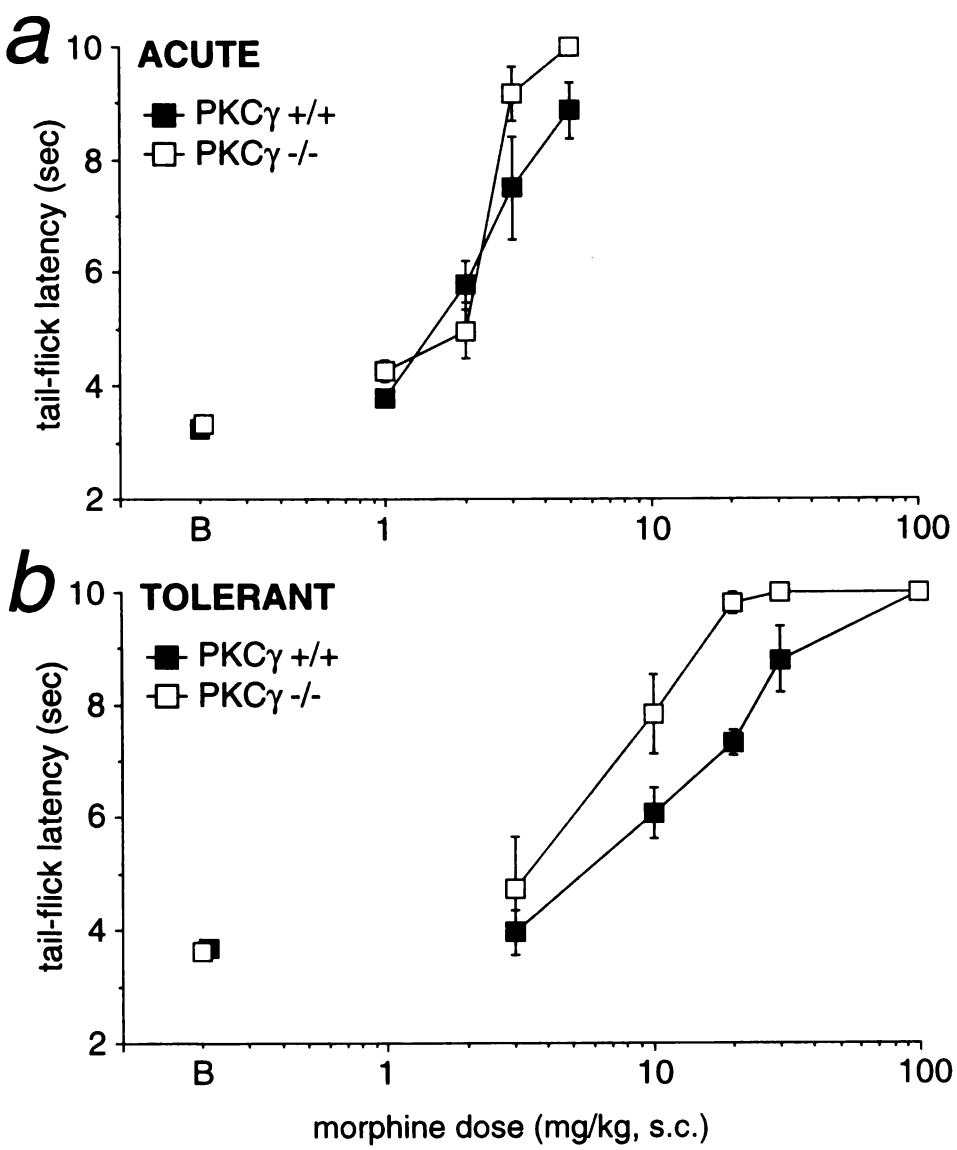
Figure 1: Effect of morphine on tail-flick latencies in (a) naive and (b) morphine-tolerant wild-type and PKCγ mutant mice. Data are presented as mean response time ±SEM in the tail-flick test; n = 3-5 per dose. For the study, we used 14 wild-type and 16 mutant mice. Mutant mice displayed decreased tolerance to the analgesic effect of morphine.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ACUTE
PKCγ +/+
PKCγ -/-
tail-flick latency (sec)
10
8
6
4
2
B
1
10
100
b
TOLERANT
PKCγ +/+
PKCγ -/-
tail-flick latency (sec)
10
8
6
4
2
B
1
10
100
morphine dose (mg/kg, s.c.)

