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CALCIUM-PERMEABLE ION CHANNELS IN PAIN SIGNALING

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Laboratories of Excellence, Ion Channel Science and Therapeutics, Institut de Genomique Fonctionelle, Centre National de la Recherche Scientifique, Montpellier, France; Department of Physiology and Pharmacology, University of Calgary, Calgary, Canada; Neuroscience & Mental Health Program, Hospital for Sick Children, and Department of Physiology, University of Toronto, Toronto, Canada; and Department of Neuroscience, Carleton University, Ottawa, Canada

Bourinet E, Altier C, Hildebrand ME, Trang T, Salter MW, Zamponi GW. Calcium-Permeable Ion Channels in Pain Signaling. *Physiol Rev* 94: 81–140, 2014; doi:10.1152/physrev.00023.2013.—The detection and processing of painful stimuli in afferent sensory neurons is critically dependent on a wide range of different types of voltage- and ligand-gated ion channels, including sodium, calcium, and TRP channels, to name a few. The functions of these channels include the detection of mechanical and chemical insults, the generation of action potentials and regulation of neuronal firing patterns, the initiation of neurotransmitter release at dorsal horn synapses, and the ensuing activation of spinal cord neurons that project to pain centers in the brain. Long-term changes in ion channel expression and function are thought to contribute to chronic pain states. Many of the channels involved in the afferent pain pathway are permeable to calcium ions, suggesting a role in cell signaling beyond the mere generation of electrical activity. In this article, we provide a broad overview of different calcium-permeable ion channels in the afferent pain pathway and their role in pain pathophysiology.

I. INTRODUCTION

Acute pain is an essential sensory input that protects individuals from harmful environmental stimuli such as heat, extreme cold, chemical irritants, and mechanical tissue damage (803, 956). Nociception alerts us to internal threats such as infections, broken bones, and torn tendons. Without the ability to feel acute pain, there would be little stopping an individual from continuing to engage in harmful behavior. This is exemplified in patients with congenital insensitivity to pain (CIP), a condition that has been linked to several different genes (214) and which has been featured in popular literature, perhaps most notably in the form of villain Ronald Niedermann in Stieg Larsson's novel *The Girl Who Played with Fire*. Just like the Niedermann character, real life CIP patients are unable to feel acute pain, while maintaining a normal sensation of touch (214). Children with CIP are at risk of self-mutilation without realizing the associated tissue damage (124). An intriguing report on a group of six Pakistani children (aged 6 to 14 yr) further highlights the dangers associated with CIP. These children were completely insensitive to pain associated with physical injury (204) and, as a result, had endured a host of physical injuries such as burns and fractures. All six Pakistani children shared a null mutation in the Nav1.7 sodium channel, thus losing all ability to sense thermal and mechanical pain (204). CIP patients that survive childhood can lead productive lives, but constant vigilance is necessary for protecting against injury.

In contrast to acute, nociceptive pain, there are chronic pain conditions that do not appear to fulfill a useful physiological function, such as inflammatory and neuropathic pain (912). These painful conditions are often difficult to manage and negatively impact not only the patient's quality of life (704), but the associated reduced ability to work also results in an economic burden that is conservatively estimated to be $600 billion in the United States alone (419). It thus remains a high priority to identify novel analgesics that target chronic (undesired) pain, while sparing an individual's ability to detect noxious stimuli. Chronic pain involves changes in expression and/or function of a number of different types of ion channels in peripheral pain-sensing neurons and the central nervous system (CNS) (912) including upregulation of *N*-methyl-D-aspartate receptors (NMDARs) and voltage-gated calcium channels among many others (954). Numerous ion channels contribute to the detection and processing of pain signals. A subset of these channels are permeable to calcium ions (266), which in turn

mediate a host of cell signaling functions such as the release of neurotransmitters (644), the activation of calcium-dependent enzymes (330), and calcium-dependent changes in gene expression (249, 640, 946). Thus aberrant calcium signaling is a key step that alters activity of neural networks engaged in the modulation of pain; changes in these networks form the cellular underpinnings of chronic pain. Here, we review the role of calcium-permeable ion channels in the detection, transmission, and processing of pain signaling in the primary afferent pain pathway.

II. ANATOMY OF THE AFFERENT PAIN PATHWAY

Pain signaling is initiated by the detection of noxious stimuli through specialized primary nociceptors located in peripheral endings within the skin and in internal organs. The cell bodies of these neurons are contained within the dorsal root ganglia (DRG) or in the trigeminal ganglia (for cephalic sensory innervation), whereas their nerve terminals are localized in the superficial layers (laminae I and II) of the dorsal horn of the spinal cord (for DRG) or in the brain stem (for trigeminal ganglia) (67). In humans, 29 pairs of DRG (at each vertebral level) and 1 pair of trigeminal ganglia contain sensory neurons. These neurons have a peculiar morphology with a pseudo unipolar axonal projection arising from the cell body and bifurcating in two branches: one very long projection targets the peripheral receptive fields, and a second projection connects to the CNS in the spinal cord or brain stem (835) **(FIGURE 1)**. Therefore, the vast majority of the afferent neuron is comprised of axonal structures (more than 99% of the cell membrane; Ref. 236). The distal parts detect external stimuli that give rise to action potentials propagating along the axonal fibers up to central synapses in the CNS. The role of the sensory neuron cell body in coding sensory information is less defined (236). More globally, peripheral sensory neurons convey a diversity of sensory modalities including pain and itch, discriminative touch, and perception of body muscle tension (proprioception). The classification of sensory fiber subtypes depends both on their function [i.e., conduction velocity (CV)] and on their anatomical features (such as axonal fiber size and myelination; Refs. 274, 275, 697, 1024). Fast-conducting Aα and Aβ fibers (CV: Aα 70–120 m/s, Aβ 70–30 m/s) have large calibers (5–20 μm) and a large cell body (>40 μm). They are heavily myelinated and correspond to proprioceptive neurons (Aα) and proprioceptive and tactile neurons (Aβ). Some subclasses of Aβ fibers also support nociceptive signals (246, 281). Lightly myelinated Aδ fibers, with a slower CV (12–30 m/s) and a smaller diameter (2–5 μm) and cell body size (30–40 μm), convey tactile and nociceptive information. Finally, slow-conducting C fibers (CV 0.5–2 m/s) with unmyelinated thin axons (0.4–1.2 μm) and small soma size (<25 μm) are mainly involved in detecting pain and itch signals, but also participate in light touch related to tickling (447, 1024). Studies using skin-nerve preparations (727, 1023) or in vivo single-unit recording of peripheral nerve axons with microneurography (727, 771, 894, 1023) further highlight their diversity. To mirror these functional data gathered over the years, detailed description of the anatomy of the distal and central nerve endings required novel technical approaches. The use of genetically modified mice for specific labeling of fiber subtypes allowed researchers to address this issue, revealing that the structural organization of distal fibers in the skin (958) and the central fibers in the dorsal horn of the spinal cord (532) is extremely complex. Thus, based on these criteria, nociceptive neurons encompass a highly heterogeneous population of neurons with respect to their morphological, anatomical, and electrophysiological properties (507, 580, 726).

Over the past few years the molecular characterization of nociceptive neurons has been intensively explored, revealing that a number of factors/markers define specific subsets of neurons. For example, nociceptive neurons in the adult animal have been classified into two major categories according to their expression of neurotrophin receptors: *1*) neurons dependent on the neurotrophin nerve growth factor (NGF) that express TrkA receptors and *2*) neurons responsive to members of the glial-derived neurotrophic factor (GDNF) family that express Ret receptors (613, 614). These populations are even more diverse than just two ensembles, since they originate from distinct lineages during development with early and late TrkA or Ret neurons (50, 562). Therefore, subcategories can be separated by distinct molecular markers. Among these markers the TrkA-positive neurons express calcitonin gene-related peptide (CGRP) and substance P (SP) and are thus referred to as peptidergic nociceptors. In contrast, nonpeptidergic nociceptors are mainly comprised of Ret positive neurons (614). These two classes of neurons anatomically project to distinct laminae in the dorsal horn with TrkA fibers innervating the outermost region (lamina I) and the Ret fibers innervating distinct layers of lamina II (507, 532, 613). In addition, these two populations are not homogeneous and contain distinct cytological markers that reflect their specific roles in detecting sensory information. For example, the Ret-positive cells contain a population of neurons that express cell surface glycol conjugates that are specifically recognized by isolectin B4 (IB4) from *Griffonia simplicifolia* (613). A specific subgroup of Ret-positive and IB4-negative cells corresponds to low-threshold mechanoreceptive C fibers that specifically express tyrosine hydroxylase and the vesicular glutamate transporter VGlut3 (532, 767). A number of studies have revealed that small nociceptive IB4 positive and negative neurons play distinct roles in pain (245, 809, 964). Going forward, it will be important albeit challenging to identify nonoverlapping molecular markers of the different subpopulations of sensory neurons and link these to specific pain responses or tactile sensations.

Some of the markers associated with specific afferent fiber populations include calcium-permeable ion channels reviewed here. The cold/menthol receptor TRPM8 (689) and the heat/vanilloid receptor TRPV1 (138) segregate into nonoverlapping classes of nociceptors, although TRPV1 and TRPM8 coexpression has been observed in cultured neurons (237). The

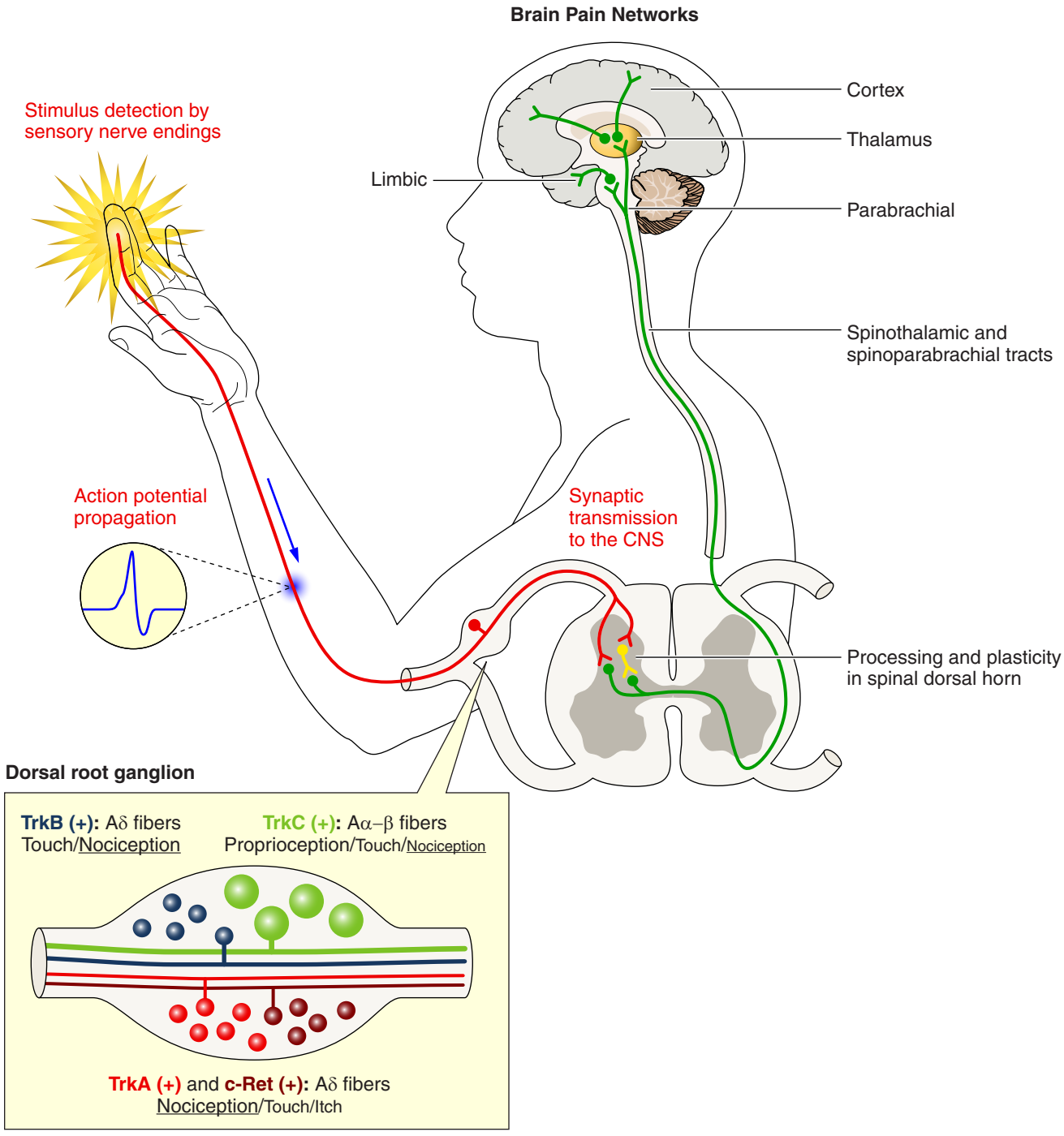


FIGURE 1. Ascending pain neuraxis. Pain sensing neurons in the peripheral nervous system have their soma located in the dorsal root ganglia (DRG). These neurons have a peripheral axon innervating the distal territories (skin, viscera, etc.) where they detect painful stimuli leading to an action potential that travels along the fibers up to the DRG and then to the first relay in the dorsal spinal cord. Sensory neurons within the DRGs are diverse and can be separated based on the expression of neurotrophin receptors. The majority are TrkA- and c-Ret-positive small-diameter sensory afferents that correspond to unmyelinated C-fibers mainly involved in nociception. TrkB- and TrkC-positive myelinated larger diameter afferents correspond to A-δ and A-α/β fibers, respectively. They convey touch and proprioception signals, although both of these subclasses contain nociceptive neurons. The sensory information is processed locally in neuronal circuitry within the dorsal horn of the spinal cord before being sent to the thalamus to convey nociceptive information. Following thalamic filtering, the information is sent to the cortical structures of the pain matrix.

mustard oil receptor TRPA1 and the purinergic receptor P2X3 are predominantly expressed in IB4-positive neurons (55, 109). Neurons that express low voltage-gated calcium channels appear to be negative for μ-opioid receptor expression (963). Altogether, this illustrates that calcium-permeable channels can be useful markers of specific primary afferent fiber types.

Multiple other signaling proteins such as Mrgpr/SNSR class G protein-coupled receptors (GPCRs) are largely expressed in a mutually exclusive fashion (553). As another molecular twist of diversity, distinct splice variants of a single gene can be specifically expressed within a subpopulation of sensory neurons. It has been demonstrated for example that for the N-type calcium channels encoded by the Cav2.2 subunit, the expression of the exon 37a variant is restricted to nociceptive neurons and acts as a molecular switch that tailors the channel toward specific roles in pain perception and modulation by GPCRs (25, 36, 82). Deciphering how these subpopulations of nociceptive neurons are molecularly specified and functionally diversified will greatly expand the understanding of pain biology, but this also represents a challenge in many laboratories working on molecular pain physiology.

The spinal cord dorsal horn is the essential CNS sensory processing hub connecting the periphery to the brain. In this nociceptive pathway, dorsal horn neurons integrate inputs from peripheral nociceptors, local interneurons, and descending projections and transmit processed signals to the brain pain network (851) **(FIGURE 1)**. Neurons in the superficial layers of the dorsal horn (laminae I and II) primarily receive nociceptive-specific inputs through high-threshold Aδ- and C-fiber primary afferents. Lamina I and II neurons display considerable heterogeneity in molecular, functional, and morphological properties and can be divided into subpopulations based on their morphological, biochemical, and electrophysiological profiles (338). Excitatory and inhibitory interneurons predominate in lamina I and II, while a subset of lamina I neurons project directly to brain pain centers which include the lateral parabrachial area, the periaquedecutal grey matter, and the thalamus. Within deeper laminae of the dorsal horn (lamina V), wide dynamic range neurons respond to both innocuous and noxious inputs and project to brain pain networks through the spinothalamic tract. Combining recently developed optogenetic approaches with spinal cord imaging and recording techniques has the potential to unlock remaining mysteries regarding how innocuous and noxious sensory information is processed within the complex synaptic circuitry of the spinal cord dorsal horn during normal and pathological pain conditions.

III. MAJOR TYPES AND KEY ANIMAL MODELS OF PAIN

As stated above, nociceptive pain is primarily a protective mechanism. Acute pain is therefore a physiological phenomenon that does not involve abnormal expression of ion channels and receptors. It has been investigated in clinical studies involving human patients, and in animal models with the use of pharmacological strategies, and gene knockout or overexpression experiments in rodents or simpler organisms, such as *Drosophila* or zebrafish (518, 519, 555, 864). Testing of acute pain responses can be achieved through application of a range of stimuli (electrical, thermal, mechanical, or chemical) that can be more or less controlled in time and intensity. Cutaneous somatic or cephalic nociception (stimulation of skin nerve endings) is by far the most widely used approach to investigate acute pain in animals, but visceral pain has also been explored through stimulation of nerve endings in hollow organs (gastrointestinal tract, bladder) (174, 325). Acute reactions due to excessive nociceptive pain can be induced by thermal and mechanical stimuli, or via chemical stimuli such as subcutaneous Formalin injection (559, 632) and by application of chemical agonists of ion channels that are involved in the detection of nociceptive signals (for example, capsaicin, mustard oil, acid). In the case of the Formalin test, the behavioral consequence of this strong tonic nociceptive stimulation is a biphasic response reflecting the initial stimulation of peripheral nociceptive sensory neurons, and a delayed second phase linked to a facilitation of dorsal horn responses (central sensitization) produced by a marked inflammatory reaction (405).

