

# Signaling Pathways in Sensitization: Toward a Nociceptor Cell Biology

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DOI 10.1016/j.neuron.2007.07.008

*Clinical pain is a serious public health issue. Treatment of pain-related suffering requires knowledge of how pain signals are initially interpreted and subsequently transmitted and perpetuated. This review article is one of three reviews in this issue of Neuron that address our understanding of the pain process and possible solutions to the problem from both cellular- and systems-level viewpoints.*

The electrophysiological properties of peripheral neurons activated by noxious stimuli, the primary afferent nociceptors, have been investigated intensively, and our knowledge about the molecular basis of transducers for noxious stimuli has increased greatly. In contrast, understanding of the intracellular signaling mechanisms regulating nociceptor sensitization downstream of ligand binding to the receptors is still at a relatively nascent stage. After outlining the initiated signaling cascades, we discuss the emerging plasticity within these cascades and the importance of subcellular compartmentalization. In addition, the recently realized importance of functional interactions with the extracellular matrix, cytoskeleton, intracellular organelles such as mitochondria, and sex hormones will be introduced. This burgeoning literature establishes new cellular features crucial for the function of nociceptive neurons and argues that additional focus should be placed on understanding the complex integration of cellular events that make up the “cell biology of pain.”

## Introduction

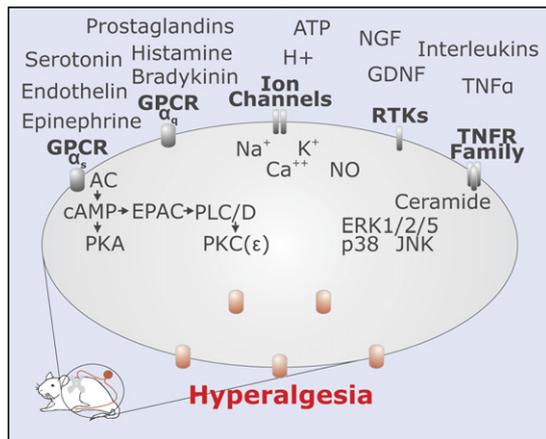
From prokaryotes to higher eukaryotes, the complexity of cellular organization expanded greatly. Cell compartments such as membrane-enclosed organelles and protein clusters at functional sites evolved. In higher eukaryotes, a multitude of specialized cells interact in a time- and tissue-dependent manner. This increase in organizational complexity is the necessary basis for the ability to integrate increasing quantities and qualities of information about the external as well as internal condition of the organism.

Sensory neurons in vertebrates connect peripheral tissues with the central nervous system, occupying a particularly prominent position in acute information reception, transduction, and integration. But they also show the potential to undergo long-lasting plastic changes. This plasticity, often in the form of sensitization, memory for prior injury, or desensitization, can be beneficial in the case of avoidance of physical stimulation of injured tissue. In contrast, in cases of chronic inflammatory or neuropathic pain, these changes produce an often disabling burden on the organism.

Nociceptive neuron sensitivity is modulated by a large variety of mediators in the extracellular space. These mediators activate a large number of receptor classes, which in turn activate a plethora of signaling cascades (Julius and Basbaum, 2001; Lewin et al., 2004; Scholz and Woolf,

2002; Figure 1). How this multitude of cascades mediates nociceptor sensitization and pain is only beginning to be understood. As function relies upon structure, investigation of the cellular components involved in this process has greatly enhanced the understanding of nociceptive mechanisms and their modulation and opened surprising new fields of research.

We review the existing data establishing the importance of classical intracellular signaling components in inflammatory and neuropathic pain. Often in nociceptive neurons the cascade-initiating receptor(s) has not been fully characterized. Thus, we sort data according to the core components of the respective signaling cascade. This also avoids confusion, as signaling pathways can be activated and/or modulated by more than one receptor. This overview is then followed by the introduction of two complementary models that might integrate this data. The models intrinsic logic highlights the need for furthering our understanding of the basic cell-biological mechanisms that impact nociceptor signaling. And indeed, as exemplified by pioneering works, the complex cellular organization of nociceptive neurons (e.g., subcellular compartmentalization, signaling cascade plasticity, extracellular matrix components, the cytoskeleton, intracellular organelles, and sex hormones) is essential for its function. We will argue that a thorough investigation of these



**Figure 1. Signaling Components in Nociceptors**

A large number of extracellular mediators modulate nociception. They act through several receptor classes. Thereby, a plethora of intracellular signaling cascades is initiated. So far, research has concentrated on verifying the involvement of core components of these pathways mostly neglecting the identification of upstream as well as downstream signaling components. Only few downstream effectors (red ovals) such as ion channels have been identified, the discussion of which is beyond the scope of this review. As none of the components characterized so far fully explains the process of sensitization, further cellular components have to be investigated.

components of the intracellular signaling machinery is essential in order to fully understand how nociceptive cascades function. Additionally, by elucidating new components of this signaling cascade, such basic cell-biological research should also help to identify potential new therapeutic targets for management of pain.