Figure 2: Effect of clonidine on tail-flick latencies in (a) naive and (b) clonidine-tolerant wild-type and PKCγ mutant mice. Data are presented as mean tail-flick time ±SEM; n = 3 per dose, n = 9 mice per group. The mutant mice displayed significantly decreased tolerance to the analgesic effect of clonidine.

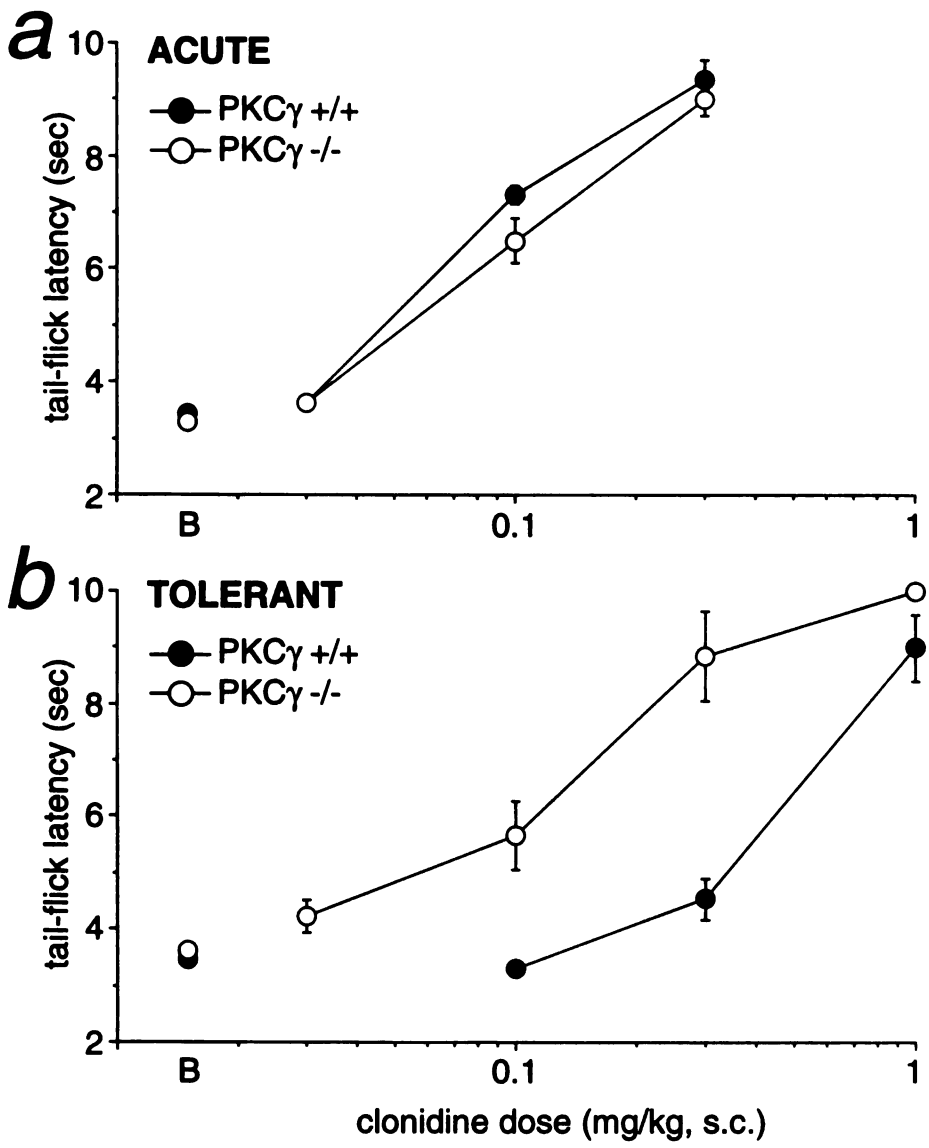

a
ACUTE
PKCγ +/+
PKCγ -/-
tail-flick latency (sec)
10
8
6
4
2
B
0.1
1
b
TOLERANT
PKCγ +/+
PKCγ -/-
tail-flick latency (sec)
10
8
6
4
2
B
0.1
1
clonidine dose (mg/kg, s.c.)

Figure 3: Naloxone-induced withdrawal responses in morphine-treated mice. (a) Jumping behavior during the first 15 minutes after naloxone injection. Data are presented