Sensitization of the nociceptive system is a hallmark of inflammatory responses. Upon tissue damage due to an injury, a burn, an infection, or a tumor, inflammatory responses ensue that alter pain responses. In the short term, sensitization may serve as a protective process aimed at preserving the injured part of the body, as an increase in pain intensity would prevent further damage of the inflamed region through overstimulation. It is important to note that excessive nociceptive stimulation itself gives rise to a phenomenon termed neurogenic inflammation. Indeed, activation of peripheral sensory terminals by local depolarization, axonal reflexes, or dorsal root reflexes, releases bioactive substances, including pro-inflammatory neuropeptides, SP, and CGRP. These molecules, in turn, act on peripheral target cells including immune cells (e.g., mast cells) and vascular cells (see Ref. 568 for review), leading to mast cell degranulation, a change in vascular permeability, and neutrophil infiltration. These neurotransmitters are located in lightly myelinated Aδ and unmyelinated C fibers that are sensitive to capsaicin and immunoreactive for the TRPV1 channel (198, 701, 822). Therefore, ablation of TRPV1-positive nociceptive fibers with systemic administration of a capsaicin analog causes SP and CGRP depletion in peripheral tissue (290), leading to immunosuppression. SP has been identified as a particularly important mediator of neurogenic inflammation, as it enhances immune cell activation and recruitment, promotes the release of proinflammatory cytokines, and induces the production of new immune cells (98). SP also stimulates the release of histamine from mast cells, further exacerbating inflammatory responses.

Pain sensitization gives rise to "allodynia" (i.e., perception of an innocuous stimulus as painful) and "hyperalgesia" (exacerbated pain in response to a noxious stimulus). Numerous factors that are released upon inflammatory reactions [such as prostaglandins, bradykinin, protons, CGRP, histamine, NGF, interleukins, tumor necrosis factor-α (TNF-α)] can participate in this sensitization, and some of these mediators trigger short-term modulation of effectors including the calcium-permeable channels reviewed here. This can occur in the form of upregulation of functional channel activity (1015), increased plasma membrane channel insertion (761), modulation of channel transcription (575), and possibly many other posttranslational mechanisms involved in promoting recycling or preventing degradation of these channels. Distinct models of inflammation are used in laboratory animals at the level of the skin [e.g., subcutaneous delivery of carrageenan or complete Freund's adjuvant (CFA) into the paw], the knee joint (CFA monoarthritis model), or the gut [such as dextran-sodium sulfate (DSS)-induced colitis]. Although many animal models have been developed, they all share a hallmark of sensitization of primary afferent neurons due to altered ion channel function (among other factors).

When the nervous system is damaged, the nociceptive circuitry can become pathological in the sense that it can abnormally and chronically produce pain in the absence of peripheral harmful stimuli. These conditions are classified as "neuropathic pain." Peripheral neuropathic pain (PNP) is the most common type of neuropathic pain afflicting ~8% of the population in Europe and North America. PNP may arise from nerve trauma, diabetes, postherpetic neuralgia, multiple sclerosis, anticancer chemotherapy, or anti-viral therapy. Chronic pain is the scourge of PNP, and it is the most challenging form of pain for clinicians to manage. Despite an increasing knowledge in pathophysiology, there remains a dearth of efficacious treatment options. Even treatments specifically dedicated to PNP, such as antidepressants or gabapentinoids, are often unsuccessful. Although pregabalin is a blockbuster drug, when given in PNP patients, the analgesic effect is weak (~1.5 to 2 on a 10-point scale). Therefore, there is clearly a need for more efficacious molecules. A range of neuropathic preclinical models has been developed in rodents (66). These include traumatic alterations of peripheral nerves such as ligatures, transection, and compression, most commonly involving the sciatic nerve (83, 227, 476, 478, 769), infraorbital nerve, and trigeminal nerve roots (913, 984). In addition, metabolic alterations (type 1 or 2 diabetes models, Refs. 203, 513) and administration of toxic compounds such as chemotherapy treatments and anti-HIV therapy are used as animal models of neuropathic pain (22, 230, 443, 444, 541, 825). In the case of the action of toxic drugs such as chemotherapy treatments, the etiology observed within rodent models is remarkably similar to what is observed in the clinic. As in inflammatory pain, neuropathic pain conditions give rise to phenomena such as allodynia and hyperalgesia, and as discussed below, calcium-permeable ion channels play important roles in this process.

IV. VOLTAGE-GATED CALCIUM CHANNELS

A. Calcium Channel Subtypes and Molecular Composition

Voltage-gated calcium channels constitute the predominant pathway for depolarization-mediated calcium entry into neurons. The calcium channel family consists of a number of different channel subtypes that can be divided broadly into two groups based on their voltage dependence of activation: low voltage activated (LVA) and high voltage activated (HVA) channels (76, 78, 666). The HVA channel family is more diverse and can be further subdivided, based on pharmacological and functional properties, into L-, N-, P-, Q-, and R-types (78, 143). Indeed, these different HVA channel subtypes can be distinguished by their sensitivities to specific antagonists: N-type channels are potently blocked by ω-conotoxins GVIA and MVIIA (671, 730), P- and Q-type channels are blocked with different affinities by the spider toxin ω-agatoxin IVA (9, 106, 608), L-type channels are sensitive to both dihydropyridine agonists and antagonists (873), and R-type channels are inhibited by the spider toxin SNX-482 (648), although SNX-482-insensitive R-type channels have also been identified in certain types of neurons (862). Different calcium channel isoforms show distinct cellular and subcellular distributions and fulfill specific functional roles. Initially, N-, P-, and Q-type calcium channels were thought to be expressed predominantly on presynaptic nerve terminals (940–942) where they support the release of neurotransmitters (945). L-type channels support excitation-contraction coupling in muscle and heart (163, 834, 1018), and in neurons they are often expressed at cell bodies (368), where they may partake in the activation of calcium-dependent enzymes and gene transcription (118, 946). However, the exact roles and distributions of each channel subtypes are neuron subtype dependent, such that most types of calcium channels are expressed at various subcellular loci. For example, both N-type and L-type channels can be expressed in dendrites (486, 1010) and do in fact support a wider range of functions. These diverse functional roles ultimately pose a challenge when designing new calcium channel therapeutics with a low risk of side effects.

At the molecular and biochemical level, T-type calcium channels are formed by a single Cavα1 subunit, a ~250-kDa protein that is comprised of four membrane domains that are connected by cytoplasmic regions and whose NH_2 and COOH termini are also cytoplasmic (142). Each membrane domain contains six membrane spanning helices (S1 though S6) that include a voltage sensor region (141) plus a re-entrant P-loop motif that lines the pore of the channel and controls ion selectivity (976). The mammalian genome encodes three distinct T-type calcium channel α1 subunits, termed Cav3.1, Cav3.2, and Cav3.3 (205, 520, 695, 696), each of which is subject to alternate splicing (149, 219, 770) and which show distinct brain tissue distributions (591). In contrast, HVA channels are heteromultimeric protein complexes that are

formed through the assembly of Cavα1, Cavβ, and Cavα2δ subunits in a 1:1:1 stoichiometry (143) **(FIGURE 2)**. In addition, calmodulin appears to be part of all HVA calcium channel complexes (607). Certain types of HVA channels such as the skeletal muscle L-type channel also contain a Cavγ subunit (41, 826). The HVA Cavα1 subunits fall into two major families (143). The Cav1 family has four members (Cav1.1 through Cav1.4), all of which encode L-type calcium channels, whereas the Cav2 family includes Cav2.1 (encoding P- and Q-types), Cav2.2 (encoding N-type), and Cav2.3 (encoding R-type). All of the known Cavα1 subunits undergo alternate splicing, in some cases giving rise to channels with dramatically different functional behavior (23, 543, 775, 830, 837). This is exemplified in Cav2.1 channels where alternate splicing in two regions of the channel appears to determine whether a channel behaves like a P- or Q-type channel (106).

In vertebrates, there are four genes that encode Cavβ subunits (121). These are (with the exception of one palmitoylated Cavβ2 splice variant) cytoplasmic proteins that associate with the Cavα1 subunit at the domain I-II linker region (706). They promote membrane expression of the channel by interfering with ER retention and ubiquitination (26, 916), in addition to possibly regulating gene transcription independently of calcium channel activity (739). There are also four different types of Cavα2δ subunits (250, 487). They are each single gene products that are posttranslationally cleaved and then relinked via a disulfide bond. The α2 portion is an extracellular protein, whereas the δ portion appears to be glycophophatidylinositol (GPI) anchored to the extracellular leaflet of the plasma membrane (221). Like the Cavβ subunit, Cavα2δ promotes membrane expression of the Cavα1 subunit, although likely through a distinct mechanism (865, 982). In addition to regulating membrane trafficking, the coexpression of ancillary calcium channels subunits can also alter the functional and pharmacological properties of the calcium channel complex, and may alter its susceptibility to second messenger regulation (287, 595, 627, 798). The Cavγ subunit is encoded by one of eight different genes and contains four transmembrane helices (264, 455). Unlike skeletal muscle L-type calcium channels, it remains unclear whether neuronal calcium channels are in fact associated with a Cavγ subunit.

Primary afferent neurons express multiple types of voltage-gated calcium channels, including P-, N-, L-, R-, and T-type Cavα1 subunits, and several of the ancillary calcium channel

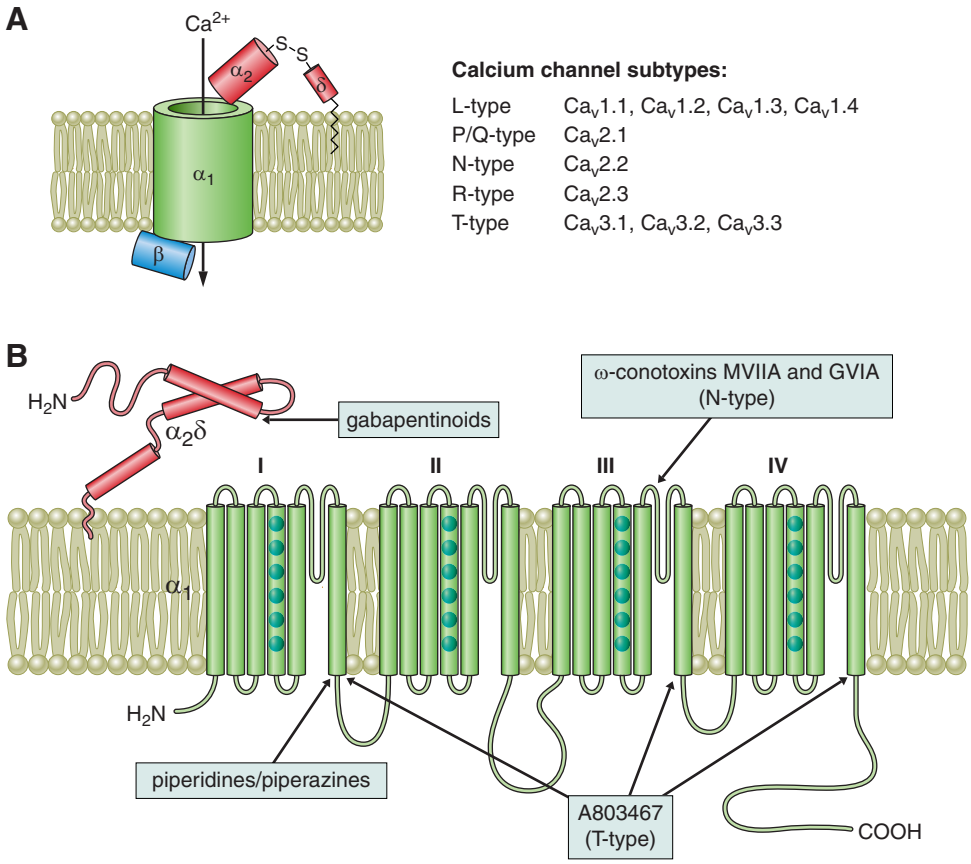


FIGURE 2. Structure and molecular assembly of voltage-gated calcium channels. *A*: high voltage activated (HVA) calcium channels are formed through the assembly of Cavα1, Cavβ, and Cavα2δ subunits. The nature of the Cavα1 subunits determines the calcium channel subtype. *B*: membrane topology of calcium channel subunits that are known targets for analgesics, and localization of key calcium channel antagonist binding sites. Gabapentinoids such as gabapentin and pregabalin bind to the Cavα2δ subunit, whereas classical calcium channel antagonists such as conotoxins (269, 289) and piperidines (1006) interact with the Cavα1 subunit.

isoforms (80, 998). Below, we will highlight the roles of these channel types in pain transmission and their usefulness as targets for analgesics.

B. Role of N-type Channels in the Afferent Pain Pathway

N-type calcium channels are almost exclusively expressed in neuronal tissue (666) and enriched at presynaptic nerve terminals where they trigger the release of neurotransmitters (940, 945) via physical association with the synaptic release machinery (1000). This also applies to terminals of primary afferent fibers whose synapses are localized in the dorsal horn of the spinal cord (346). Calcium entry into these synaptic nerve terminals releases neurotransmitters such as glutamate, CGRP, and SP (278, 569, 788). Consequently, inhibiting N-type channel activity results in reduced neurotransmission and thus analgesia (45). N-type channel knockout mice are hyposensitive to pain (365, 474, 745, 747), and there is only a relatively mild CNS phenotype in these animals that includes reduced anxiety levels and reduced alcohol withdrawal symptoms (649, 745). Altogether, these findings suggest that N-type channels are potentially viable drug targets for the development of novel analgesics.

Like many of the other calcium channel isoforms, the pore-forming Cav2.2 α1 subunit undergoes alternate splicing at a number of different loci (454, 540, 680), including exon 37 which exists as either the exon 37a or the exon 37b variant (82). Interestingly, channels including exon 37a are almost exclusively confined to small nociceptive neurons that are positive for Nav1.7 channels (82). In transient expression systems, inclusion of exon 37a results in increased whole cell current density, shifts in the half activation voltage, and altered regulation of channel activity by G proteins and tyrosine kinases (134, 577, 723). In vivo siRNA knockdown experiments indicate that basal nociception and inflammatory pain are mediated by channels containing exon 37a (25). Tactile allodynia in response to sciatic nerve ligation appears to rely on both splice variants, whereas in the same animal model, thermal hyperalgesia is again predominantly dependent on the exon 37a variant (25). Experiments with transgenic mice which express exclusively exon 37a are more sensitive to morphine-induced analgesia compared with mice expressing exon 37b (36). Altogether, these data indicate that channels containing exon 37a may be a more suitable target for analgesics compared with exon 37b containing channels. In practical terms, however, selective targeting of exon 37a channels via drugs poses a significant challenge, as exon 37a differs from exon 37b in only 14 amino acid residues within the cytoplasmic COOH-terminal tail of the channel (82). It may, however, be possible to exploit the differences in gating properties of the two channels via the use of state-dependent inhibitors, as we outline below.

C. Analgesic Effects of Direct N-type Channel Inhibitors

One of the distinguishing characteristics of N-type calcium channels is their sensitivity to ω-conotoxin GVIA, a peptide toxin isolated from the fish hunting mollusk *Conus geographus* (672). This 27-amino acid peptide has a rigid backbone structure due to the formation of three disulfide bonds, and blocks current flow by occluding the outer vestibule of the Cav2.2 pore (269, 289, 730). This blocking action is poorly reversible, but strong membrane hyperpolarizations appear to favor unblock (288, 805). When delivered intrathecally into rodents, this peptide elicits potent suppression of pain (240, 673, 766). A structurally related 25-amino acid N-type channel blocking toxin has been isolated from the *Conus magus* snail and is termed ω-conotoxin MVIIA (671). As with GVIA, this peptide causes potent analgesia when delivered intrathecally (107, 148, 766, 928). MVIIA can be synthesized in vitro and undergoes correct disulfide bond formation and folding, thus retaining blocking activity (966). This has allowed the toxin to be used as a therapeutic for treating pain in humans under the trade name Prialt, and has been approved for use in humans to treat patients with refractory cancer pain (45, 605, 796, 920). However, because this peptide does not readily cross the blood-brain barrier, it must be delivered intrathecally via implantation of a minipump (845, 899). In addition, a number of side effects of Prialt have been reported, including dizziness, blurred vision, hypotension, and memory problems (694, 725), and as a result, this drug has a narrow therapeutic window (762). This then begs the questions as to why acute delivery of a selective N-type channel inhibitor can produce adverse effects, when total knockout of the Cav2.2 protein in mice yields only a mild phenotype. While it is possible that MVIIA could have unknown off-target actions, the more likely explanation is that gene knockout may result in compensation from other synaptic calcium channel subtypes (such as P/Q-type channels as has been reported in response to chronic N-type channel block; Ref. 341), whereas acute block eliminates calcium entry without time for the establishment of compensatory mechanisms. A series of related peptides from *Conus fulman* and *Conus catus*, ω-conotoxins FVIA, CVID, CVIE, and CVIF also potently inhibit N-type calcium channels and display antinociceptive effects (8, 87, 524, 527, 625, 788). CVID has been tested in human trials and appears to have a larger therapeutic window than Prialt (764).