**Nociceptor Signaling Pathway Components**  
**cAMP and Protein Kinase A (PKA)**

The first cellular second messenger discovered, cAMP, was also the first implicated in pain and nociceptor sensitization. Indeed, intradermal injection of membrane-permeable cAMP analogs (Ferreira et al., 1990; Taiwo et al., 1989) or the adenylyl cyclase activator forskolin (Taiwo and Levine, 1991), produce robust sensitization toward physical stimuli (hyperalgesia) and sensitization of nociceptive fibers (Kress et al., 1996). Inflammatory mediators such as prostaglandins result in increased intracellular cAMP and lead to hyperalgesia, which can be blocked by the inactive cAMP analog, Rp-cAMP (Taiwo and Levine, 1991). The inflammation-induced increase in cAMP also induces cellular correlates of pain such as increased evoked transmitter release (Hingtgen et al., 1995) and modulates voltage (England et al., 1996; Gold et al., 1998) and ligand-gated ion channels important in pain (Lopshire and Nicol, 1998; Pitchford and Levine, 1991). Not only the onset but also the duration of hyperalgesia is dependent on continuously elevated cAMP levels. Thus, Rp-cAMP reduces hyperalgesia even if injected after hyperalgesia is already established (Aley and Levine, 1999; Taiwo and Levine, 1991). The intracellular cAMP

concentration in nociceptive neurons can be reduced by activation of endogenous  $\mu$ -,  $\delta$ -, and  $\kappa$ -opioid receptors, which may be, in part, responsible for the peripheral antinociceptive actions of morphine and other opioids (Aley and Levine, 1997; Collier and Roy, 1974; Ferreira and Nakamura, 1979; Stein et al., 1989). Long-term exposure to opioids results in desensitization of the opioid receptors and compensatory production of cAMP accompanied by loss of the antinociceptive effect of opioids (Nestler, 2004).

cAMP signaling is widely held to be synonymous with the activity of its binding partner, protein kinase A (PKA). Pharmacological as well as genetic inhibition of PKA, results in a reduction of inflammatory mediator-induced hyperalgesic behavior (Aley and Levine, 1999; Malmberg et al., 1997), in reduced nociceptor discharge (Cui and Nicol, 1995; Zhang et al., 2002a), as well as in attenuated stimulus-induced peptide release (Oshita et al., 2005). While the exact mechanisms of these PKA-mediated effects are not fully understood, mutation of PKA phosphorylation sites on effector ion channels such as the primary afferent nociceptor specific, tetrodotoxin-resistant sodium channel, NaV1.8 (TTX-R I<sub>Na</sub>) (Fitzgerald et al., 1999) and the ligand-gated ion channel TRPV1 (Bhave et al., 2002) results in ablation of channel modulation by PKA.

It has recently become clear that cAMP can also activate molecules other than PKA such as calcium channels (reviewed by Kaupp and Seifert, 2002) and the GDP/GTP exchange factor Epac (de Rooij et al., 1998; Kawasaki et al., 1998; see discussion below). Therefore, some of the established data on cAMP signaling pathways in nociceptor sensitization must also take these additional targets into consideration (Hucho et al., 2005). While cAMP/PKA signaling is important for inflammatory hyperalgesia, it is also clear that other second messenger pathways play critical roles in this process.

**Protein Kinase C (PKC)**

There is an extensive literature documenting a role of PKC in nociceptor activation as well as sensitization. PKC activating phorbol esters and inflammatory mediators cause long-lasting nociceptive behaviors (Souza et al., 2002) and depolarize as well as activate nociceptors (Burgess et al., 1989b; Dray et al., 1988; Rang and Ritchie, 1988).

Also, sensitization of nociceptors can be induced in a PKC dependent manner, as measured by thermal and mechanical hyperalgesia (Souza et al., 2002), mechanically induced nociceptor activity in knee joint afferents (Schepelmann et al., 1993), thermally induced secretion of neuropeptides from the peripheral terminals of afferents (Cesare et al., 1999; Kessler et al., 1999), as well as increase of TTX-R I<sub>Na</sub> in DRG neurons (Cesare and McNaughton, 1996; Gold et al., 1996). Treatment of TRPV1 expressing cells with phorbol esters lowers the heat-threshold of TRPV1 below body temperature and sensitizes TRPV1 expressing cells to stimulation by capsaicin (Crandall et al., 2002; Premkumar and Ahern,

2000). The mechanism of this effect appears to involve direct phosphorylation (Bhave et al., 2003; Mandadi et al., 2006; Numazaki et al., 2002) leading to, among other changes, PKC-dependent insertion of TRPV1 channels into the plasma membrane (Morenilla-Palao et al., 2004; Van Buren et al., 2005).

At least six PKC isoforms ( $\alpha$ ,  $\beta$ I,  $\beta$ II,  $\delta$ ,  $\epsilon$ ,  $\zeta$ ) have been detected in DRG neurons. But only PKC $\epsilon$ , a member of the calcium-independent novel PKCs, has been shown to be activated by the inflammatory mediators bradykinin, epinephrine, carrageenan, tumor necrosis factor alpha (TNF $\alpha$ ), and the protease-activated receptor (PAR2) and to mediate sensitization to mechanical and thermal stimuli (Amadesi et al., 2006; Cesare et al., 1999; Khasar et al., 1999a; Olah et al., 2002; Parada et al., 2003a). In a PKC $\epsilon$  knockout mouse, the basal threshold to mechanical as well as thermal stimulation was unchanged. In contrast, sensitization in response to inflammatory mediator treatment was much reduced (Khasar et al., 1999b). Indeed, sensitization of the nociceptor specific TTX-R I $_{Na}$  was dependent on PKC $\epsilon$  activity (Khasar et al., 1999b), and enhanced activity of another ion channel important in inflammatory pain, TRPV1, was shown to require direct phosphorylation of TRPV1 by PKC $\epsilon$  (Bhave et al., 2003; Mandadi et al., 2006; Numazaki et al., 2002).