as mean number of jumps ± SEM. (b) Percent body weight loss ± SEM sixty minutes following naloxone injection; n =13 wild-type mice and 18 mutant mice.

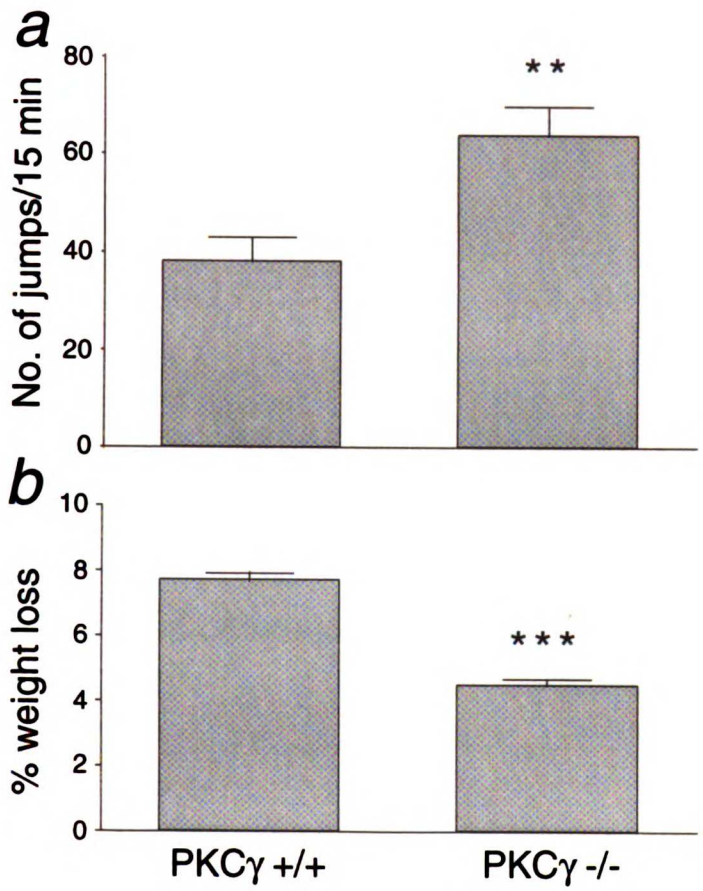

a
No. of jumps/15 min
80
60
40
20
0
**
b
% weight loss
10
8
6
4
2
0
***
PKCγ +/+
PKCγ -/-

Figure 4: The effect of an i.pl. 1% formalin injection in placebo or morphine pelleted wild-type and PKCγ mutant mice. The data are presented as mean licking time ± SEM during phase 1 (0-9 min) and phase 2 (10-40 min) of the formalin test; n=5 mice per group. The asterisks indicate significant difference between the groups; *: $p<0.05$; ***: $p<0.001$, ANOVA followed by Student Newman-Keuls test.