In addition to their restricted route of delivery, pore-blocking peptide toxins also have the disadvantage of interacting with multiple gating states of the N-type channel (288), which is perhaps not an ideal property for a drug designed to target excessive neuronal activity. Indeed, some lessons may be learned from sodium channel blocking anticonvulsants, local anesthetics, and antiarrhythmic drugs which preferentially interact with inactivated channels, thus selectively targeting hyperexcitable cells, while sparing channel activity in normally functioning tissues (377, 722, 950). It thus stands to reason that a similar property may be useful towards normalizing the

function of aberrantly active pain fibers. The drug discovery sector has been actively engaged in identifying small organic use-dependent inhibitors of N-type calcium channels. A number of compounds such as aminopiperidine-sulfonamide (772), pyrazolpiperidines (810), TROX-1 (3), and cilnidipine (491) are examples of different classes of small organic state-dependent inhibitors of N-type channels with efficacy in various pain models. A series of rationally designed piperazine derivatives with strong inhibition of inactivated calcium channels have also been reported to induce potent analgesia (678, 679, 1003). One of these compounds, Z160, is currently being evaluated in phase 2 clinical trials in the United States and shows oral bioavailability as well as strong frequency-dependent inhibition of N-type channels (678, 679, 1003).

D. Modulation of N-type Calcium Channels by G Protein-Coupled Receptors

The activity of N-type calcium channels is strongly regulated by a number of different GPCRs, including opioid, dopamine, and metabotropic glutamate receptors, among many others (77, 207, 251, 261, 262, 842). Upon receptor activation, nucleotide exchange on the Gα subunit results in a conformational change in the G$\alpha\beta\gamma$ trimer that generates two independent signaling entities: Gα-GTP and G$\beta\gamma$ (364, 374) **(FIGURE 3)**. In the case of N-type (and P/Q-type) calcium channels, the G$\beta\gamma$ subunit (372, 412) physically associates with a binding pocket formed by the domain I-II linker and NH_2-terminal regions of the α1 subunit of the channel (11, 225, 1001), and this binding interaction results in the stabilization of the closed state of the channel (688). As a result, larger depolarizations are required to open the channel, and channel activity within the physiological voltage range therefore becomes inhibited (77). Upon strong membrane depolarizations, or in response to rapid trains of action potentials, the G$\beta\gamma$ subunits transiently dissociate from the channel, resulting in a temporary disinhibition (119, 1005). At the whole cell level, G$\beta\gamma$ effects on N-type channel activity are seen as a reduction in peak current amplitude, as well as a slowing of the time course of activation, and an apparent slowing of the time course of inactivation (262).

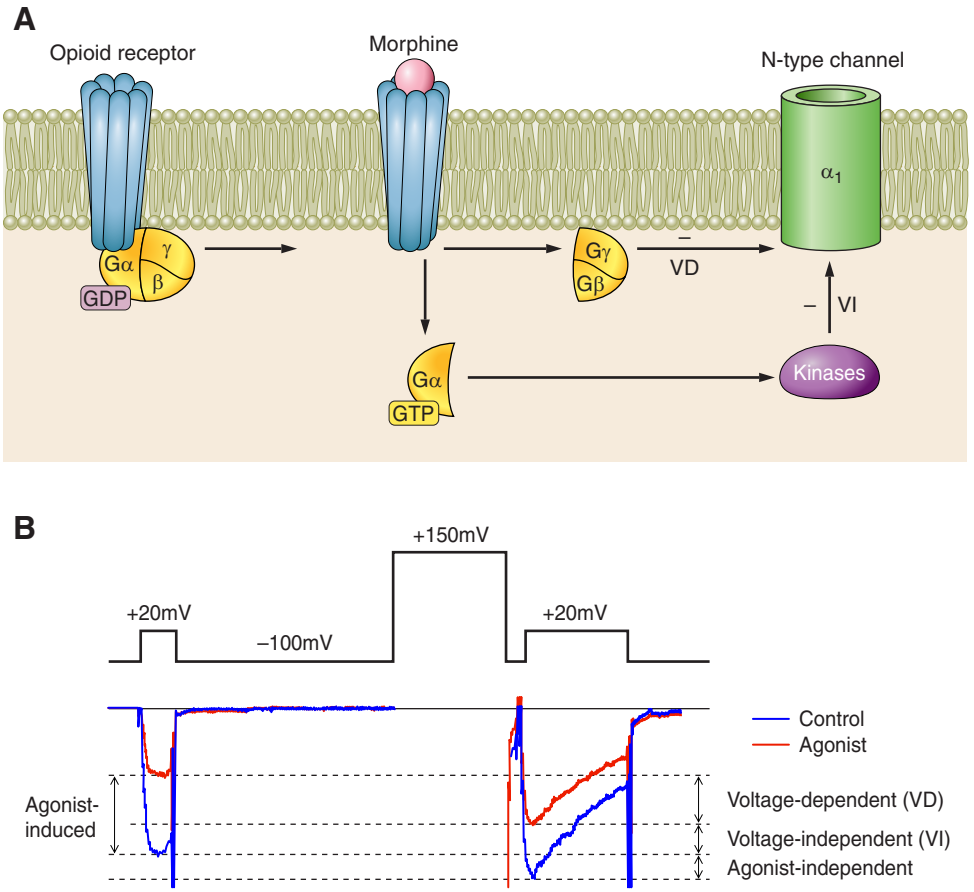


FIGURE 3. G protein inhibition of N-type calcium channels. *A*: activation of opioid receptors by its agonist morphine results in the binding of G protein $\beta\gamma$ subunits to the N-type calcium channel Cavα2.2 α1 subunit to mediate voltage-dependent (VD) inhibition, whereas second messenger pathways activated by Gα mediate voltage-independent (VI) modulation. *B*: dissection of VD and VI modulation by electrophysiology. Application of a strong depolarizing voltage pulse prior to a test depolarization (see voltage protocol at the top) results in partial relief of agonist-induced inhibition of N-type channel currents. The prepulse-sensitive inhibition corresponds to VD modulation, whereas the one that is remaining after the prepulse is VI. In the absence of agonist, the small enhancement of current activity in response to the prepulse is a reflection of agonist -independent (tonic) G protein inhibition that is observed with certain types of receptors.

At the single-channel level, these effects can be primarily attributed to an increased first latency to channel opening (688). The inhibitory effects of G$\beta\gamma$ subunits are dependent on a wide range of factors, including the calcium channel subtype (N-type channels are more potently inhibited than P/Q-types) (42, 105, 208), the Cavβ subunit isoform (254, 287), and the Gβ subunit subtype (287, 318, 436). In addition, other second messenger pathways such as protein kinase C activity modulate the functional effects of G$\beta\gamma$ (357, 367, 820, 1001). This heterogeneity in N-type channel modulation by G$\beta\gamma$ subunits needs to be considered when interpreting inhibitory actions of various GPCRs on channel activity in different cells/tissues. In addition to this voltage-dependent, direct inhibition of N-type channel activity, the activation of GPCRs can also trigger a voltage-independent modulation that is linked to Gα-mediated signaling and may involve phosphorylation of the channel by kinases such as tyrosine kinase and protein kinase A (451, 486, 758) **(FIGURE 3)**. For more details on the GPCR modulation of voltage-gated calcium channels, we refer the reader to a number of comprehensive review articles (251, 842, 1002).

In the context of pain modulation, the μ-opioid receptor (MOR) is perhaps the most extensively studied receptor subtype (947). It is the pharmacological target of morphine, one of the most potent analgesics (610). MORs act both by activating G protein-coupled inwardly rectifying potassium (GIRK) channels in spinal neurons (579), as well as by inhibiting presynaptic N-type calcium channels in primary afferent fibers (367). The resulting reduction in presynaptic calcium influx is assumed to reduce neurotransmitter release (79, 493). In addition, opioids may act directly on the neurotransmitter release machinery (367). Together with reduced excitability of postsynaptic dorsal horn neurons due to GIRK activation, this then produces analgesia. In addition, there is a major role of supraspinal MORs in the analgesic properties of morphine (241, 335, 756). While highly effective, morphine has a number of adverse effects including constipation, respiratory depression, and itch, and it can lead to tolerance, dependence, and abuse (304, 503, 599, 789). Furthermore, prolonged use of morphine results in the development of tolerance which remains a major problem in the clinic (947). Other members of the opioid receptor family [i.e., δ (DOR) and κ-opioid receptors (KOR)] also inhibit N-type calcium channels (279, 343, 611, 626, 861) and have analgesic properties in various rodent models of pain (294, 603, 667), with KOR agonists having the advantage that they may not cause respiratory depression (294, 305). Knockout mice lacking the DOR show increased mechanical allodynia, whereas selective activation of these receptors with the DOR agonist SNC80 elicits analgesia in response to both thermal and mechanical pain (323). The KOR agonist pentazocine (332) is used clinically to treat pain, whereas to our knowledge there are no DOR agonists that are approved for use as analgesics in human patients. An elegant study using transgenic mice in which DORs were fused to a GFP epitope revealed that DORs and MORs are expressed on distinct subtypes of primary afferent fibers, and regulate distinct aspects of pain signaling, with DORs regulating mechanical pain, whereas MORs appear to preferentially regulate heat pain (757). The absence of DORs in heat-sensing fibers, however, seems at odds with the analgesic effects of SNC80 in thermal pain (323), suggesting perhaps a more complex relative contribution of different opioid receptor subtypes to pain signaling. Further complicating matters is the fact that MORs, KORs, and DORs can form heterodimers (199, 685) with altered agonist responses and perhaps altered signaling to N-type channels (329, 333). Notably, the formation of such heterodimers appears to change dynamically in response to prolonged MOR receptor activation by morphine (354). Finally, a recent study has implicated specific MOR splice isoforms in analgesia and morphine-induced itch, with the MOR1D isoform triggering an itch response via interactions with the gastrin releasing peptide receptor (552). Whether the itch response is related to N-type channel modulation is not known. Nonetheless, receptor subtype and splice isoform specificity is a key consideration in the development of new opioid analgesics.

Prolonged use of opioids can result in opioid-induced hyperalgesia, i.e., a condition where chronic opioid treatment can in fact result in increased pain (523). The underlying mechanisms appear to be complex and multifactorial. It has been reported that opioid-induced hyperalgesia may involve central sensitization mediated by upregulation of NMDARs, through alterations of chloride homeostasis in spinal lamina I neurons (293) or through modulation of descending pathways (733, 900). It has also been shown that opioids such as dynorphin may have off-target effects on bradykinin receptors, which in turn activates voltage-gated calcium channels (506, 561), thereby mediating proalgesic effects.

A fourth member of the extended opioid receptor family is the nociceptin (NOP) receptor (612, 844). It is expressed in both the CNS and in afferent nerve terminals (12, 576). This receptor is insensitive to classical opioid receptor ligands (286) but is activated via its endogenous agonist orphanin-FQ (also known as nociceptin) (598). Like other members of the opioid receptor family, NOP receptor activation by orphanin-FQ mediates voltage-dependent inhibition of N-type channel activity (4, 5, 511, 621, 742, 985). When delivered intrathecally, orphanin-FQ induces analgesia (202, 215, 483), whereas pronociceptive effects have been described upon activation of NOP receptors in the brain (297, 734, 847). An endogenous biologically active antagonist of NOP receptors, nocistatin, inhibits the analgesic actions of orphanin-FQ (670), although reportedly through an NOP receptor-independent mechanism (12). Even though NOP receptors are not sensitive to morphine, there is an intriguing crosstalk between the NOP and MOR systems. Chronic administration of morphine leads to increased expression of NOP receptors (337, 885) and, conversely, mice lacking NOP receptors exhibit decreased morphine tolerance (884, 886). The molecular basis for this crosstalk is not fully understood. The NOP receptor has been

shown to form a physical signaling complex with N-type calcium channels in both DRG neurons and in transient expression systems (28, 80). This has two important implications. First, because the NOP receptor appears to show a low level of constitutive activity, the formation of N-type channels/NOP receptor complexes triggers an agonist-independent G$\beta\gamma$ modulation of channel activity which increases with increasing receptor densities (80). Second, NOP receptor interaction with the N-type channel enhances forward trafficking of the receptors to the plasma membrane, and allows for agonist-mediated internalization of channel/receptor complexes (27) (although it should be noted that a nociceptin receptor-mediated loss of surface N-type channels in DRG neurons has not been observed in a recent study; Ref. 630). The agonist-independent modulation coupled with NOP receptor-mediated forward trafficking of N-type channels could potentially result in an enhanced population of N-type channels in the plasma membrane that is under tonic G protein inhibition, and therefore insensitive to further modulation by other GPCRs such as the MOR. It is thus possible that this regulation could contribute to a form of morphine resistance under conditions where NOP receptor density is upregulated (as is known to occur in response to chronic morphine administration; Refs. 337, 885). However, this has not been demonstrated experimentally, and such a mechanism may be further confounded by the notion that MORs, NOPs, and N-type channels can form larger complexes with altered channel trafficking properties and regulation (279).

$GABA_B$ receptors are another class of GPCRs expressed in primary afferent fibers and are known to inhibit N-type calcium channels via both voltage-dependent and voltage-independent pathways (758). Intrathecal delivery of the $GABA_B$ receptor agonist baclofen induces analgesia; however, the use of systemic $GABA_B$ receptor agonists to treat pain is not possible due to CNS side effects, such as increased short-term food intake and increased seizure activity (102, 754). On the other hand, it may be possible to selectively target $GABA_B$ receptors in peripheral neurons. Indeed, Vc1.1 is an α-conotoxin that was originally thought to selectively inhibit nicotinic receptors (187, 647). However, this toxin and a related peptide, Rg1A, remarkably inhibit the activity of N-type calcium channels through $GABA_B$ receptor activation (125, 126, 206). This in turn is responsible for the analgesic action of this peptide (707). Interestingly, a cyclized version of the Vc1.1 peptide has been generated and shown to be orally effective for treating pain (132). Whether this oral route of administration may result in side effects similar to those observed with baclofen remains to be seen.

Altogether, the regulation of N-type calcium channels by GPCRs is a potent modifier of pain transmission and can be exploited pharmacologically towards the development of analgesics. The normal physiological role of these receptors may be an intrinsic mechanism to depress pain through elevation of endogenous receptor ligands such as endorphins.

E. N-type Calcium Channel Trafficking and Pain

Under conditions of chronic pain, there is an upregulation of N-type channel expression in primary afferent fibers and the dorsal horn of the spinal cord (182, 987) as well as a change in the expression of specific N-type channel splice isoforms lacking exon 18a, which corresponds to a region in the domain II-III linker of the channel (44). Furthermore, exposure of cultured DRG neurons to an inflammatory cocktail results in an increased proportion of N-type channels at the cell surface (Altier and Zamponi, unpublished observation). Real-time PCR analysis of mouse DRG neurons shows that N-type channel mRNA is unaltered in conditions of diabetic neuropathic pain (890), suggesting that chronic pain-induced changes in N-type channel membrane expression may occur at the protein, rather than the mRNA, level. This also fits with observations that N-type channels in superior cervical ganglion neurons are subject to regulation by ubiquitination and proteasomal degradation (916), and that the Cav2.2 exon37b splice variant is more susceptible to internalization in response to ubiquitination (577).

A recent study has reported an association of N-type calcium channels with the collapsin response mediator protein 2 (CRMP2) (117). This protein is involved in cell growth, but has also been linked to synaptic function. In CRMP2-overexpressing neurons, N-type calcium channel cell surface density appears to be increased, suggesting a potential role in N-type channel stability (117). Furthermore, in DRG neurons, overexpression of CRMP2 results in an increase in N-type channel-mediated secretion of CGRP, suggesting a potential role in pain signaling (171). Indeed, uncoupling of CRMP2 from N-type calcium channels using a TAT peptide based approach results in suppression of both inflammatory and neuropathic pain (116, 445, 951) and migraine pain (732). This effect likely occurs by interference with N-type calcium channel stability in the plasma membrane that is mediated by CRMP2 association with the channel, and may be regulated by SUMOylation (446). However, at this point, it is not clear if chronic pain conditions result in an increased association of N-type channels with CRMP2, and if so, what the underlying cell signaling mechanisms are. Nonetheless, interfering with N-type calcium channel trafficking may be an effective alternative means of regulating pain transmission, perhaps selectively in neurons where an aberrant upregulation of channel activity has occurred.