Beyond inflammatory mediator-induced sensitization PKC $\epsilon$  is involved in various models of neuropathic pain, such as that associated with diabetes (Joseph and Levine, 2003b), chronic alcoholism (Dina et al., 2000), and cancer chemotherapy (Dina et al., 2001b; Joseph and Levine, 2003a). In addition, PKC $\epsilon$  activity widens the generally accepted dichotomy of naive and sensitized nociceptors by defining the novel "primed state" (see discussion below and Figure 3). Interestingly, PKC $\epsilon$  signaling in primary afferent nociceptors was found to depend on cytoskeleton and cell membrane microdomains (expanded upon below), exemplifying the need to investigate other cellular aspects than electrophysiological properties and kinase activation states for nociceptor sensitization.

#### **Mitogen-Activated Protein Kinases (MAPK)**

Mitogen-activated protein kinases (MAPKs) have also recently been implicated in nociceptor sensitization associated with inflammation and peripheral neuropathy. Activation of ERK1/2 by  $\beta_2$ -adrenergic agonists contributes to mechanical hyperalgesia (Aley et al., 2001). In nociceptors, ERK is also activated by NGF (Averill et al., 2001; Delcroix et al., 2003; Malik-Hall et al., 2005), capsaicin, electrical stimulation (Dai et al., 2002; Ji et al., 1999), Freund's adjuvant (Obata et al., 2003), and nerve transection (Obata et al., 2003).

MAPK p38 is activated in response to peripheral inflammation (Ji et al., 2002), and activation of TRPV1 leads to p38-dependent hyperalgesia (Mizushima et al., 2005). Inflammation, axotomy, and spinal nerve ligation similarly activate p38 in spinal cord and DRG neurons, contributing to neuropathic pain (Jin et al., 2003; Kim et al., 2002). In the spinal nerve ligation model of painful peripheral neuropathy, TNF $\alpha$  was central to p38 phosphorylation and me-

chanical hyperalgesia (Jin and Gereau, 2006; Pollock et al., 2002; Schafers et al., 2003). But also receptor activation and retrograde transport of locally produced NGF resulted in p38 activation leading to increased expression of TRPV1 (Ji et al., 2002).

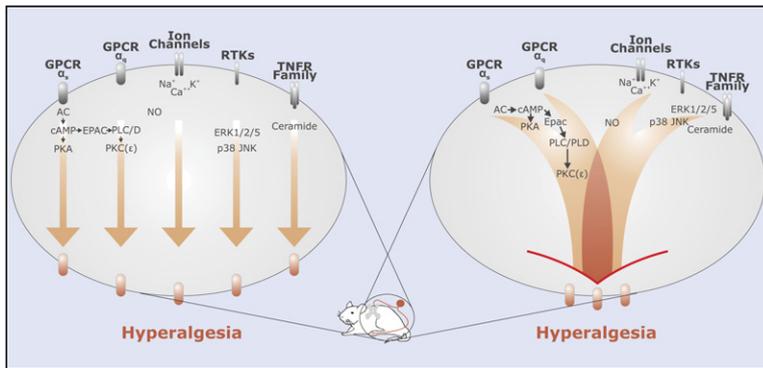
Additional members of the MAPK family, c-Jun amino-terminal kinase 1 (JNK) and ERK5, are also implicated in nociception. Nerve transection results in chronic activation of JNK in DRGs in a process that appears to require retrograde transport (Kenney and Kocsis, 1998), and TNF $\alpha$  induces activation of JNK in cultured sensory neurons (Pollock et al., 2002).

#### **Nitric Oxide (NO)**

In addition to the kinases, reviewed above, the second messenger nitric oxide (NO) contributes to induction of pain and sensitization in humans (Holthusen and Arndt, 1994), rats (Aley et al., 1998; Chen and Levine, 1999), as well as *Aplysia* (Lewin and Walters, 1999). The NO-producing enzyme, nitric oxide synthase (NOS), was localized immunohistochemically in small- and medium-diameter, nociceptive DRG neurons in rat and monkey (Zhang et al., 1993). NOS expression is prominent during development and after nerve lesion (Majewski et al., 1995; Qian et al., 1996). Its immunoreactivity is increased in DRG neurons by noxious irritants (Vizzard et al., 1995, 1996) as well as nerve injury (Choi et al., 1996; Steel et al., 1994; Zhang et al., 1993). Also, production of the downstream effector of NO, cGMP, sharply increases in response to exposure to inflammatory mediators (Burgess et al., 1989a). In turn, inhibition of NOS suppresses activity in dorsal roots originating from sciatic neuromas (Wiesenfeld-Hallin et al., 1993) and reduces thermal hyperalgesia established by chronic constriction injury or hindpaw inflammation (Moore et al., 1993; Thomas et al., 1996). Also, in cultured DRG neurons, the Prostaglandin E $_2$  (PGE $_2$ )-induced increase of TTX-R I $_{Na}$  was partially suppressed by NOS inhibitors (Aley et al., 1998).

However, unlike the other second messengers, NO has the potential to induce opposing effects. Thus, antinociceptive effects of NO are also reported (Duarte et al., 1992; Kawabata et al., 1994), potentially due to differential dosing (Kawabata et al., 1994) or depth of injection into the animals skin (Vivancos et al., 2003).

The exact function and interrelationship of the different second messengers discussed above as well as others (e.g., calcium influx, ceramide, caspases, BH4, ...) in nociceptor signaling remains to be established. Establishment of these relationships must also take into account the extent to which these signaling components are activated in the same cell and additionally whether they are activated within the same or distinct cellular compartments. Below, we expand upon these points in an effort to highlight the importance of taking a broader cell-biological approach to nociceptor signaling in order to understand both the physiological and pathophysiological consequences that can arise via the plethora of signaling cascades potentially activated in response to noxious stimuli.