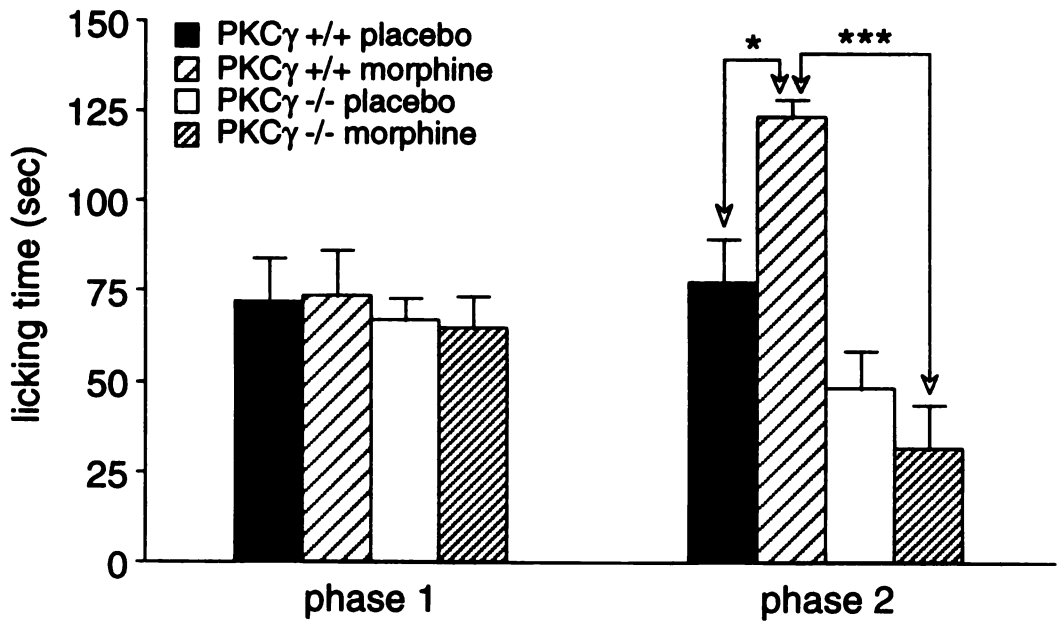

150
125
100
75
50
25
0
licking time (sec)
PKCγ +/+ placebo
PKCγ +/+ morphine
PKCγ -/- placebo
PKCγ -/- morphine
*
***
phase 1
phase 2

Figure 5: Formalin-evoked Fos immunoreactivity. Data are presented as mean number of Fos-immunoreactive neurons ± SEM on the formalin injected side at the L4-L5 level; n=5 mice per group. The asterisks indicate significant difference between the groups; *: $p<0.05$; ***: $p<0.001$, ANOVA followed by Student Newman-Keuls test..