As noted earlier, the Cavα2δ subunit is an important accessory subunit for all HVA calcium channels, and typically promotes the trafficking of HVA α1 subunits to the plasma membrane. There is evidence that Cavα2δ1 expression is enhanced during neuropathic pain states such as those triggered by mechanical nerve injury or diabetes (100, 101, 563) and that this upregulation is linked to the development of tactile allodynia (528, 564). Along these lines, in transgenic animals overexpressing Cavα2δ1, trigeminal sensory neurons show hyperexcitability

due to an enhancement of whole cell calcium channel activity (529). Altogether, this fits with an enhancement of N-type calcium channel cell surface density as a result of increased cell surface trafficking. The Cavα2δ1 subunit is the key pharmacological target for gabapentinoids such as gabapentin and pregabalin (296), a class of drugs that is highly effective in the treatment of neuropathic pain (740). Gabapentin directly binds to the Cavα2δ1 subunit (326). A transgenic mouse carrying a point mutation (R217A) in the Cavα2δ1 subunit that abolishes pregabalin binding is insensitive to the analgesic actions of pregabalin (295). In this mouse, binding of tritiated pregabalin to brain tissue is virtually abolished, indicating that the Cavα2δ1 subunit is indeed the primary in vivo target of this drug compound. The Cavα2δ subunit has recently been shown to act as a thrombospondin receptor (276) and shown to be involved in regulation of synaptic morphology (719). Furthermore, thrombospondin 4 has been implicated in spinal sensitization during neuropathic pain (475), thus raising the question as to whether gabapentinoids act exclusively via calcium channel regulation, or perhaps via multiple cellular pathways that rely on the Cavα2δ subunit. Although acute gabapentin effects on voltage-gated calcium channels have been observed under some conditions (220, 581, 816, 883), such actions have little relevance to their clinical effect that takes days or weeks to develop (168, 773). However, upon chronic exposure to pregabalin, transiently expressed Cav2.1 calcium currents become reduced, as do native whole cell calcium currents in DRG neurons (371), indicating a mechanism involving channel trafficking. This effect is abolished upon mutating the gabapentinoid binding site on the Cavα2δ1 subunit (366). Along these lines, pregabalin inhibits the synaptic targeting of Cavα2δ (71) and abolishes the increased membrane expression of Cavα2δ in DRG neurons from rodents under neuropathic pain conditions (70). Altogether, these findings suggest that, by as yet an unknown mechanism, there is an upregulation of Cavα2δ subunits in afferent pain fibers that in turn promotes enhanced expression of synaptic N-type calcium channels, thus facilitating the transmission of pain signals. Gabapentinoids appear to interfere with Cavα2δ subunit trafficking, thus promoting normal N-type channel trafficking activity and synaptic transmission (370) to produce analgesia.

F. Role of T-type Calcium Channels in Pain Signaling

By virtue of their hyperpolarized voltage activation range and window current, T-type (Cav3) calcium channels are ideally suited to regulate neuronal excitability, as evident from their role in the development of spike and wave discharges in the epileptic brain (for review, see Refs. 473, 1004). In addition, T-type channels also support secretion from neuroendocrine cells (311, 669) and are capable of associating with the synaptic vesicle release machinery (934). Along these lines, T-type calcium channels have been implicated in synaptic release in the dorsal horn of the spinal cord (425, 852). Cav3.2 calcium channels are expressed in various subpopulations of primary afferent neurons (104, 853), altogether suggesting a role of these channels in pain processing. Consistent with this idea, systemic or intrathecal delivery of T-type calcium channel blockers such as ethosuximide and mibefradil produce analgesia in rodents (165, 248, 300, 358, 629). On the flip side, T-type calcium channel activity is increased in afferent pain fibers in a number of chronic pain conditions, such as after spinal nerve injury (996), diabetic neuropathy (131, 427), and mechanical nerve injury (426, 938). At least in the case of diabetic neuropathy, blocking T-type channel activity restores a normal pain phenotype (513, 597). In vivo silencing of Cav3.2 calcium channels (but not other T-type calcium channel isoforms) via siRNA reduces mechanical nociception, and tactile allodynia arising from nerve injury (104). This fits with observations showing that Cav3.2 channels regulate mechanosensitivity of D-hair receptors (259, 924). Furthermore, in a rodent model of colonic hypersensitivity, in vivo knockdown of Cav3.2 channels reverses pain hypersensitivity in response to colorectal distension (578). Collectively, these data indicate that T-type channel membrane expression is dynamically regulated and increased under conditions of chronic pain, and that counteracting this aberrant upregulation may constitute an effective means of mediating analgesia.

It is interesting to note that mice lacking Cav3.2 show hyposensitivity to Formalin-induced but not neuropathic pain (173). They do, however, exhibit significant developmental abnormalities such as deformed trachea (154). While this could potentially limit clinical applications, it may be possible to exploit state dependence of drug action as a means to preferentially inhibit T-type calcium channels in highly active pain fibers, akin to our discussion on N-type channel blockers. Indeed, new generation state-dependent blockers such as TTA-P2 and TTA-A2, which appear to interact preferentially with inactivated T-type calcium channels, both elicit analgesia in rodent models of pain (172, 303). Z123212, a mixed blocker of voltage-dependent sodium channels and T-type calcium channels, induces analgesia by selectively targeting the slow inactivated state of these channels (376). In this context it is interesting to note that the local anesthetic binding domain of voltage-gated sodium channels is partially conserved in T-type calcium channels (95). Finally, Z944, another state-dependent T-type channel inhibitor, is currently in phase I clinical trials for pain.

A number of questions concerning the role of T-type channels in pain remain unresolved. First, it is unclear precisely how T-type channels contribute to pain signaling. Possibilities include the following: *1*) a lowering of the firing threshold for afferent pain fibers, *2*) a direct contribution to neurotransmitter release at primary afferent synapses (852), *3*) a direct function of T-type channels as mechanosensors, *4*) activation of pathways such as ERK which in turn is linked to increased pain (160), and *5*) perhaps via interactions with other types of ion channels such as voltage- and calcium-activated potassium channels as described for different types of CNS neurons (34,

271, 882). Second, the mechanism by which T-type calcium channel activity is enhanced in chronic pain conditions remains to be determined. Posttranslational modifications such glycosylation, phosphorylation (406, 407, 674, 933), redox modulation (687), or potentially ubiquitination could potentially contribute to enhanced T-type channel activity/density, as could interactions with other regulatory proteins such as Kelch-like 1 protein as described for cerebellar Purkinje neurons (43). This could potentially include the Cavα2δ subunit, which has been shown to increase T-type channel amplitude in heterologous expression systems (258). Finally, it is possible that T-type channel expression is aberrantly regulated at the transcriptional level via regulatory elements such as early growth response 1 and repressor element protein silencing transcription factor (897) or TrkB receptors (375).

G. Direct Inhibition of T-type Channels by GPCR Ligands

T-type calcium channels are regulated through a number of different second messenger pathways in response to activation of various GPCRs (400, 407). It is well established that activation of some GPCRs such as opioid or cannabinoid receptors induces antinociception (829), whereas others such as bradykinin receptors (127) or CCR2 receptors (2, 808) are pronociceptive. Some GPCR agonists have been shown to directly regulate T-type channel activity, rather than acting via G protein signaling. For example, the endocannabinoid anandamide potently blocks T-type channels (150). Naturally occurring anandamide derivatives also inhibit T-type channels, and by doing so, produce analgesia (57) in normal mice, but not in Cav3.2 channel knockout mice. Along these lines, mixed T-type channel/cannabinoid receptor ligands have been shown to be efficacious in inflammatory pain (990). The CCR2 receptor agonist monocyte chemoattractant protein-1 (MCP-1) also directly and selectively inhibits Cav3.2 channels (989). T-type channel inhibition occurs at nanomolar concentrations of this ligand and is partial with a plateau of ~50% inhibition of current activity. MCP-1 activation of CCR2 receptors is proalgesic (606), whereas CCR2 receptor antagonists elicit analgesia (808). Some of these CCR2 receptor antagonists also block T-type channels (989), and it is thus possible that such a mixed Cav3.2 channel/CCR2 receptor antagonist may have synergistic effects in treating pain.

H. R-, P/Q-, and L-type Channels and Afferent Pain Signaling

Among all HVA calcium channel subtypes, R-type calcium channels are most similar to T-type calcium channels at the functional level, including a hyperpolarized activation and inactivation range, and rapid inactivation kinetics (791). Like T-type channels, R-type calcium channels have been linked to the regulation of neuronal excitability in a number of neuronal subtypes including DRG neurons (544, 683, 999). R-type channels have also been reported to contribute to neurotransmitter release at certain synapses (135, 322, 452, 634, 638, 960). Given that R-type channels are expressed in DRG neurons (282, 283), it stands to reason that R-type channels could be involved in pain signaling. Several lines of experiments implicate R-type calcium channels in pain transmission. Mice lacking Cav2.3 show hyposensitivity to inflammatory pain (746) through alterations in both ascending and descending pathways (747). Like N-type channels, R-type channels are upregulated during spinal nerve ligation (979), whereas intrathecal delivery of the R-type channel blocker SNX-482 (a peptide isolated from the venom of the Tarantula *Hysterocrates gigas*) elicits analgesia in models of neuropathic pain (584). Along these lines, the mixed R-type and P/Q-type channel blocker TX3.3 (isolated from the venom of an armed Brazilian spider) produces antinociception in conditions of neuropathic pain. Altogether, these findings support a role of R-type calcium channels in the development of neuropathic pain and implicate Cav2.3 as a potential target for analgesics.

Although the role of P/Q-type calcium channels in migraine is well established (700), the participation of these channels in afferent pain signaling is much less clear. Mice lacking Cav2.1 display hyposensitivity to inflammatory and neuropathic pain, but curiously, increased acute thermal nociception (when tested at young ages to minimize knockout-induced motor deficits) (565). Along these lines, the rolling Nagoya mutant mouse that carries a loss of function mutation in Cav2.1 shows a reduced inflammatory pain phenotype (307). It has also been suggested that gabapentin may decrease P/Q-type calcium channel activity in dorsal horn synapses, potentially contributing to the analgesic properties of this compound (75). Finally, topical application of the P-type blocker ω-AGA-IVA appears to inhibit inflammatory pain processing in neurons innervating the knee joint (643). Altogether, although there is evidence indicating that P/Q-type channels may contribute to nociceptive signaling in the afferent pain pathway, but in a much more limited role compared with N- and T-type channels.

There is only scant evidence of a role of L-type calcium channels in the afferent pain pathway. Mice lacking Cav1.3 channels display a normal pain phenotype (186). In contrast, an upregulation of Cav1.2 channels in spinal cord neurons in chronic pain conditions has been reported (285). Notably, this upregulation was shown to involve a change in translational regulation by a specific species of microRNA (mir-103) that normally downregulates Cav1.2 expression. When mir-103 was knocked down in rats, the rats developed pain hypersensitivity, consistent with an involvement of Cav1.2 channels in pain transmission. Other roles of Cav1.2 have been described at the central level in the anterior cingulate cortex, where region specific knockout of this channel not only altered fear learning, but also reduced pain responses (435) and modulated the effects of morphine. Direct pharmacological block of spinal L-type channels has been shown to interfere with mor-

phine hyperalgesia and morphine tolerance (247, 601, 1007), and conversely, chronic treatment of rodents with morphine elevates L-type calcium channel expression levels in the spinal cord (89, 904). Collectively, these studies suggest a postsynaptic role of L-type calcium channels in afferent pain signaling, although it is not clear to what extent these channels can be targeted therapeutically.

In summary, multiple types of voltage-gated calcium channels are involved in primary afferent pain signaling. Among the calcium channel family, the N- and T-type calcium channels appear to have the most critical role, and as a result are the two calcium channel subtypes that are most vigorously pursued as therapeutic targets.

V. NMDA RECEPTORS

A. NMDA Receptor Subtypes, Molecular Composition, and Function

The glutamate receptor family includes three pharmacologically and genetically distinct ionotropic receptor types, named based on their selective pharmacological ligands: *N*-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid receptors (AMPARs), and kainate receptors. Compared with AMPARs and kainate receptors, NMDARs have unique functional properties that include high calcium permeability, blockade by extracellular magnesium ions at physiological resting membrane potential, slow activation (~10 ms) and deactivation (>100 ms) kinetics, and a high degree of allosteric modulation by endogenous molecules. NMDARs function as integrators of synaptic inputs and intra- as well as intercellular signaling. Thereby, NMDARs are critical in physiological mechanisms of synaptic plasticity underlying learning and memory (193) and in pathological mechanisms of neuronal hyperexcitability underlying a number of neurological disorders (450). NMDARs are well-recognized to play essential roles in pain signaling and are the focus here. Other calcium-permeable glutamate receptors, i.e., AMPARs that lack edited GluA2, may contribute to pain signaling (312, 348, 793, 857, 906, 910) and have been reviewed in detail elsewhere (839).

The NMDAR is a tetrameric assembly that forms the core of a computational complex consisting of many different proteins, including scaffolding proteins, peptide regulators of NMDAR function, and signaling proteins (270) **(FIGURE 4)**. At a molecular level, seven homologous genes code for NMDAR subunits and are categorized into three major classes: GluN1/NR1 (*Grin1*), GluN2/NR2 (*Grin2A*, *Grin2B*, *Grin2C*, and *Grin2D*), and GluN3/NR3 (*Grin3A* and *Grin3B*) (180, 216, 420, 505, 619, 624, 811). As for all ionotropic glutamate receptors, an individual NMDAR subunit is composed of four domains, including an NH_2-terminal domain and an agonist-binding domain in the extracellular region, a pore-forming transmembrane domain consisting of three transmembrane (M1, M3, and M4) segments and a short re-entrant pore loop (M2), and an intracellular COOH-terminal domain. The variability in length and sequence of the NMDAR COOH terminus generates much of the diversity in function and modulation across NMDAR subtypes (570, 571; for reviews, see Refs. 681, 871). The pore of the NMDAR is permeable to monovalent cations such as sodium and potassium and divalent cations including calcium **(FIGURE 4*A*)**. Activation of NMDARs is not voltage dependent and only requires the binding of coagonists glycine and L-glutamate. However, at physiological resting membrane potential, the pore is largely blocked by extracellular magnesium ions, and this blockade is relieved by membrane depolarization to greatly increase inward current through the receptor (586). Regulators that are physically incorporated into the macromolecular NMDAR membrane complex (270) also enhance NMDAR function, through mechanisms that include phosphorylation and membrane trafficking. Under basal physiological conditions in the CNS, including the afferent nociception pathway, the naive state of NMDARs is the nonpotentiated state (399, 691) **(FIGURE 4*B*)**. The net inward flow of sodium and calcium ions through NMDARs modulates synaptic plasticity and integration by depolarizing the synaptic membrane to facilitate excitatory postsynaptic potentials (EPSPs) and by causing an increase in intracellular calcium concentration **(FIGURE 4*C*)**. Although these two distinct events are often not experimentally separated, it is the NMDAR-mediated rise in intracellular calcium beyond threshold levels that activates multiple downstream effectors through signaling molecules associated with the NMDAR complex (189). In addition to increasing intracellular calcium, occupancy of the glutamate or glycine sites on the receptor may signal independently of ion flux (635, 660, 661), a concept that is only beginning to be explored.