**Figure 2. Parallel versus Convergent Signaling Models**

The observed multitude of signaling pathways, all of which lead to the induction of hyperalgesia, raises the question of the relationships between the signaling cascades. Two complementary models have to be considered: (1) the signaling components define parallel pathways, leading to the modification of distinct effector molecules (orange arrows, red ovals, left). Alternatively, (2) at least partial convergence occurs, leading necessarily to the formation of a nociceptive module (orange arrows, red area, right). Currently, data do not falsify any of the two. Detailed analysis of the signaling pathways, their downstream targets, as well as modulatory sites in the primary afferent nociceptive neuron is required.

### A Cell-Biological View of Nociceptor Signaling Parallel versus Convergent Signaling

The studies reviewed above have established the involvement of selected classical signaling molecules in sensitization of primary afferent nociceptors (Figure 1). But even with a fairly solid understanding of the contribution of a number of signaling components a synthesis of the pathways mediating sensitization is lacking. Having many stimuli all leading to sensitization, a priori two complementary models of signaling have to be considered: (1) the signals resulting in nociceptor sensitization are separate and involve distinct and nonoverlapping signaling components which modify separate effector molecules (Figure 2, left). (2) The signal cascades are not separate, i.e., the initiated cascades converge (partially or completely) (Figure 2, right). Both of these models bear important implications. If the signaling is parallel, the current phenotypic distinction between mechanical and thermal hyperalgesia would have to be further differentiated, as necessarily the involvement of distinct effector molecules defines also mechanistically distinct phenotypes. On the other hand, if convergence occurs, a common “nociception module” would be defined with intriguing possibilities for novel therapeutic interventions. But, illustrating the insufficiency of the current knowledge about nociceptive signaling pathways, the important and very basic question, which of these two complementary models can be ruled out, has yet to be answered.

#### Model to Be Tested on the Level of Signaling Cascades

At the level of the initial stimulus, noxious physical stimuli and changes in the immediate tissue environment act on the nociceptors. As reviewed by others (Julius and Basbaum, 2001; Scholz and Woolf, 2002), the concentration of neurotransmitters, growth factors, hormones, fatty acid derivatives, neuropeptides, cytokines, ATP, and protons are altered, resulting in what has been referred to as an inflammatory soup. As each mediator has the potential to individually modulate sensitization, convergence at the stimulus level seems unlikely. Also, evidence for convergence at the receptor level has, so far, not emerged, as a wide variety of receptors from classes as different as G protein-coupled receptors, receptor tyrosine kinases,

TNF-family receptors, ligand-gated ion channels, and cytoplasmic/nuclear steroid hormone receptors are activated (Figure 2).

But the models have to be tested on two successive levels of signal transmission: the use of distinct versus convergent neuronal subpopulations and of distinct versus convergent intracellular signaling pathways. Potentially, separate stimuli can act on separate neurons, as, e.g., the respective receptors are not expressed ubiquitously. And, indeed, nociceptive neurons of varying subtypes are differentiated by histological, electrophysiological, and molecular characteristics (Julius and Basbaum, 2001). How these neurons are interconnected, how other neurons such as the peripheral sympathetic nervous system and central interneurons modulate them, as well as to what extent they innervate the same or distinct areas in the spinal cord is beyond the scope of this review. We would only like to note here, that while the number of nociceptive neuron subtypes is increasing, most nociceptors are, nevertheless, described as polymodal, i.e., responding to multiple kinds of stimuli (Lewin and Moshourab, 2004). Therefore, the question of parallel versus convergent use of nociceptive neuron subtypes remains open. Some of these important unanswered questions can be addressed by studying the next level, the intracellular signaling. There, to validate either of the two models, one has to follow the so-far-identified signaling components all the way down to the effector molecules. Doing this for more than one cascade, it will emerge, if the cascades and/or effectors are separate or not. Keeping markers for nociceptive subtypes included, will indicate also the use of parallel versus convergent neuronal subtypes.

Investigations to test the complementary models for validity has to consider new aspects of importance much beyond the classical signaling components discussed so far, which are established by recently emerging results discussed in the following sections.

#### Variability of Receptor Signal-Cascade Coupling

In other cellular systems, it is known that stimuli which lead to similar net effects often activate the same intracellular signaling cascades (e.g., receptor tyrosine kinases in *Drosophila* eye development [Freeman, 1996]). In nociceptive neurons, do extracellular mediators, which activate the

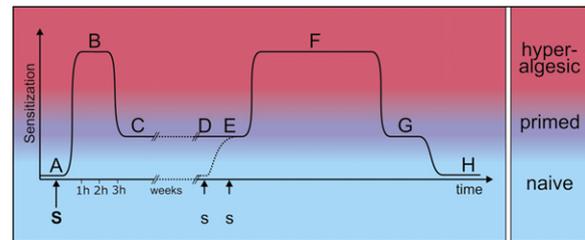
same class of receptors, share their intracellular signaling mechanism? Both epinephrine and PGE<sub>2</sub> use  $\alpha_s$ -coupled G protein receptors (GPCRs). However, while epinephrine hyperalgesia involves PKA, PKC $\epsilon$ , and ERK1/2, PGE<sub>2</sub>-induced hyperalgesia is PKA dependent only (Aley et al., 2001; Hucho et al., 2005; Khasar et al., 1999a). Therefore, stimulating a similar receptor/mediator module such as GPCRs coupled to  $\alpha_s$  does not necessarily result in the stimulation of the same downstream events. Core components such as PKC $\epsilon$  and ERK1/2 can be excluded from the sensitization process even though in both cases  $\alpha_s$  is activated.