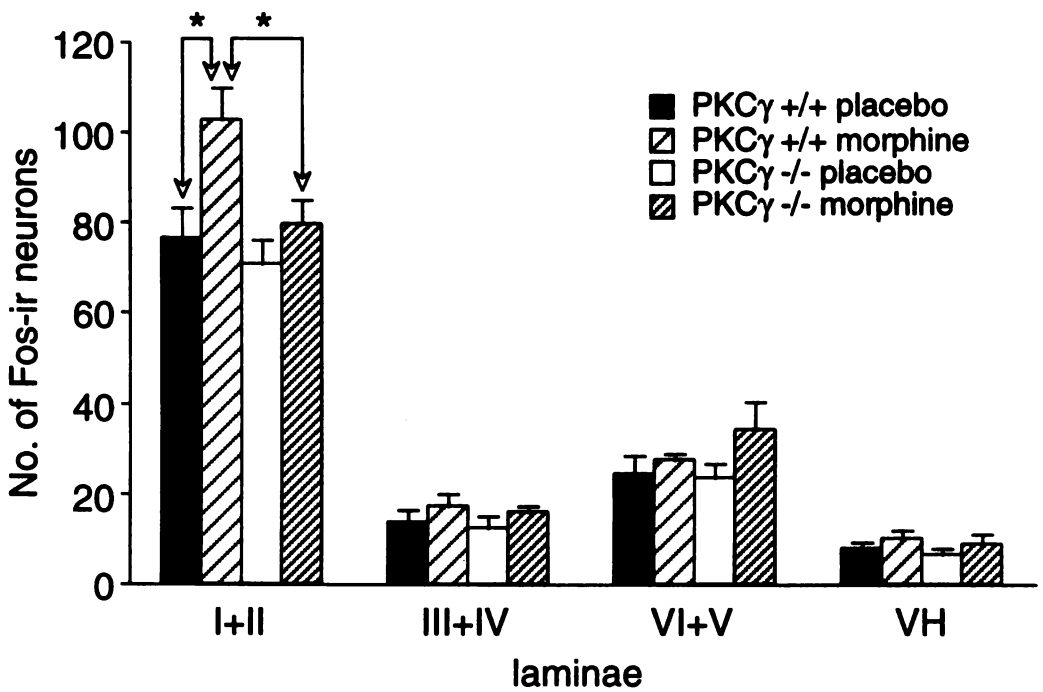
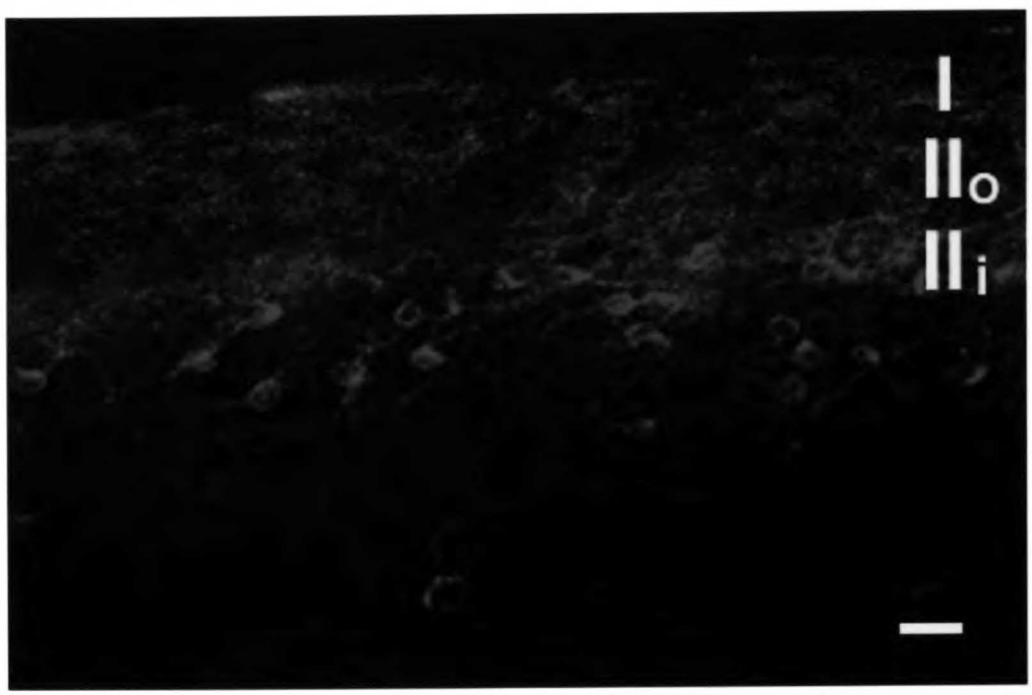

120
100
80
60
40
20
0
No. of Fos-ir neurons
*
*
PKCγ +/+ placebo
PKCγ +/+ morphine
PKCγ -/- placebo
PKCγ -/- morphine
I+II
III+IV
VI+V
VH
laminae

Figure 6: This photomicrograph illustrates PKCγ (red) and MOR (μ-opioid receptor; green) double immunostaining in a sagittal section from the dorsal horn of a mouse spinal cord. PKCγ -positive neurons are located in the most ventral portion of lamina II inner, whereas MOR staining is observed in laminae I and II outer and the more dorsal potion of lamina II inner. The almost complete segregation of PKCγ and MOR immunolabeling is consistent with the report of Polgár et al. (1999). Scale bar equals 50 μm.


I
IIo
IIi