A functional, pore-forming NMDAR complex is composed of two glycine-binding GluN1 subunits and two L-glutamate binding GluN2 or GluN3 subunits (310). The GluN1 subtype is essential for NMDAR function and is expressed in the majority of CNS neurons, while differential expression of GluN2 subtype variants account for differences in the functional properties of native NMDAR currents throughout the CNS (618). For example, GluN2A- and GluN2B-containing NMDARs have larger single-channel conductances and higher sensitivity to magnesium blockade than GluN2C- and GluN2D-containing NMDARs, while agonist-induced deactivation rates differ across GluN2 variants according to the following order: GluN2A > GluN2B = GluN2C > GluN2D (618, 715, 804). The primary function of GluN3 variants is to negatively regulate GluN1/GluN2-mediated NMDAR currents during development and in the mature CNS (180, 216, 811). In brain regions including the hippocampus and cortex, GluN2B is the predominant GluN2 variant expressed during the first week of postnatal development, followed by a robust increase in the functional expression of GluN2A (618, 718, 776, 949) and a concomitant decrease in functional expression

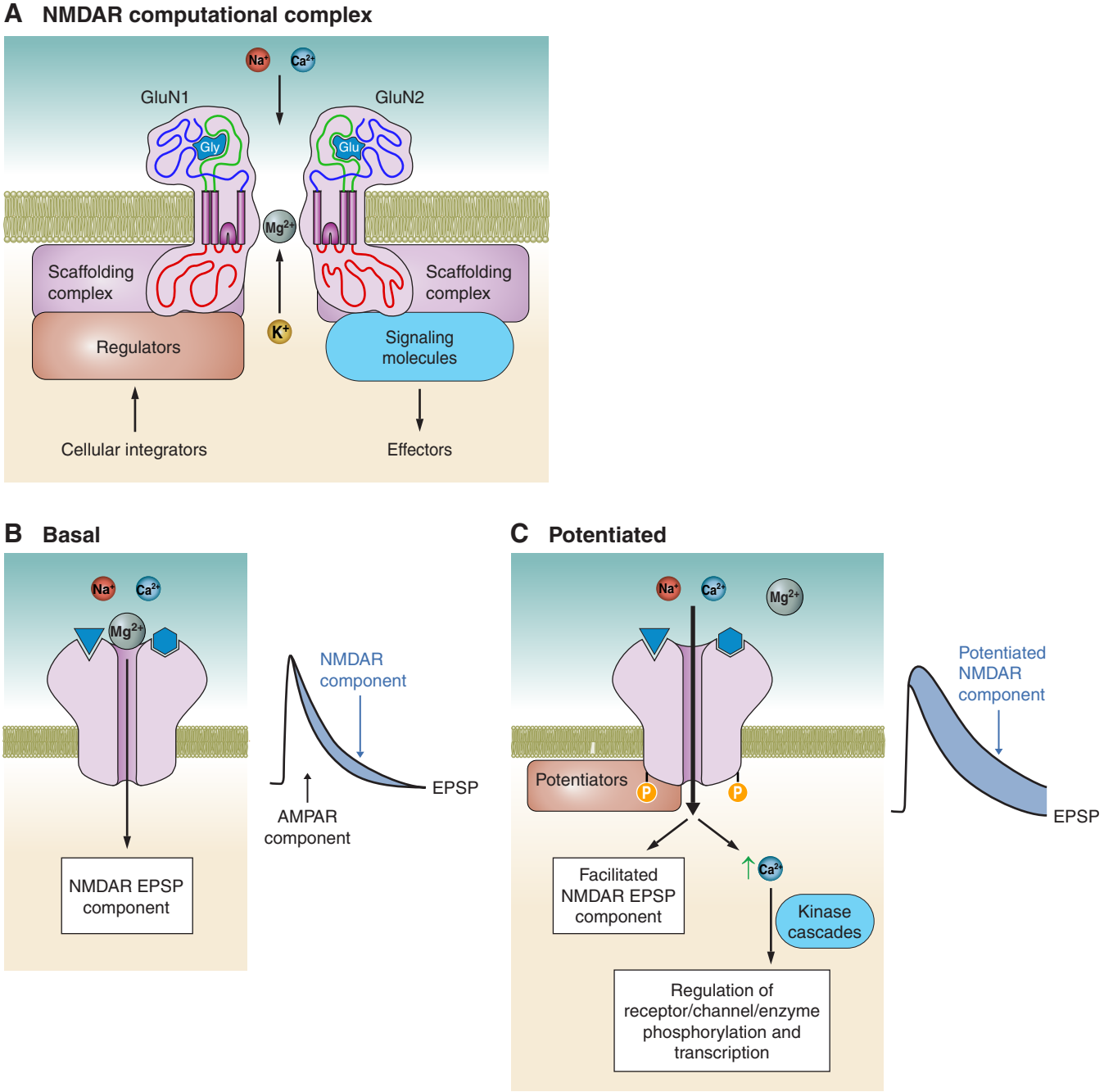


FIGURE 4. The NMDA receptor is an ionotropic glutamate receptor at the core of a synaptic computational membrane complex. *A*: functional NMDARs typically contain two GluN1 and two GluN2 genetically encoded subunits, which have conserved extracellular, transmembrane, and intracellular domains. In the postsynaptic membrane, NMDARs form the core of a macromolecular complex that includes regulators, scaffolding proteins, and signaling molecules. Cellular integrators can modulate NMDAR function by activating associated regulators, while NMDAR activity can modulate effector proteins through the activation of associated signaling molecules. *B*: under basal physiological conditions, the NMDAR is in a nonpotentiated state, with attenuation of inward sodium and calcium currents by extracellular magnesium ions. However, NMDAR activity can contribute to the slow component of EPSPs during basal synaptic transmission. *C*: NMDAR currents are potentiated through relief of extracellular magnesium blockade by membrane depolarization as well as through phosphorylation by activated membrane-associated regulators. Potentiated NMDAR activity results in both a facilitation of EPSPs as well as an increase in synaptic intracellular calcium concentration, leading to activation of associated signaling molecules, kinase cascades, and the modulation of target effector proteins.

of GluN2B (737) during subsequent postnatal development. GluN2C-containing NMDAR currents are primarily restricted to expression in the cerebellum and barrel and auditory cortex (93, 139, 265, 284, 801), while GluN2D-containing NMDAR currents are prevalent during early postnatal development in CNS regions including the substantia nigra, cerebellum, and spinal cord (120, 440, 615, 616).

In the brain, synaptic NMDAR currents are predominantly mediated by GluN2A while extrasynaptic NMDAR currents are dominated by GluN2B (363, 485, 682, 743). However, similar to voltage-gated calcium channels, the exact roles and distributions of NMDAR subunits vary, such that all GluN2 variants have been shown to contribute to synaptic NMDAR currents in specific neuronal populations at specific points in development (120, 139, 609). In contrast to voltage-gated calcium channels, alternative splicing of NMDAR subunits is much less extensive. Three independent alternative splice sites on GluN1 have been identified, creating eight distinct developmentally and regionally expressed GluN1 isoforms (517) (for review, see Ref. 681) that generate further diversity in NMDAR function and modulation.

Pharmacological probes and knockout of specific NMDAR subunits have been used to characterize the properties and functions of native NMDAR currents. To complement existing GluN2B-specific antagonists (i.e., ifenprodil and analogs; Refs. 299, 948) and the GluN2A-specific antagonist Zn^{2+} (158, 939), selective pharmacological agonists and antagonists of both GluN2A (i.e., TCN-201, TCN-213) (90, 361) and GluN2C/GluN2D (i.e., DQP-1105, CIQ) (7, 628) have recently been identified. These newer agents will help further characterize the specific functional roles of GluN2 variants and could lead to the development of novel classes of therapeutics. Along with GluN2 variant-specific antagonists, several classes of NMDAR antagonists have been developed for basic research and/or therapeutic purposes, including competitive L-glutamate binding site antagonists [i.e., D-APV, (*R*)-CPP, NVP-AAM077], competitive glycine binding site antagonists [i.e., (*R*)-HA-966, ACPC, L-683,344], noncompetitive channel blockers (i.e., memantine, ketamine, dextromethorphan, MK-801), NH_2-terminal domain binding modulators (i.e., ifenprodil, Ro25-6981, CP-101,606), negative allosteric modulators (i.e., QNZ46, TCN-201, DQP-1105), and positive allosteric modulators (i.e., spermine, UBP551, and CIQ) (for reviews, see Refs. 617, 684).

B. Expression and Function of NMDA Receptors in the Afferent Nociception Pathway

1. Primary afferent neurons

NMDARs are expressed within the central and peripheral endings of nociceptors, as well as in the soma of these cells within the DRG. GluN1 and GluN2 (especially GluN2B) mRNA and protein are localized in nociceptive DRG neurons (582, 755) and in primary afferent fibers, including their presynaptic terminals (547, 566). Whether functional NMDARs are present in DRG neurons is somewhat controversial. No NMDA-induced currents were found in the majority of acutely dissociated DRG neurons (401), while other studies have demonstrated NMDA-induced currents in cultured DRG neurons of adult rats (531, 554). The hallmark biophysical and pharmacological properties of these NMDA-evoked currents are consistent with GluN2B-containing NMDARs. The ability of DRG neurons to develop functional processes (potentially enriched with NMDARs) in culture could possibly account for differences in functional NMDAR expression between acutely dissociated and cultured DRG neurons.

Within the central terminals of primary afferents, functional presynaptic GluN2B-containing NMDARs induce SP release through a tyrosine kinase-dependent pathway (159, 546). Presynaptic NMDARs also inhibit glutamate release from terminals in the dorsal horn of infant (P6 to P12) rats (60), while having no effect on glutamate release in sham-operated adult rats (974). Electron microscopy studies in naive adult rats have demonstrated relatively sparse staining of GluN1 in presynaptic primary afferent terminals of the superficial dorsal horn (laminae I and II) (556), while GluN1 is found on presynaptic terminals of GABAergic interneurons in the superficial dorsal horn (557). Following peripheral nerve injury (974) or chronic morphine exposure (1008, 1019), presynaptic NMDAR expression is upregulated, leading to an increase in the frequency of spontaneously occurring excitatory postsynaptic currents (EPSCs). An increase in NMDAR expression has also been demonstrated in DRG neurons (530) and unmyelinated primary afferents (255) following inflammation. There is evidence that NMDARs in the periphery contribute to inflammatory pain hypersensitivity, particularly in the orofacial region (252, 422). Centrally, the majority of studies investigating a role for "presynaptic" NMDARs at primary afferent-dorsal horn neuron synapses test for effects of NMDAR agonists and antagonists on *1*) the frequency of spontaneous EPSCs, *2*) neurotransmitter or neuropeptide release, and *3*) primary afferent responses or other readouts of presynaptic activity. These approaches do not test or account for pharmacological effects on NMDARs in dorsal horn spinal cord neurons that could potentially cause presynaptic changes through retrograde signaling (267) or polysynaptic circuits (851).

2. Superficial dorsal horn neurons

NMDARs are robustly expressed in the spinal cord superficial dorsal horn. In situ hybridization studies consistently demonstrate high expression of GluN1 subunit mRNA in the superficial dorsal horn laminae (I and II) (777, 799, 854, 930). However, the relative expression of specific GluN2 variant mRNAs differs between studies. Immunohistochemical studies reveal dense staining for GluN1 and GluN2B in laminae I and II of adult rats, with moderate staining for GluN2D and minimal immunoreactivity for GluN2A and GluN2C (6, 404,

997). At a subcellular level, antigen-unmasking immunohistochemistry demonstrates GluN1, GluN2A, and GluN2B localization at synaptic sites in the dorsal horn, with GluN2B proteins being concentrated in laminae I and II and GluN2A being concentrated in laminae III and IV (637).

At a functional level, neurons in laminae I and II show robust NMDAR-mediated responses in vitro and in vivo to either primary afferent stimulation or local administration of exogenous agonists (46, 59, 615, 721, 779, 818, 856, 988). Stimulus-evoked NMDAR EPSCs in superficial dorsal horn neurons of acute spinal cord slices exhibit hallmark biophysical properties, including slow activation and deactivation kinetics, APV sensitivity, and blockade by extracellular magnesium ions at negative membrane potentials (46, 59). In lamina I neurons, the differential sensitivity to blockade by magnesium of different GluN2 variants has been used to infer that both high magnesium-sensitive (GluN2A/B) and low magnesium-sensitive (GluN2C/D) GluN2 variants are functionally expressed (856). In lamina II neurons, a combination of single-channel and whole cell recordings have been used to suggest that functional GluN2B-containing and GluN2D-containing NMDARs are located extrasynaptically (615, 779). Which GluN2 variants mediate the postsynaptic responses at primary afferent synapses onto lamina I or lamina II neurons is an open question. Primary afferent stimulation evokes direct, monosynaptic as well as indirect, polysynaptic responses which overlap temporally with the prolonged time course of NMDAR deactivation. To date, monosynaptic versus polysynaptic contributions of GluN2 variants have not been separated experimentally.

C. NMDA Receptors and Spinal Cord Neuronal Hyperexcitability

Chronic pain states involve enhanced responses in the nociceptive relay/processing circuitry of the spinal cord dorsal horn, and this phenomenon is referred to as central sensitization. Central sensitization is mechanistically and qualitatively distinct from peripheral sensitization, which includes a reduction in threshold and increase in responsiveness of peripheral nociceptors when their terminals are exposed to inflammatory mediators or damaged tissue. Peripheral sensitization is restricted to the site of injury and causes primary hyperalgesia when peripheral pathology is present. Central sensitization mediates allodynia, primary hyperalgesia, and secondary hyperalgesia and can be maintained long after the initiating cause has resolved and even when no peripheral pathology is present. The changes associated with central sensitization underlie a functional reorganization of the somatosensory system so that the low-threshold mechanosensory system feeds into the normally high-threshold nociception system, leading to pain hypersensitivity (for review, see Ref. 514).

The initial activity-dependent increases in synaptic responses that trigger central sensitization include a process known as windup. Windup is characterized by a successive increase in the output of a dorsal horn neuron produced by afferent stimuli during a repetitive train of inputs **(FIGURE 5*A*)**. Although the repetitive stimuli that induce windup can also lead to central sensitization, the two phenomena are distinct, as windup itself is rapidly reversed during the time period in which central sensitization manifests (for review, see Ref. 957). Clinically, windup is a form of physiological pain amplification, measured as enhanced pain responses during repetitive noxious stimuli (for review, see Ref. 38). The cumulative depolarization produced during windup leads to a relief of NMDAR blockade by magnesium and a subsequent feed-forward amplification of synaptic membrane depolarization (222, 242, 846). Accordingly, NMDAR antagonists abolish windup in placebo-controlled, blinded clinical studies. Administration of dextromethorphan or ketamine in healthy human subjects attenuates the facilitation of behavioral responses induced by repeated noxious stimuli while not affecting pain responses to a single stimulus (39, 708).

Central sensitization refers to a phenomenon defined by activity-dependent, persistent changes in dorsal horn neuronal excitability (955), rather than a singular neuronal process or mechanism. The sequential processes that underlie facilitation of dorsal horn neurons include *1*) cumulative depolarization of superficial dorsal horn neurons; *2*) relief of magnesium blockade of NMDARs, causing an increase in intracellular calcium (560) and synaptic depolarization **(FIGURE 4*C*)**; *3*) feed-forward depolarization and calcium influx mediated by increased NMDAR currents and voltage-gated calcium-permeable channel activity; and *4*) elevation of intracellular calcium beyond a threshold required to activate multiple intracellular signaling pathways, leading to sustained increases in synaptic transmission and neuronal excitability. Mechanisms that underlie the initial cumulative depolarization include repetitive inputs causing temporal summation through ligand-gated ion channel activity, activation of receptor tyrosine kinases by growth factors such as brain-derived neurotrophic factor (BDNF) to cause disinhibition, and activation of GPCRs by neurotransmitters and neuromodulators to inhibit voltage-gated potassium channels and facilitate nonselective cation channels (for review, see Ref. 502).

In addition to the role of NMDARs as a trigger in dorsal horn neuron sensitization, NMDARs also act as effectors in dorsal horn neuronal hyperexcitability **(FIGURE 5*B*)**. The NMDAR-mediated rise in intracellular calcium in dorsal horn neurons causes an activation of kinases including protein kinase A (PKA), protein kinase C (PKC), and extracellular signal-regulated kinase (ERK) (461). These kinase cascades lead to a phosphorylation-mediated increase in the activity of excitatory NMDARs, AMPARs, and voltage-gated calcium channels as well as an inhibition of voltage-gated potassium channels (357, 396, 521). The phosphorylation of NMDARs results in feed-forward potentiation of NMDAR function by increasing NMDAR channel open time and probability (927,

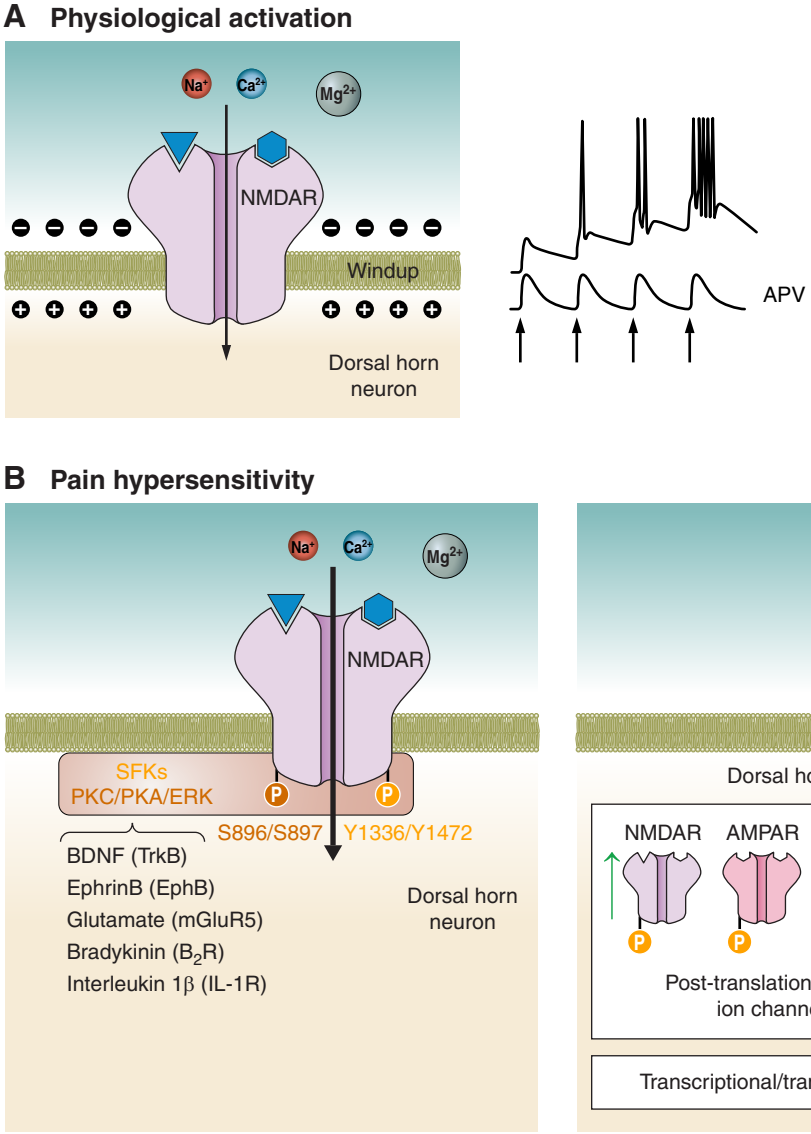


FIGURE 5. NMDA receptors contribute to dorsal horn neuronal windup and persistent hyperexcitability. *A*: repetitive presynaptic afferent fiber activity leads to a depolarization at dorsal horn neuron synapses, resulting in the relief of magnesium blockade of NMDARs, feed-forward cumulative synaptic depolarization, and neuronal hyperexcitability (*right, top*). This "windup" of dorsal horn excitability is immediately reversed upon the cessation of repetitive presynaptic firing and the return to resting membrane potentials. Blockade of NMDARs with APV attenuates the slow component of the initial EPSP and abolishes the cumulative dorsal horn neuronal hyperexcitability produced by repetitive afferent activity (*right, bottom*). *B*, *left*: in mechanisms of dorsal horn neuron sensitization, NMDAR currents are potentiated by both membrane depolarization and by phosphorylation through associated upstream regulators and membrane receptors. The GluN1 subunit of NMDARs is phosphorylated by serine/threonine kinases while the GluN2 subunit is phosphorylated by tyrosine kinases. *Right*, NMDAR activity in sensitized dorsal horn neurons causes calcium-dependent activation of associated effectors that leads to an upregulation in the activity of excitatory ion channels and a downregulation in the activity of inhibitory ion channels through posttranslational modifications. The activation of kinase cascades by NMDAR activity also results in the transcriptional and translational regulation of many proteins, resulting in persistent changes in dorsal horn neuron excitability.