### Selective Activation of Signaling Pathways

At the moment, it remains unclear what determines the use of the varying signaling pathways in nociceptive neurons. Increasingly, the importance of subcellular clustering and signaling “hub” formation is being investigated. One such class of compartments are lipid rafts. They are membrane patches enriched in sphingomyelin- and cholesterol-based lipids, accumulating a large number of proteins (Ostrom and Insel, 2004). Functional consequences of this subcellular localization have been assumed, as in response to activation protein relocalization toward or away from lipid rafts has been observed, and a variety of signaling events is abolished if lipid rafts are disrupted. Also, in nociception, lipid rafts are important. In behavioral experiments, interference with fibronectin-integrin binding attenuated PKC $\epsilon$ -mediated hyperalgesia. In contrast, PKA-mediated sensitization can be abolished by interference with laminin-integrin binding (Dina et al., 2005). While the latter effect is dependent on the integrity of lipid rafts, the former is not. And indeed, reflecting these *in vivo* observations, biochemically only the laminin-binding integrin  $\alpha_1$  is found to be localized to lipid rafts in DRG neurons, while the fibronectin-binding  $\alpha_5$  integrin is not (Dina et al., 2005). Other receptors involved in nociception such as the  $\beta_2$ -adrenergic receptor, the bradykinin receptor 2 (de Weerd and Leeb-Lundberg, 1997), and the neurokinin receptor 1 (Monastyrskaya et al., 2005) have been found to be localized to membrane subdomains in non-neuronal cells. Whether they compartmentalize to lipid rafts also in nociceptive neurons and whether localization of these receptors is relevant to nociceptor function awaits further investigation.

### The “Primed State”: A New Mode of Sensitization

Two functional modes have been well described in nociceptors: the naive or normal mode and the sensitized or hyperalgesic mode. Recent work on PKC-dependent hyperalgesia suggests that there is an additional complexity to consider, a mode referred to as the “primed state” (Figure 3; Aley et al., 2000). In the primed state, basal nociceptive thresholds are still normal (Parada et al., 2003a). Instead of being sensitized against physical stimuli, the nerve is sensitized against exposure to sensitizing agents. In this primed state, far lower concentrations of inflammatory mediators are sufficient to elicit enhanced and notably much prolonged hyperalgesia. In contrast to traditional sensitization induced by inflammatory mediators, which



**Figure 3. Three Modes of Sensitivity**

Three neuronal states can be differentiated in the nociceptive neuron, the naive, the just recently identified primed, and the hyperalgesic state. (A) Exposure of the naive neuron to noxious physical or inflammatory stimuli (bold capital S) results in hyperalgesia (red area, B) i.e., sensitization of the nerve toward future physical stimuli. Dependent on the animal model the hyperalgesic state lasts for some few hours. While the sensitivity to physical stimuli then returns to normal, the sensitivity to successive inflammatory stimuli (small s, E) remains increased. The primary afferent nociceptor remains in the primed state (C). This state can be established also by treatment with small concentrations of inflammatory mediators (small s), which do not result in hyperalgesia (D, dotted line). In contrast to the hyperalgesic state, the primed state is still present weeks later. The hyperalgesia induced in the primed state is markedly prolonged (F). The establishment as well as the maintenance of the primed state is PKC $\epsilon$ -dependent. If in the primed neuron PKC $\epsilon$  is blocked (G) the neuron returns to the naive state (H).

recovers within minutes to hours, neurons can remain in the primed state for several weeks. Both the establishment, which does not require prior hyperalgesia, and maintenance of the primed state is PKC $\epsilon$  dependent (Aley et al., 2000; Parada et al., 2003b). Inhibition or down-regulation of PKC $\epsilon$  in rats five days after the establishment of the primed state results in return to the initial nonprimed state (Parada et al., 2003b). Given the long-lasting effects of priming, this state could potentially underlie the chronicification of pain. Consistent with this idea, PKC $\epsilon$  also plays a central role in models of chronic pain (Dina et al., 2000, 2001b; Joseph and Levine, 2003a, 2003b).

### Plasticity of Signaling Cascades

Investigating the primed state not only shows the plasticity of nociceptive neurons beyond the naive/sensitized dichotomy (see above) but also exemplifies the plasticity in signaling pathways in response to the same stimulus. PGE<sub>2</sub>-induced hyperalgesia in naive animals is mediated by PKA. However, switching the nociceptive neuron from the naive to the primed state leads to a shift in the underlying signaling cascade. In the primed nociceptor, PGE<sub>2</sub> hyperalgesia is additionally mediated by PKC $\epsilon$  (Aley et al., 2000). While the mechanism underlying the switch is unknown, the site of this switch nevertheless has been narrowed down to a site downstream of adenylyl cyclase activity but upstream of PKA activation (Parada et al., 2005). This is surprising, as cAMP signaling is often considered to be synonymous with PKA activity. Obviously, one component of a cascade should not be taken to indicate involvement of the whole cascade, even if the components can directly interact with each as do cAMP and PKA. While switches of signaling pathways have been described in other cellular systems (e.g., the switch

from  $\alpha_s$  to  $\alpha_i$  coupling of the  $\beta_2$ -adrenergic receptor), their role in nociceptive signaling remain largely unexplored.

### **Network of Signaling Pathways**

Do the various pathways underlying nociception influence each other, and if so, to what extent? In cultured DRG neurons the induction of TTX-R  $I_{Na}$ -mediated sensitization by PGE<sub>2</sub> suggests PKC is a downstream effector of PKA (Gold et al., 1998). Nevertheless, in vivo this relationship is not observed (Taiwo and Levine, 1991). Similarly, an interrelationship between the downstream components of the  $\beta_2$ -adrenergic receptor (i.e., PKA, PKC $\epsilon$ , and ERK1/2) is not transparent; each on its own induces robust mechanical hyperalgesia (Khasar et al., 1999a, 1999b). But it remains unclear whether these pathways normally function in concert or whether they independently can result in the same behavioral output. In both behavioral and biochemical experiments, ERK1/2 appears not to depend on either PKA or PKC (Aley et al., 2001), and PKC $\epsilon$  does not depend on PKA (Hucho et al., 2005). However, potential convergence further downstream has still to be examined.

An additional aspect of the relationship of different signaling pathways has to be investigated. Alessandri-Haber et al. (2006) recently found a strong dependence for mechanical hyperalgesia on TRPV4 requiring both PKC $\epsilon$  as well as PKA activation. This result suggests that the utilization of combinations of signaling cascades can result in changes in effector molecule modulation.

### **Cytoskeleton**

Identification of the mechanism underlying crossactivation of PKC by cAMP adds a new class of proteins to nociceptive signaling, Epac, a small family of GDP/GTP exchange factors acting on small G proteins (Hucho et al., 2005). Small G proteins are involved in regulation of cytoskeletal elements. While a direct link between Epac and the cytoskeleton in nociceptor signaling has not been established, long-time drug treatment inducing stabilization as well as destabilization of microtubules produces painful neuropathies in patients and neuropathic pain like behavior in animals (Aley et al., 1996; Dina et al., 2001b). In contrast, short-term destruction of cytoskeletal components dramatically attenuates the establishment of mechanical hyperalgesia (Dina et al., 2003). Interestingly, interference with sensitization was only observed for the PKC $\epsilon$ -mediated epinephrine-induced hyperalgesia but not for PKA-dependent PGE<sub>2</sub>-induced hyperalgesia. But if PGE<sub>2</sub>-induced sensitization is shifted from PKA to PKC $\epsilon$  dependence by priming, then PGE<sub>2</sub>-induced hyperalgesia also becomes dependent on cytoskeletal elements. In vitro destruction of microtubules also abrogated the epinephrine-induced increase of TTX-R  $I_{Na}$  (Dina et al., 2003). Of note, PKC $\epsilon$  has been described to bind to and potentially be activated by the cytoskeleton (Prekeris et al., 1998). Indeed, a link between the cytoskeleton and nociceptive signaling components has been found also by a purely biochemical approach. The C terminus of TRPV1 binds to tubulin and thereby stabilizes microtubules specifically (Goswami et al., 2004). Activation of TRPV1, in contrast, resulted in

rapid disassembly of microtubules leaving the neurofilaments and the actin cytoskeleton intact (Goswami et al., 2006, 2007). The molecular mechanism of tubulin dependent regulation of TRP channels still has to be unraveled.

Combined, these data suggest a critical role for the cytoskeletal matrix in nociceptor signaling. Given the limited number of studies examining cytoskeletal interactions, it is clear that additional research in this area is needed before we can fully appreciate the impact that this structure has on pain propagation.

### **The Extracellular Matrix**

Extracellular as well as intracellular scaffolding proteins are involved in the modulation of nociception. Changing the interaction of the extracellular matrix with cell surface integrin receptors completely inhibits the establishment of mechanical hyperalgesia (Dina et al., 2004). Interestingly, this effect shows differential specificity depending on the intracellular signaling molecules involved. Interference with the binding of the nociceptive neuron to laminin abolishes PKA-mediated hyperalgesia. Conversely, blockade of fibronectin receptors by various mechanisms specifically attenuates PKC $\epsilon$ -dependent hyperalgesia.

Other extracellular matrix proteins may also have a role in nociception. Markers such as TrkA or CGRP versus Ret and Isolectin B4 (IB4) binding differentiate nociceptor subpopulations. Recently, the protein carrying the epitope for IB4 binding has been identified to be the extracellular matrix proteoglycan versican (Bogen et al., 2005). About 70% of the sensory neurons innervating the epidermis are positive for IB4. As the activation of PKC $\epsilon$  through  $\alpha_s$ , adenylyl cyclase, cAMP, Epac, and PLD/PLC was found to be specific for this subpopulation of nociceptive neurons (Hucho et al., 2005), it will be of interest to test if versican or other extracellular matrix molecules modulates the function of the adjacent neurons.

### **Organelles**

Important cellular functions, such as regulation of intracellular Ca<sup>2+</sup>, aerobic energy metabolism, generation of reactive oxygen species, and apoptosis are highly dependent on mitochondria (Wei and Lee, 2002). Given their critical role in cellular homeostasis and their high concentration in the peripheral terminals of sensory neurons (Heppelmann et al., 1994), it is perhaps not surprising that mitochondria were recently proposed to contribute to nociceptor function (Joseph and Levine, 2004). Indeed, interference with any of the five electron transport chain complexes of the mitochondrion resulted in marked attenuation of the mechanical hyperalgesia in models of AIDS therapy, cancer therapy, and diabetic neuropathy (Joseph and Levine, 2006). Interestingly, AIDS therapy-induced, mitochondria function-dependent hyperalgesia appears to be independent of PKA, PKC, ERK1/2, and NO. Conversely, hyperalgesia induced by epinephrine and PGE<sub>2</sub> is not affected by inhibitors of the electron transport chain (Joseph and Levine, 2006). Electron transport chain dependence was also found for sphingomyelinase-dependent and ceramide-induced hyperalgesia, downstream mediators of TNF $\alpha$  and NGF in the induction of

sensitization (Joseph and Levine, 2006; Zhang et al., 2002b). A signaling cascade including caspases appeared to be involved (Joseph and Levine, 2004). Others have suggested a role for mitochondria-derived reactive oxygen species (Kim et al., 2004) and mitochondrial-regulated intracellular calcium concentration (Kostyuk et al., 1999; Shishkin et al., 2002) in nociceptor function. While details have to be worked out, these data indicate the need to investigate nociceptive signaling in the context of normal cell function and the neurons attempt to maintain homeostatic balance.