992) and by modulating NMDAR trafficking (151). Phosphorylation effects combine with membrane depolarization and the activation of extrasynaptic NMDARs by glutamate and glycine spillover during repetitive firing (13, 651) to enhance the contribution of NMDARs to EPSPs. Potentiated NMDAR activity further increases intracellular calcium, leading to downstream activation of CaMKII, CREB, and c-*fos* as well as NO-mediated activation of guanylate cyclases, ultimately resulting in changes in synaptic strength mediated by de novo gene transcription and translation of synaptic proteins

(reviewed in Refs. 512, 551, 744). The essential and ongoing role of potentiated NMDAR activity in neuronal hyperexcitability and pathological pain is demonstrated in the fact that intrathecal injection of NMDAR antagonists attenuates and reverses hyperalgesia and allodynia following peripheral inflammation or nerve injury (see below).

Long-term potentiation (LTP) at primary afferent-dorsal horn neuron synapses constitutes a specific form of synaptic facilitation that leads to pain hypersensitivity (for review, see Ref. 753). At C-fiber-lamina I neuron synapses, NMDAR activation is required for the induction but not maintenance of LTP that is elicited by either low-frequency or high-frequency stimulation, both in vitro and in vivo (85, 410, 411, 549). This form of LTP is referred to as homosynaptic, as the activating synaptic input and the potentiated synaptic output are restricted to the same synapse(s). However, homosynaptic LTP does not offer a mechanistic explanation for the generation of allodynia nor the spreading of hyperalgesia to uninjured regions (secondary hyperalgesia) in chronic pain states and also does not explain why NMDAR antagonists reverse pain hypersensitivity when administered long after sensitization mechanisms have been initiated. A distinct form of plasticity, heterosynaptic potentiation, occurs when one population of synapses increases the responses of a distinct synaptic population and is thought to be the predominant mechanism underlying neuronal sensitization and pathological pain (for reviews, see Refs. 514, 515, 752).

It has been proposed that NMDAR-mediated plasticity in spinal cord superficial dorsal horn neurons is similar in mechanism to the LTP observed at glutamatergic synapses in other regions of the CNS, including the hippocampus (reviewed in Refs. 512, 744). However, synapses of superficial dorsal horn neurons contain unique molecular and functional properties that set them apart from other CNS synapses. For example, GluN2B proteins rather than GluN2A proteins predominate at mature synaptic sites in the superficial dorsal horn, which is the reverse of hippocampal synapses (637, 682). NMDAR-dependent hippocampal LTP is persistent and is maintained by insertion of new AMPARs into the postsynaptic membrane (470), while plasticity in the superficial dorsal horn only lasts for hours and involves ongoing functional contributions of potentiated NMDARs to facilitated EPSCs, including the NMDAR-dependent recruitment of normally mechanosensory Aβ fiber inputs (397, 515, 860). Moreover, non-Hebbian synaptic plasticity induced exclusively by postsynaptic depolarization and calcium entry has recently been described in superficial dorsal horn neurons (639). Thus hippocampal LTP and dorsal horn neuroplasticity are distinct phenomena.

D. Changes in NMDAR Subtype Expression and Phosphorylation in Chronic Pain Models

A wealth of literature exists on the changes in NMDAR phosphorylation and function that underlie dorsal horn neuronal hyperexcitability in inflammatory and neuropathic chronic pain states (514, 551, 744). This section focuses on genetic, molecular, biochemical, pharmacological, and behavioral evidence for the involvement of specific potentiated NMDAR subtypes and variants in chronic pain, with an emphasis on recent studies that have not been comprehensively reviewed to date.

1. GluN1

Phosphorylation of the GluN1 NMDAR subtype by serine/threonine kinases is enhanced in superficial dorsal horn neurons during pathological pain signaling. In response to continual noxious stimulation but not innocuous stimuli, GluN1 is rapidly and reversibly phosphorylated (112) by PKC and ERK at S896 and by PKA at S897 in the superficial dorsal horn, leading to pain hypersensitivity in models of neuropathic, inflammatory, visceral, and cancer pain (130, 785, 848, 889, 1011, 1012) **(FIGURE 5*B*)**. Increases in GluN1 phosphorylation during sensitization mechanisms are reversed by in vivo administration of NMDAR antagonists (i.e., MK-801), indicating that the activation of NMDARs leads to feedforward mechanisms resulting in enhanced phosphorylation of GluN1 itself (112, 217, 218). Thus intrathecal administration of NMDAR antagonists attenuates both NMDAR activation and potentiation to reverse behavioral hypersensitivity in chronic pain states (217, 218).

The expression of total GluN1 protein within the dorsal horn does not change during pathological pain signaling (130, 218, 889, 980); however, an increase in GluN1 immunoreactivity is observed in the synaptic membrane fraction of dorsal horn neurons during the development of mechanical allodynia in inflammatory pain states (130, 980). As NMDAR phosphorylation can modulate both channel opening and receptor trafficking (reviewed in Ref. 151), it is likely that increased phosphorylation of GluN1 contributes to enhanced synaptic targeting during pathological dorsal horn plasticity (130).

The essential role of NMDARs in dorsal horn sensitization and pathological pain is demonstrated in GluN1 conditional knockout mice. As all functional NMDARs contain two GluN1 subunits (310), attenuated GluN1 expression results in decreased functional expression of NMDARs. Mice with an inducible knockout of GluN1 in superficial dorsal horn neurons exhibit no deficits in acute thermal or mechanical pain sensation but have significantly attenuated pain hypersensitivity in models of chronic inflammatory pain (164, 795, 944). Similarly, antisense siRNA knockdown of GluN1 expression in the superficial dorsal horn attenuates mechanical allodynia produced by inflammation but has no effect on acute thermal and mechanical pain sensation (320). Consistent with a role for NMDAR calcium currents in activating downstream effector kinases during dorsal horn sensitization, knockout of GluN1 abolishes inflammation-induced increases in PKC and ERK phosphorylation (at 24 h) within superficial dorsal horn neurons (164, 944). Selective knockout of GluN1 in primary

afferent neurons also decreases pain hypersensitivity produced by inflammation, while not affecting acute pain sensation (594).

2. GluN2A

No alterations in the phosphorylation state or synaptic targeting of GluN2A in the dorsal horn have been observed following peripheral nerve injury or NMDA injection models of chronic pain (534, 693). Furthermore, global GluN2A knockout mice display no significant differences in pain behavior produced by models of chronic postoperative, inflammatory, and neuropathic pain compared with wild-type mice (6, 658, 698), and acute thermal and mechanical pain sensation are not significantly different between wild-type and GluN2A knockout mice (6, 698). However, one study has reported that global GluN2A knockout mice do exhibit a reduced secondary inflammatory pain response compared with wild-type mice (379).

3. GluN2B

Phosphorylation of the GluN2B NMDAR variant by tyrosine kinases (Src-family kinases, SFKs) is critical for dorsal horn neuronal hyperexcitability and pain hypersensitivity. Increased phosphorylation of GluN2B at Y1336 and Y1472 residues occurs in superficial dorsal horn neurons in rodent models of neuropathic and visceral pain and in some models of inflammatory pain (6, 693, 784, 959, 975) **(FIGURE 5*B*)**. As for GluN1, the overall expression of GluN2B protein in the superficial dorsal horn is not altered in chronic pain states (6, 784, 959), while there is an increase in synaptic GluN2B protein and GluN2B-like synaptic NMDAR currents in disinhibition, inflammatory, and neuropathic chronic pain states (130, 423, 975). Furthermore, mechanical allodynia induced by intrathecal injection of NMDA is sufficient to increase phosphorylation of GluN2B at Y1472 as well as synaptic targeting of GluN2B (and GluN1) (534). Thus feedforward NMDAR-dependent mechanisms promote both the phosphorylation and synaptic targeting of GluN1 and GluN2B in the spinal cord superficial dorsal horn.

Specific SFK members, including Src and Fyn, are involved in the tyrosine phosphorylation of GluN2B, critical for initiating and maintaining pathological pain signaling. Genetic deletion of either Src or Fyn (6, 550) as well as pharmacological inhibition by general SFK antagonists (353, 692, 784) results in reduced GluN2B phosphorylation and attenuated behavioral hypersensitivity in rodent models of visceral, inflammatory, and neuropathic pain. The specific interactions between Src or Fyn and GluN2B can also be targeted to reverse pain hypersensitivity. Blockade of the Src-ND2-NMDAR interaction through intrathecal injection of a specific peptide disrupter (Src40–49Tat) (1, 331) prevents pain behaviors in rodent models of both inflammatory and neuropathic pain, while having no effect on acute pain sensation or cardiovascular, respiratory, locomotor, and cognitive functions (550; reviewed in Ref. 750). Alternatively, transgenic GluN2B Y1472F knock-in mice have impaired phosphorylation at Y1472 by SFKs and do not exhibit neuropathic pain produced by peripheral nerve injury or postherpetic neuralgia, while displaying unaltered acute and inflammatory pain signaling (583, 891). The observation that SFK inhibitors attenuate Aβ fiber-mediated NMDAR mEPSCs (but not AMPAR mEPSCs) in nerve-injured and not sham-operated rats also supports the conclusion that the potentiation of GluN2B by SFKs is critical for chronic but not acute pain (397).

GluN2B and Src proteins also interact with other proteins in the macromolecular membrane complex to modulate NMDAR functional expression and downstream synaptic signaling **(FIGURE 4)**. One such protein family is the membrane-associated guanylate kinases (MAGUKs), composed of four distinct members (PSD-95, PSD-93, SAP102, and SAP97) that form intracellular scaffolding proteins. Both the PSD-93 and PSD-95 MAGUK proteins have been shown to interact with the COOH-terminal of GluN2 and are expressed in the superficial dorsal horn (reviewed in Ref. 840). Genetic deletion of PSD-93 in transgenic mice results in reduced expression of GluN2A/B in the superficial dorsal horn, reduced NMDAR-mediated synaptic currents, and reduced pain hypersensitivity in neuropathic and inflammatory pain states, with preserved acute nociceptive responsiveness (536, 841). Transgenic mice with a truncated version of PSD-95 also fail to develop allodynia and hyperalgesia in the peripheral nerve injury model of neuropathic pain (321); however, PSD-95 truncation does not affect synaptic NMDAR expression or NMDAR-mediated synaptic currents (602). Thus PSD-95-NMDAR interactions likely induce neuronal hyperexcitability and pathological pain by modulating NMDAR-mediated effector signaling and not NMDAR-mediated depolarization **(FIGURE 5*B*)**. Indeed, an increase in phosphorylation and colocalization of PSD-95, SHP2, Shank1, and GluN2B at superficial dorsal horn synapses has been implicated in mediating pain hypersensitivity in models of inflammatory and neuropathic pain (353, 604, 693). The activation of neuronal nitric oxide synthase by NMDAR is a likely effector downstream of PSD-95-GluN2B interactions (926), and disruption of the association between PSD-95 and nNOS reverses neuropathic pain (301). Finally, spinal delivery of peptides that disrupt interactions between GluN2B and PSD-95 reduce dorsal horn neuronal hyperexcitability and pain hypersensitivity in vivo (210, 838). Further peptides that disrupt interactions between NMDARs and PSD-95 are currently under development as potential novel therapeutics for the treatment of chronic pain (49).

Given that GluN2B is highly expressed in laminae I and II of the spinal cord and is potentiated during mechanisms of dorsal horn neuronal hyperexcitability, considerable efforts have been made in developing GluN2B-specific antagonists and in testing their efficacy in attenuating chronic pain signaling. Global GluN2B knockout mice are embryonic lethal, but

knocking down GluN2B expression in the dorsal horn with siRNA attenuates behavioral hypersensitivity in the Formalin-induced inflammatory pain model and has no effect on acute pain responsiveness (833). Intrathecal administration of GluN2B-selective antagonists including Ro25-6981, ifenprodil, and CP-101,606 reverse allodynia and hyperalgesia in rodent models of cancer, inflammatory, and neuropathic pain, while causing minimal deficits in motor function or acute pain sensation (108, 349, 479, 716, 819, 967, 1013). Furthermore, intrathecal injection of Ro25-6981 and ifenprodil also inhibits activity-dependent plasticity of nociceptive dorsal horn neurons in vivo (716). In models of neuropathic pain, Glu2B-specific antagonists exhibit a significantly greater separation between antinociceptive doses and doses that produce behavioral and motor side effects when compared with nonselective NMDAR antagonists (108, 819). Thus GluN2B-specific antagonists have high efficacy in specifically attenuating spinal cord hyperexcitability and pain hypersensitivity as well as a potentially preferable side effect profile compared with nonselective NMDAR antagonists.

4. GluN2C/D

Expression of the GluN2C NMDAR variant is thought to be predominantly restricted to the cerebellum (618), with minimal GluN2C mRNA and protein identified in superficial dorsal horn neurons (458, 997). Transgenic GluN2C knockout mice do not display deficits in acute pain sensitivity and have not been tested in models of chronic pain (378). In contrast, GluN2D proteins are specifically localized to superficial dorsal horn neurons (404) and the low magnesium-sensitive, putative GluN2D-containing NMDAR currents in laminae I and II spinal cord neurons (779, 856) could potentially contribute to slow EPSCs and resultant slow EPSPs (482, 988) in mechanisms of superficial dorsal horn transmission and plasticity. Experiments on GluN2D knockout mice have yielded conflicting results, with no deficits in pain hypersensitivity in the L5 spinal nerve transection, plantar incision, and plantar Formalin injection models of chronic pain (6, 379, 658). In contrast, mechanical allodynia is completely abolished in GluN2D knockout mice with the partial sciatic nerve ligation model of neuropathic pain (379). Given the recent development of GluN2D-selective antagonists and agonists (reviewed in Ref. 617), the specific roles of GluN2D-containing NMDARs in superficial dorsal horn plasticity and pathological pain signaling should be investigated.

E. Upstream Regulators of NMDA Receptors Linked to Dorsal Horn Neuronal Hyperexcitability

Inflammation- or nerve injury-mediated activation of many different GPCRs, receptor tyrosine kinases, and intracellular signaling pathways converge onto the potentiation of NMDARs to cause dorsal horn neuronal facilitation and pain hypersensitivity **(FIGURE 5*B*)**. Activation of the TrkB receptor tyrosine kinase in lamina I projection neurons by BDNF causes a downregulation of a chloride transporter (KCC2), leading to attenuation of GABAergic inhibition and neuronal hyperexcitability (201, 467). However, NMDAR activity may be required for the downregulation of KCC2 by BDNF (1020) and, conversely, the BDNF-mediated disinhibition pathway may induce NMDAR potentiation to facilitate excitation in lamina I projection neurons. In support of this idea, the increase in BDNF expression produced in nerve injury or bone cancer models of chronic pain is paralleled by an increase in the expression and/or phosphorylation of NMDAR subunits in superficial dorsal horn neurons (328, 923). Blocking NMDARs with antagonists including APV and Ro25-6981 abolishes BDNF-mediated behavioral hypersensitivity in chronic pain models (328, 344), while knocking down BDNF expression with RNAi attenuates increases in GluN1 phosphorylation induced by a bone cancer pain model (923). Increases in BDNF and NMDAR expression and phosphorylation in the superficial dorsal horn are correlated with activation of downstream PLC-, PKC-, SFK-, and ERK-mediated signaling pathways (319, 785; reviewed in Refs. 729). At a functional level, application of exogenous BDNF facilitates EPSCs and NMDA-evoked currents in neonatal lamina II neurons in vitro (319, 469). However, recordings of synaptic NMDAR activity in lamina I neurons are required to test for a direct and causative functional linkage between BDNF disinhibition and NMDAR potentiation.