### **Sex Hormones**

Another emerging and still ill defined layer of regulation is the actions of sex hormones. Both estrogen and androgen receptors are present in DRG neurons (Patrone et al., 1999). Indeed, sex-dependent differences in pain and analgesia, both in humans and in animal models, are well established (Berkley, 1997; Coyle et al., 1996; Dina et al., 2001a; Gear et al., 1996). This sexual dimorphism includes differences in pain as well as involvement of different signaling pathways mediating sensitization of the primary afferent nociceptor. In behavioral experiments, systemic estrogen has been found to play a crucial role in the establishment of sex differences (Aloisi et al., 2002; Dina et al., 2001a; Joseph et al., 2003). One aspect that has been investigated in greater detail is the role of the  $\beta_2$ -adrenergic receptor. In male rats, activation of the  $\beta_2$ -adrenergic receptor induces PKC $\epsilon$ - as well as PKA- and ERK1/2-dependent mechanical hyperalgesia. In contrast, in female rats,  $\beta_2$ -adrenergic receptor-mediated sensitization does not require PKC $\epsilon$  (Dina et al., 2001a). This phenotype is dependent on systemic estrogen levels. A similar dependency was found on the cellular level, establishing that estrogen can act on the nociceptive neuron directly (Hucho et al., 2006). Surprisingly, in cultured DRG neurons, the action of estrogen is very fast. One minute preincubation with estrogen abolishes the translocation of PKC $\epsilon$  in cultured, male-derived sensory neurons, suggesting that a transcription-independent mechanism is involved.

Fast actions of sex hormones have been shown also in other systems (Falkenstein et al., 2000). A physiological role for such fast concentration changes might exist in pain pathways. The estrogen-producing enzyme aromatase is present in the spinal dorsal horn, at sites where peripheral nociceptive neurons terminate (Evrard and Balthazart, 2003). Aromatase activity was recently found to be involved in the establishment of thermal nociceptive threshold (Evrard and Balthazart, 2003). Having the estrogen-producing enzyme and the estrogen receptors adjacent to each other opens the possibility that concentration changes occur rapidly and only on a very local level, which therefore might not be reflected in changes of the more constant plasma levels. Indeed, a local rise in estrogen by injection into the hindpaw results in complete abolition of mechanical hyperalgesia (Hucho et al., 2006). Thus, hormones could potentially have fast and local regulatory functions beyond their classical organism-wide actions on gene transcription.

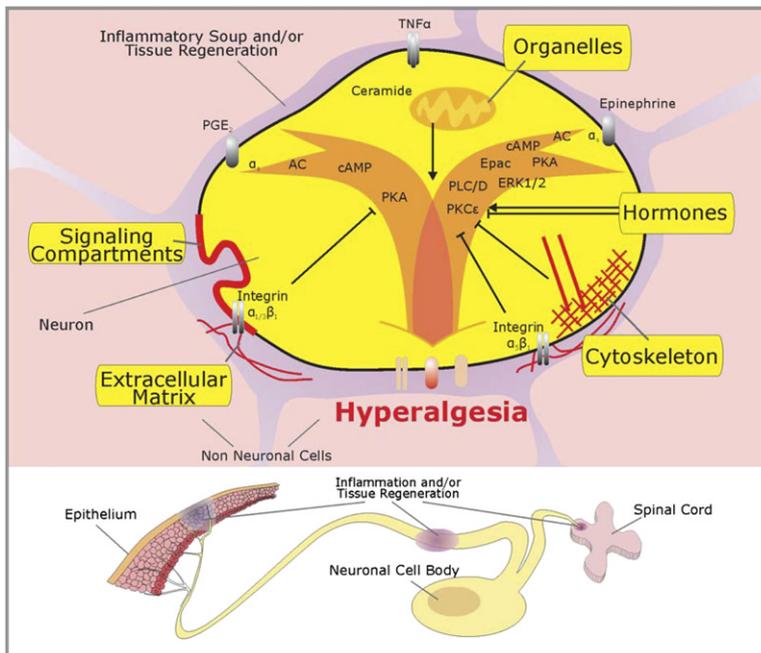
### **Nociceptive Signaling Is Context Dependent**

As evident from the above, nociceptive signaling is dependent on and can be modulated by a multitude of intracellular second messenger pathways, ancillary proteins, and organelles. Obviously, signaling is first of all dependent on the presence of all components of the respective pathway. But despite the presence of the initiating receptor in all DRG neurons, PKC $\epsilon$  translocates only in IB4-positive neurons (Hucho et al., 2005). Similarly, the NaV1.8 sodium channel behaves differently in the context of IB4-positive versus IB4-negative sensory neurons, both of which express this channel endogenously (Choi et al., 2007). What defines the mechanistic specificity of this subgroup of nociceptive neurons remains to be defined. Acknowledging the apparent importance of the cellular context, studies in cell lines have to be viewed with care (Vellani et al., 2001). For example, when comparing different cell lines transiently expressing TRPV1, phorbol ester-induced sensitization of TRPV1 did not occur in cells with low endogenous PKC $\epsilon$  concentration but could be "rescued" by the overexpression of PKC $\epsilon$  (Mandadi et al., 2006). Furthermore, even if the same inducer seemingly activates the same signaling cascade *in vitro* as *in vivo*, the receptor subtypes involved can differ significantly (e.g., bradykinin receptor 1 versus 2 and P2Y2 versus P2Y1; Moriyama et al., 2003; Sugiura et al., 2002; Tominaga et al., 2001; Vellani et al., 2004). It therefore is essential that, for the pain field in particular, data derived from expression systems must be confirmed by experiments in animals and primary cells.

Apart from differential involvement of signaling components, the importance of temporal context is also critical. For example, activation of PKC $\epsilon$  leads to mechanical hyperalgesia (Hucho et al., 2005; Khasar et al., 1999a). Surprisingly, if two hyperalgesia-inducing stimuli are applied consecutively, only the first establishes sensitization, while the second aborts it, bringing the nociceptive threshold back to baseline (Hucho et al., 2006). Apparently as in other plastic neuronal systems, stimulus context must be taken into account.

### **Nociceptor Cell Biology**

Historically, research on peripheral and especially sensory neurons led to the establishment of essential concepts in neurobiology such as the understanding of sensors of physical stimuli (e.g., rhodopsin), identification of nerve growth factors and their receptors, the concept of target-derived neurotrophins, neuronal differentiation through a process of consecutive growth factor dependence, and many others. Further investigation of the fully differentiated, very specialized, and highly plastic primary afferent nociceptor still bears the potential not only to provide a better understanding of nociceptive but also of neuronal mechanisms in general. For example, work on these neurons recently led to the realization that cAMP and PKC, critical for neuronal plasticity and so far considered to act in parallel, are in fact interconnected (Aley et al., 2000; Hucho et al., 2005; Parada et al., 2005). Additionally, the enormous recent excitement surrounding TRP channels,



**Figure 4. A Cell-Biological Perspective on Nociceptive Signaling**

The primed state can develop and be maintained without overt hyperalgesia. Thus, sensitization goes potentially beyond the acute modification of ion channels, which define the electrophysiological properties of nociceptors. The cell-biological perspective led to the realization that organelles and cytoskeleton, as well as subcellular compartmentalization, are important components underlying sensitization. It also helped focus on the influence of the surrounding environment such as hormones, the extracellular matrix, and neighboring cells. Thereby, new pathways that produce hyperalgesia (black arrow-headed lines) and more importantly of four new endogenous inhibitory mechanisms (black blunt-ended lines) could be identified. These pathways show specificity for interference with PKA versus PKC $\epsilon$ -dependent hyperalgesic signaling. Thus, the interference occurs upstream of the hypothetical nociceptive module (large arrow, dark red area). To what extent intracellular nociceptive signaling occurs at the nerve terminus in the periphery, along the nerve, in the cell body, or in the spinal cord mostly remains to be defined.

which was sparked by research on sensory neurons, has now begun to extend to other tissues and cells, where these molecules are also found to be expressed.

A surprisingly large number of important conceptual questions still remain to be solved. Given the function of nociceptive neurons in acute tissue protection, why are they the slowest conducting cells in the somatosensory system? Why, given the poor spatial localization of pain, are there so many nociceptors in peripheral tissues? Why, in contrast to all other sensory systems, is the sensation still ongoing and sometimes increasing when a constant stimulus is applied and even after the stimulus is gone? And how is long-lasting potentiation as the result of complex associative stimuli accomplished in a neuronal system without classical input stimuli and without classical input structures such as neurotransmitters and synapses?

By asking for clarification of the subcellular organization and thus the exact molecular mechanisms of nociception, the emerging cell-biological picture of nociceptor function may provide us with a fresh perspective and new avenues for research (Figure 4). Most of the known extracellular sensitizers are inflammatory mediators. As noxious stimuli are potentially tissue damaging, resulting in inflammation, could the nociceptor be first of all a surveillance tool of tissue integrity? As proof of principal supporting this notion, the importance of integrin-extracellular matrix interaction has been shown. What other cell-cell and cell-matrix component interactions might play a role? Is there, as in other systems, not only outside-in but also inside-out signaling? Furthermore, the cytoskeleton, the mitochondrion, and the submembrane organization of signaling components in signaling hot spots (e.g., lipid rafts) have been shown to play a role in nociception. What other organelles, intracel-

lular subcompartments, and second messengers are involved and in which cellular context? And, last but not least, will it be possible to define more mechanism-based phenotypes, if signaling occurs in parallel, or can one characterize a nociceptive module if convergence occurs?

For addressing these questions, methodological approaches must evolve. Such evolution is in progress. For example, sensory neurons, notoriously resistant against transfection, can now be molecularly altered through new electroporation methods as well as viral techniques. The broad surveillance of transcriptional changes in small subsets or even single cells is also increasingly feasible, as are imaging techniques enabling visualization of signaling activity. And, not last, genetic analysis of pain phenotypes in model organisms as well as in humans will provide a rich field of data that should help to complement the classical pharmacological approaches in pain research.

Still 100 years after Santiago Ramón y Cajal won the Nobel Prize for his pioneering work on sensory neurons, among others, these cells still harbor many important mysteries and potential answers about fundamental questions in cellular neurobiology. Pain research bears the burden to bridge molecular and cellular events, and a complex phenotype. In this way it is hoped that the combined efforts from different areas of neuroscience will ultimately result in overcoming current therapeutic stumbling blocks, helping to address a major unmet medical need.

#### ACKNOWLEDGMENTS

Many thanks to Professors Michael Gold and Ferdinand Hucho for critically reading versions of this manuscript and making constructive comments. Apologies for not being able, due to space constraints, to more fully reference all investigators who have contributed to the field.

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