EphB receptor tyrosine kinases and their endogenous ligands (EphrinBs) are presynaptic and postsynaptic membrane proteins, respectively, that are essential for mechanisms of synaptic scaffolding, including the maintenance of NMDAR clustering at the synapse (213). Activation of EphB induces allodynia and hyperalgesia in chronic pain states while not affecting acute nociception (69, 790). Mechanistically, EphB activation causes a SFK-mediated phosphorylation of GluN2B at Y1336 and Y1472 and an enhancement of synaptic plasticity and dorsal horn neuronal excitability in chronic pain states, while not altering acute pain transmission (181, 784, 790, 959, 1017).

Activation of GPCRs can also potentiate NMDARs in pathological mechanisms of dorsal horn neuroplasticity. In addition to the activation of ionotropic AMPARs and NMDARs, repetitive synaptic glutamate release also activates metabotropic glutamate receptors (mGluRs) in superficial dorsal horn neurons to induce prolonged EPSPs and neuronal hyperexcitability (reviewed in Ref. 551). In inflammatory pain states, activation of group I mGluRs causes the tyrosine phosphorylation of GluN2B through a PKC-/SFK-dependent pathway as well as an enhancement of NMDAR EPSCs and inflammatory hyperalgesia (353, 978). Bradykinin, another inflammatory mediator, acts on B2 GPCRs to potentiate NMDARs in lamina II neurons through a pathway that includes PKA, PKC, and ERK activation, leading to thermal hyperalgesia (492, 921).

The activity of dorsal horn NMDARs is also facilitated by other proinflammatory cytokines to potentially mediate neuronal hyperexcitability and pain hypersensitivity. Activation of the IL-1R receptor by interleukin-1β potentiates NMDA-induced currents in lamina II neurons (462, 548), where GluN1 and IL-IR are colocalized (1011, 1012). Furthermore, intrathecal administration of the IL-1R antagonist IL-1Ra reverses GluN1 phosphorylation and mechanical hyperalgesia in rodent models of inflammatory and bone cancer pain (1011, 1012). A similar reduction in inflammation-induced pain hypersensitivity and GluN1 phosphorylation is observed following intrathecal administration of an antibody against the proinflammatory cytokine interleukin-17 (596).

The contributions of NMDAR antagonism to morphine analgesia are influenced by sex differences (645). A greater understanding of how sex differences shape dorsal horn NMDAR function is of clinical interest, as opioids and NMDAR antagonists can have complimentary analgesic effects (298, 355, 495). Application of exogenous estrogen (17β-estradiol) induces an NMDAR-dependent increase in GluN2B phosphorylation, facilitates NMDAR-mediated currents, and increases LTP in superficial dorsal horn neurons (1016). Furthermore, estrogen activates feedforward phosphorylation of GluN1 via PKA activity and induces behavioral hypersensitivity in a rodent visceral pain model (836).

F. Lipid Signaling Molecules Also Modulate NMDAR Activity in the Superficial Dorsal Horn

Leptin, a pronociceptive adipocytokine, increases spinal cord GluN1 expression and potentiates NMDA-induced currents in spinal cord lamina II neurons through a pathway that includes the leptin receptor and downstream JAK2/STAT3 signaling (539, 849). The allodynia and hyperalgesia induced by intrathecal infusion of leptin is abolished by coadministration of MK-801 and reversed by subsequent administration of MK-801 (849). Furthermore, spinal administration of a leptin antagonist prevents and reverses hyperalgesia and allodynia in a rodent peripheral nerve injury model of neuropathic pain (539). In contrast to leptins, resolvins are a family of lipid mediators with efficacy in attenuating inflammatory pain. Spinal administration of resolvin E1 blocks the facilitation of NMDARs by the inflammatory cytokine TNF-α in superficial dorsal horn neurons (972). Intrathecal administration of resolvin D1 also attenuates the increase in phosphorylation of spinal cord GluN1 and GluN2B and reverses mechanical allodynia in a rat model of chronic pancreatitis, while having no effect on pain sensation or NMDAR phosphorylation in sham-operated rats (717).

G. NMDA Receptors as Clinical Targets for Pain Therapeutics

Preclinical evidence demonstrates that NMDARs are essential for spinal cord facilitation in chronic pain states. Inhibiting the activation or potentiation of NMDARs attenuates dorsal horn neuronal hyperexcitability and pathological pain, while leaving acute pain transmission intact. Blinded, placebo-controlled clinical trials with healthy, unmedicated volunteers have been used to test the efficacy of NMDAR antagonists in reversing pain hypersensitivity produced by relatively minor injury models. Administration of either low-dose oral dextromethorphan or intravenous ketamine in volunteers with a first degree burn injury significantly reduced mechanical allodynia and secondary hyperalgesia yet had no effect on heat pain detection thresholds in undamaged skin (415, 416). Similarly, intravenous administration of ketamine attenuated secondary hyperalgesia and had no effect on thermal pain thresholds in volunteers with topical application of 1% capsaicin to the foot (33). Thus NMDAR antagonists reverse dorsal horn sensitization and pain hypersensitivity in human models of pathological pain but do not affect basal pain transmission.

Testing the clinical efficacy of NMDAR antagonists in chronic pain syndromes is complicated by heterogeneity in underlying genetic, etiological, and environmental factors (912); variability in patient drug metabolism (600); and potentially confounding comorbidities (84, 497). For neuropathic pain, a recent meta-analysis identified 28 blinded, randomized, placebo-controlled clinical trials that tested the efficacy of NMDAR antagonists in reversing pain hypersensitivity (194). While administration of NMDAR antagonists caused no significant pain relief in a subset of studies, potentially due to the factors listed above, significant reductions in pain hypersensitivity were observed in clinical trials testing the efficacy of amantadine, dextromethorphan, magnesium salts, ketamine, and memantine in specific neuropathic pain syndromes. Overall, the meta-analysis study stated that "evidence in favor of the effectiveness of NMDAR antagonists for the treatment of neuropathic pain, of which ketamine seems to be the most potent, is accumulating" (194). It should be noted that most of the currently available clinical NMDAR antagonists are moderate-affinity NMDAR antagonists with multiple potential mechanisms of action that may contribute to their therapeutic efficacy. For example, micromolar concentrations of riluzole and dextromethorphan inhibit NMDAR activity (226, 646) but also inhibit excitatory voltage-gated sodium and/or calcium channels (646, 968).

Broad-spectrum inhibition of all NMDAR function by clinical antagonists such as ketamine can cause an inhibition of physiological NMDAR activity, leading to CNS-related adverse effects that include drowsiness, restlessness, hallucinations, headaches, and impairment of memory and motor functions. In an effort to develop therapeutics that treat chronic pain with greater efficacy and fewer side effects, new strategies are emerging that target specific pronociceptive NMDAR vari-

ants, their potentiators, and their downstream effectors (FIGURE 4). Due to the critical role of GluN2B in spinal cord sensitization, high-affinity antagonists of GluN2B are under preclinical and clinical development for the treatment of chronic pain (reviewed in Ref. 962). However, inhibition of GluN2B-mediated plasticity in physiological brain functions may cause psychotomimetic effects and limit the utility of Glu2B-specific therapeutics. Given the restricted expression of GluN2D in the mature CNS and the putative functional expression of GluN2D in the superficial dorsal horn, it will be interesting to explore whether recently developed GluN2C/D-specific antagonists (617) are efficacious in attenuating neuronal hyperexcitability and chronic pain. Another promising therapeutic strategy for treating pathological pain is to block upstream potentiators of NMDAR function or downstream GluN2B-coupled effectors (FIGURE 5*B*). Recent candidate approaches include the use of peptide disrupters of peptide-peptide interactions to specifically target the upstream dysregulated Src enhancement of NMDARs (550) or the downstream coupling of GluN2B with PSD-95 (210, 838). Further study of the functional roles of NMDARs in the afferent nociception pathway could reveal further upstream enhancers of NMDAR activity and downstream NMDAR-coupled effectors that may constitute novel therapeutic targets for pain treatment.

VI. PURINERGIC RECEPTORS

A. ATP and Pain

ATP is the prototypical energy source that fuels virtually all cellular processes. The first clue that ATP might possess an alter ego, one that mediates nociceptive signaling between cells, was the observation that it is released from sensory nerves following electrical stimulation (382, 383). This initial finding explicated an extracellular role for an otherwise intracellular molecule that was consistent with reports of pain evoked by intradermal ATP injection in both humans (96, 359) and animals (433). Subsequent studies demonstrated that exogenous application of ATP causes neuronal excitation in the spinal cord and DRG (428, 499). A mechanistic link between extracellular ATP release and pain signaling was realized when the P2X3 receptor was cloned and shown to be preferentially expressed on nociceptive neurons together with the P2X2/3 heterodimer receptor (152, 526). Identification of the P2X3 receptor spawned an intense search for other ATP receptors which led to the discovery of a number of additional P2 purinergic receptor family members.

B. Purinergic Receptor Subtypes and Molecular Composition

The P2 family of receptors is comprised of P2Y metabotropic and P2X ionotropic receptors. Activation of these receptors is the modus operandi for ATP-mediated intercellular signaling implicated in a wide range of physiological processes, including neurotransmission, neuromodulation, chemoattraction or chemotaxis, and pain (123). Unlike P2Y receptors, which are G protein-coupled, P2X receptors are nonselective cation channels permeable to calcium, sodium, and potassium ions. Seven P2X (P2X1-P2X7) receptor subunits have been cloned (472, 662). Assembly of three of these subunits as either homomeric or heteromeric complexes forms the core P2X receptor channel (650, 905). To date, six homomeric (P2X1-P2X5 and P2X7) and eight heteromeric (P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/5, P2X2/6, P2X4/6, and possibly P2X4/7) receptors with unique molecular and cellular properties have been identified (195, 432, 858, 859). Each subunit shares a common topology with two transmembrane helices that link the intracellular NH_2 and COOH termini to a large extracellular ligand binding region (110, 662, 893). The NH_2 terminus of all P2X receptor subunits is similar in length (20–30 amino acids) and contains a protein kinase C phosphorylation site (103). In contrast, the COOH terminus can differ between 26 (P2X6) and 239 (P2X7) amino acids; this intracellular region contains several distinct motifs that confer subunit specific properties related to trafficking, internalization, and protein-protein interactions (448). The COOH terminus of the P2X4 receptor in particular appears to be important for agonist-induced desensitization (302), binding of phosphoinositide PIP_2 (88), and constitutive internalization (97, 306, 720).

The trimeric architecture of P2X receptors is distinct from that of other ligand-gated cation channels, such as tetrameric glutamate receptors (741) and pentameric acetylcholine receptors (731, 892). However, the precise arrangement of P2X receptor subunits remained enigmatic until the recent crystallization of the zebrafish P2X4 receptor, which revealed hidden details about its extracellular domain, transmembrane regions, and ion permeation pathway (463, 464). Elucidation of the P2X4 crystal structure also uncovered three ATP binding pockets (463, 464) validating earlier predictions that at least three ATP molecules are required to induce conformational changes that open the cation channel (65, 650). Influx of calcium through the opened P2X channel engages specific intracellular signaling cascades that are now beginning to be understood within the context of physiological and pathological processes. The idea that extracellular ATP is significantly increased in response to inflammation and tissue trauma (504, 652, 813) is consistent with growing evidence that P2X receptors are causally implicated in chronic inflammatory and neuropathic pain (868, 874). The converging lines of evidence that P2X receptors are cellular substrates for pain pathology has generated considerable therapeutic interest, with the most promising of these targets being P2X3, P2X2/3, P2X4, and P2X7 receptors. However, a major challenge that has plagued the study of P2X receptors is the dearth of selective agonists, antagonists, and modulators. A lack of research tools has made it difficult to parse the specific contribution of each P2X receptor subtype based on pharmacological strategies alone. Rather, a combination of pharmacological and genetic approaches has

been necessary to build the argument that P2X3, P2X2/3, P2X4, and P2X7 receptors are key components that drive chronic pain signaling in the central and peripheral nervous systems.

C. Role of P2X3 Receptors in Pain Signaling

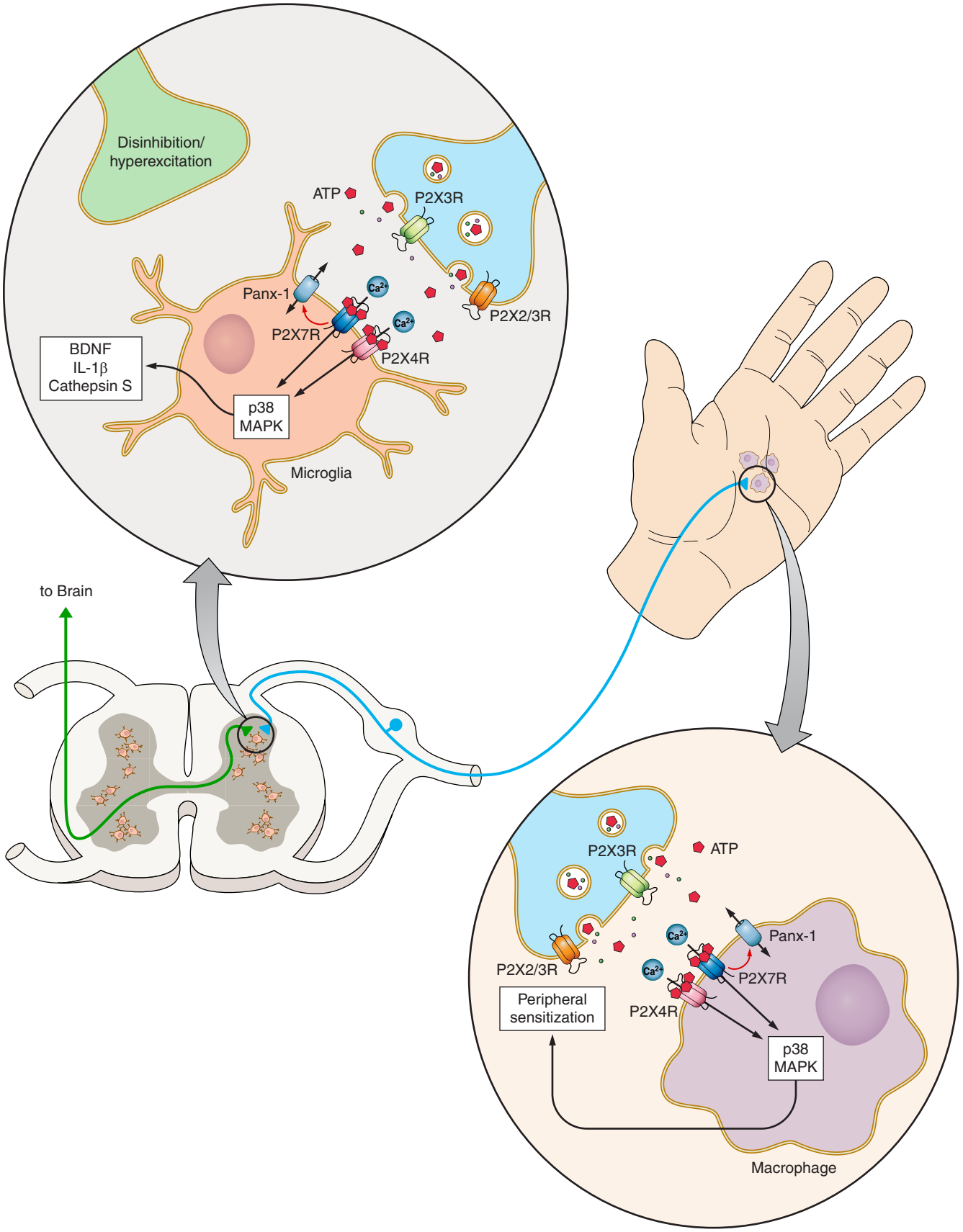
The P2X3 receptor is a homomeric trimer composed of three P2X3 monomers. Two subunits of P2X3 monomer can also assemble with one P2X2 monomer to form the P2X2/3 heteromeric receptor (128, 526). Both P2X3 and P2X2/3 receptors are localized on a subset of primary afferents (152, 526), with the highest expression being on small to medium-sized nonpeptidergic afferents (915). In addition, P2X3 receptor subunits are found in supraspinal regions involved in pain modulation (188, 768, 981). P2X3 receptors are therefore crucial nodes for transmitting nociceptive signals from the periphery to the CNS. However, it appears that these receptors are not required for acute responses to noxious mechanical or thermal stimuli as these modalities appear to be unaffected in the P2X3 receptor knockout mice (191, 794). Rather, there is growing evidence that P2X3 receptors are critically involved in pain caused by chronic inflammation or nerve injury (133, 388, 590, 876). Such injuries alter P2X3 receptor expression leading to increased spontaneous firing of wide dynamic range and nociceptive specific neurons in the spinal cord (431, 665, 971). Pharmacologically blocking P2X3 function or suppressing its expression with antisense oligonucleotides attenuates pain behaviors in rodent models of inflammation, nerve injury, and bone cancer (388, 389, 774). These results have been confirmed by a new generation of drugs with improved selectivity against P2X3 and P2X2/3 receptors (128, 351, 971) as well as in P2X3 knockout mice which display an attenuated inflammatory and neuropathic pain phenotype (190, 191, 794). Altogether these findings provide pharmacological, genetic, and behavioral evidence towards a causative role of peripheral and central P2X3 receptors in mediating the sequelae of chronic pain. However, deciphering the specific contribution of homomeric versus heteromeric P2X3 receptors has not been possible because both receptor types are suppressed in the P2X3 knockout mice or blocked by the current pharmacological repertoire of antagonists.

D. P2X4 Receptors Are Cellular Hubs for Pain Signaling in Microglia

In the CNS, P2X4 receptors have emerged as key cellular players involved in neuropathic pain signaling (81, 866). The specific role of P2X4 receptors was first teased out from pharmacological responses to P2X receptor antagonists: it was demonstrated that intrathecal injection of 2′,3′-*O*-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP), an antagonist of P2X1–4 receptors, reversed mechanical allodynia in nerve-injured rats, whereas pyridoxalphosphate-6-azophenyl-2′,4′-disulphonoic acid (PPADs), an antagonist of P2X1–3,5,7 receptors, had no effect on mechanical allodynia (879). On the basis of the pharmacological profiles of these antagonists, it was deduced that the essential P2X receptor subtype involved in central responses to peripheral nerve injury is the P2X4 receptor, and that persistence of mechanical allodynia requires tonic P2X4 receptor activation. A causative link between P2X4 receptors and neuropathic pain was supported by direct targeting of P2X4 receptors with antisense oligonucleotides (879) and by genetically deleting the *P2rx4* gene (877, 887). Both of these approaches produced a marked reduction in mechanical allodynia, demonstrating the necessity of P2X4 receptors in neuropathic pain. Furthermore, it was discovered that the onset of mechanical allodynia coincides with a progressive increase in spinal P2X4 expression, which is typically present at low levels in the uninjured CNS (879, 887). In an unexpected twist, this increase was confined to microglia (which are immunocompetent cells) residing in the spinal dorsal horn (879, 887). Delivering P2X4 stimulated microglia into the spinal cord of an uninjured animal mimicked the neuropathic pain phenotype, and recapitulated the altered nociceptive output of lamina I neurons induced by peripheral nerve injury (201, 467, 879). As such, P2X4 receptors expressed on microglia provide a neural basis for the etiology of neuropathic pain **(FIGURE 6)**.

E. Modulators of P2X4 Receptor Expression

Building on the discovery that P2X4 receptors are causally implicated in neuropathic pain, a catalog of signaling molecules has been discovered to modulate P2X4 expression in microglia. Cytokines, chemokines, and extracellular matrix molecules are among the cellular substrates that engage the P2X4 receptor response in spinal microglia (418, 558, 870). In particular, chemokines CCL21 and CCL2 released from injured neurons are known to affect microglial P2X4 receptors through distinct intracellular mechanisms; CCL21 is an upstream activator of de novo P2X4 synthesis (91, 223, 224), whereas CCL2 increases cell surface expression of P2X4 receptors without changing total P2X4 protein levels (863). Other signaling molecules that promote P2X4 expression include interferon-γ, a cytokine released following nerve injury (878), and tryptase, a protease secreted from activated mast cells (995). In addition, extracellular matrix molecule fibronectin signaling through Lyn kinase has been found to regulate the transcription and translation of P2X4 receptors (642, 880, 881). Activation of MORs by morphine can also increase microglial P2X4 receptor expression; this response opposes morphine analgesia (391, 392) and paradoxically enhances pain sensitivity (293). Thus several signaling molecules are implicated in the regulation of microglial P2X4 receptors. The next steps will be to determine the significance of this diverse, yet convergent modulation of P2X4 expression, and to elucidate how they are causally interconnected in P2X4 receptor mediated pain signaling.



F. Convergent P2X4 Receptor Signaling Gates Inflammatory and Neuropathic Pain

The intracellular convergence point for P2X4 receptor regulation and the key effector of P2X4 receptor signaling is p38 mitogen-activated protein kinase (MAPK) (869, 875, 888). In microglia, influx of calcium through the P2X4 receptor couples activation of p38 MAPK to the synthesis and release of BDNF, a critical microglia-to-neuron signaling molecule implicated in aberrant nociceptive processing in the spinal cord (201, 867). The requirement for P2X4 receptors in BDNF release is consistent with evidence that P2X4 knockout mice have impaired microglial BDNF release, altered BDNF signaling in the spinal cord, and abrogated development of mechanical allodynia following peripheral nerve injury (887). A causative link between P2X4 receptors and p38 MAPK has also been uncovered in peripheral macrophages **(FIGURE 6)**. Like microglia, influx of calcium through stimulated P2X4 receptors is an essential mechanistic step that is permissive for macrophage mediated pain signaling; however, in this cell type the released signaling molecule is prostaglandin E_2, which sensitizes peripheral nociceptors leading to inflammatory pain hypersensitivity (888). Thus central and peripheral P2X4 receptors control the release of distinct factors from spinal microglia (BDNF) and peripheral macrophages (prostaglandin E_2) which differentially contribute to the sequelae of neuropathic and inflammatory pain.

G. Role of P2X7 Receptors in Pain Signaling

P2X7 receptors are predominantly expressed on immune cells in both central and peripheral tissues (402, 780, 783, 911). Their activation provides the critical signal that engages the inflammasome response, initiating synthesis and release of proinflammatory cytokines (291, 490). Concomitant with increased activation is the upregulation of P2X7 receptor expression, which is particularly striking in DRG and injured nerves isolated from chronic neuropathic pain patients (170). Genetically suppressing or pharmacologically blocking the P2X7 receptor blunts inflammatory and neuropathic pain behaviors in rodents, as well as abolishes ATP evoked release of the cytokine interleukin-1β, a mediator of inflammation (170, 387, 459). The cellular contribution of P2X7 receptors to pain pathology also encompasses the regulation of neuronal P2X3 receptors (162), downregulation of glutamate transport (622), and release of matrix metalloproteinase-9 (347) and prostaglandin E_2 (58). In the CNS, the P2X7 receptor is a locus for microglia-mediated chronic pain signaling. Activating microglial P2X7 receptors drives the release of interleukin-1β, cathepsin S, and TNF-α, which contribute to the development and maintenance of mechanical pain hypersensitivity (184, 185, 631). Thus extensive and converging lines of preclinical evidence clearly support P2X7 receptors as being essential players in chronic pain and inflammation.

Because of the potential implications for treating chronic pain, P2X7 receptors are considered high-priority targets for drug development. Drugs that have entered clinical testing have a mechanism of action that blocks P2X7 cation channel function (169, 350, 351). However, P2X7 receptors possess two distinct modes of action; not only do they function as a cation-selective channel, but they can also form large nonselective pores (also referred to as pore formation) that allow molecules up to 900 Da to pass through the cell membrane (437, 690, 814). Pore formation is regulated by the COOH-terminal domain of the P2X7 receptor (10, 408). In this region, a proline to leucine substitution at amino acid 451 of the P2X7 receptor differentially impairs pore formation while leaving cationic channel function completely intact (10, 408). In mice, the pore-disabling P2X7 variant (451L) is associated with an attenuated inflammatory and neuropathic pain phenotype (792). In humans, the *P2RX7* gene is highly polymorphic (309, 806), and genetic differences within *P2RX7* that affect P2X7 pore formation have been associated with two distinct types of persistent pain: chronic post-mastectomy pain and osteoarthritis pain (792). Individuals in these cohorts carrying an allele encoding for heightened P2X7 pore function reported more intense pain, whereas those with a low-functioning allele reported lower pain. Taken together, the most parsimonious explanation is that genetically determined P2X7 pore formation critically controls variability in chronic pain sensitivity in both mice and humans. Targeting pore formation might therefore provide a focused therapeutic strategy with the potential for fewer side effects associated with indiscriminate P2X7 inhibition. Moreover, identification of specific *P2RX7* haplotypes has potentially important implications for predicting an

FIGURE 6. Critical role of P2X receptors in central and peripheral chronic pain signaling. P2X3, P2X2/3, P2X4, and P2X7 receptors are causally implicated in inflammatory and neuropathic pain. Localized on primary sensory afferents, P2X3 and P2X2/3 receptors are nodes for transmitting nociceptive signals from the periphery to the CNS. P2X4 and P2X7 receptors, on the other hand, are expressed predominantly on immune cells. In particular, P2X4 receptors are cellular hubs for microglia-mediated chronic pain signaling: influx of extracellular calcium through ATP-stimulated P2X4 receptors activates p38-MAPK and gates the release of BDNF. Acting via its cognate receptor, TrkB, BDNF signals to increase spinal nociceptive output through neuronal disinhibition or hyperexcitation. Likewise, inflammatory challenges that stimulate P2X4 receptors on macrophages trigger a calcium and p38-MAPK-dependent release of prostaglandin E_2 (PGE_2), which sensitizes peripheral nociceptors. P2X7 receptors expressed on microglia and macrophages are also key players in chronic pain signaling. Activation of these receptors releases interleukin-1β (IL-1β) and cathepsin S which contribute to the development and maintenance of chronic pain. A unique feature of the P2X7 receptor is its ability to form large nonselective cytosolic pores (pore formation) that allow molecules up to 900 Da to pass through the cell membrane. P2X7 pore formation mediated by recruitment of an accessory protein, pannexin-1 channels, which form the pore, is critically implicated in experimentally induced pain in mice and in chronic pain conditions in humans.

individual's risk of developing chronic pain. The most salient prediction is that individuals with pore-impaired P2X7 receptors would not benefit from pharmacological interventions aimed at this target. Hence, a therapeutic strategy that involves *P2RX7* genotyping, or functionally assessed pore formation, has great potential for guiding and individualizing the treatment of chronic pain.

In summary, P2X3, P2X2/3, P2X4, and P2X7 receptors have emerged as the core P2X receptor subtypes involved in central and peripheral pain pathology. Several recent discoveries have begun to unravel the molecular composition of these receptors and build a comprehensive mechanistic framework for understanding the intracellular components that control P2X receptor-mediated pain signaling. A large body of evidence now suggests that strategies directed against P2X3, P2X2/3, P2X4, or P2X7 receptors is of high value for designing novel and more effective pain therapies. Although there are P2X-based drugs currently in clinical trials for rheumatoid arthritis, pain, and cough (663), there remains a pressing need for a new generation of P2X receptor antagonists with improved selectively and pharmacological profiles, both as research tools and as realized clinical pain therapies.

VII. TRANSIENT RECEPTOR POTENTIAL CHANNELS

A. Physiological Role and Molecular Compositions Of Transient Receptor Potential Channels

The identification of the superfamily of transient receptor potential (TRP) cation channels has led to many important insights into the molecular basis of pain signaling. In particular, the family of thermosensitive TRP channels has been of utmost interest due to their polymodal activation. They are nonselective cation channels and act as molecular transducers of noxious temperatures (heat or cold), as well as mechanical and chemical stimuli (292, 654, 668). Calcium and sodium influx through these channels convert these stimuli into locally spreading membrane depolarizations, propagating action potentials to the spinal cord and higher brain centers (827). These channels can also contribute to changes in the intracellular calcium concentration that subsequently activates calcium-dependent enzymes involved in the pain pathway, such as the neuronal nitric oxide synthase (nNOS) (421; for review, see Ref. 327). Thermosensitive TRPs allow us to experience the burning of chili peppers or the cooling of menthol, as they can be activated not only by temperature, but also by endogenous molecules and synthetic substances known to trigger thermal and pain sensation. While these channels are of tremendous importance in somatosensory perception, their dysregulation (i.e., increased expression and sensitivity) is often associated with inflammatory and neuropathic pain (748). Along these lines, growing evidence of the regulation of these channels by pro-inflammatory mediators such as serotonin, bradykinin, prostaglandins, proteases, chemokines, and growth factors has confirmed how essential these channels are in the sensitization of the afferent pain pathway (for review, see Ref. 898).

The TRP channel superfamily is a large family of ion channels with 28 mammalian members identified, separated into 7 distinct subfamilies (183). TRP channels are found in a wide variety of cell types, including smooth muscle, epithelium, and immune cells as well as neurons. Since the cloning of the first vanilloid receptor (TRPV1), six subfamilies have been described: the vanilloid (TRPV), canonical (TRPC), melastatin (TRPM), ankyrin (TRPA), polycystin (TRPP), and mucolipin (TRPML) (655). While TRP channels share little sequence homology among subfamilies, they exhibit a similar membrane topology. Each TRP subunit contains six membrane spanning helices (termed S1-S6) as well as a pore-lining loop between S5 and S6 that enables distinct cation selectivity and permeability among TRP channels. The assembly of four subunits is generally required to form a functional channel (465, 759), yet both homo- and heteromultimerization of channel complexes have been reported (52, 166, 167, 369). The NH_2 and COOH termini are intracellular regions that contain sites of regulation by protein kinases, chaperones, and scaffold proteins. These interactions either facilitate channel trafficking to the cell surface or lower the threshold of channel activation, both leading to sensitization of nociceptive transduction.

TRP channels are regulated by a wide variety of molecules and stimuli, from protons to arachidonic acid (AA) metabolites or even changes in osmolarity. Thus TRP channels are ideally suited towards detection of pathophysiological inflammatory conditions: heat, acidosis, changes in osmolarity (mechanical stimulus), as well as the release of inflammatory mediators that might accompany these conditions (686, 922). These channels can also be intrinsically voltage-dependent, as shown for TRPV1 (352), TRPM3 (342), TRPM4 (656), TRPM5 (381, 828), TRPM8 (907), TRPV3 (179), TRPM3 (342), TRPA1 (1025), and TRPP3 (778). Finally, phosphorylation is an important regulator of TRP channel function (705, 931).

While the TRPC and TRPM families have been characterized in sensory neurons, analysis of mice lacking various thermosensitive TRP genes has revealed a crucial role of TRPV1–4, TRPM8, TRPM3 (914), and TRPA1 channels in pain signal transduction and integration, and these channels have been highlighted as molecular players in nociceptive and neuropathic pain. Here we will review their molecular identity, their mechanism of activation by nociceptive stimuli, their regulation by proinflammatory mediators, and changes in their expression patterns in physiopathological pain states. To date, drug companies have dedicated considerable efforts towards targeting TRP channels for pain management (197, 620, 822). However, the development of new analgesic molecules has encountered limitations due to undesired on-target side-effects. In particular, early TRPV1 antagonists elicited marked

hyperthermia in animal models (315, 324, 386, 952) and in clinical studies (471). As discussed here, the task is not insurmountable, and analgesic molecules targeting nociceptive TRP channels may soon find their place in the therapeutic arsenal (641).

B. TRPV1

The first characterized nociceptive TRP channel was the vanilloid type 1 (TRPV1), the receptor for the vanilloid irritant capsaicin. This channel was cloned in 1997 using an expression-cloning screening strategy (138). Structure-function analysis indicates that several phosphorylation sites involved in sensitizing actions of PKA and PKC are located in the cytoplasmic NH_2 and COOH termini (702). In addition, the NH_2-terminal region of TRPV1 holds six cytosolic ankyrin repeat domains (ARDs) that form a multiligand-binding site for calmodulin (CaM) and ATP (545) (see **FIGURE 7**). While the CaM-binding site in the NH_2-terminal region was described as an important regulator of TRPV1 desensitization, the high-affinity CaM-binding site isolated in the COOH-terminal region of the channel showed a minor contribution (516).

TRPV1 is a noxious heat-sensitive channel with polymodal activation (low pH, osmolarity changes, arachidonic acid metabolites) and voltage-dependent properties (352). Expression of the TRPV1 channel was reported in small to medium DRG neurons that respond to the pungent ingredients of hot pepper as well as heat (138, 239). It is also widely expressed within the peripheral and central nervous systems, in the gastrointestinal tract, and the epithelium of the bladder and skin (620, 633, 702, 728, 908). In rodents, TRPV1 has been described primarily in small- to medium-diameter primary sensory ganglia (DRG, trigeminal and nodose ganglia). TRPV1 is expressed in nociceptive Aδ- and C-fibers (138) which project to the superficial dorsal horn (855) and is found in 70% of small (<50 μm) neurons from human cervical DRG (31). TRPV1 is also distributed in trigeminal and vagal afferents immunoreactive for SP, CGRP, and the NGF receptor TrkA (138, 178, 1021, 1022). Consequently, sensitization of the channel during inflammation or ectopic discharge may contribute to the development of autonomic dysregulation in visceral tissue innervated by the vagus nerve (935). Electrophysiological characterization of the channel has revealed its intrinsic heat sensitivity in DRG neurons (146) and in reconstituted artificial liposomes (129). Importantly, the temperature threshold that gates the channel can be lowered by proinflammatory mediators released during inflammation. As discussed below, numerous studies have proposed a pivotal role of TRPV1 in hypersensitive states that result from tissue inflammation, including thermal and mechanical hyperalgesia.

Due to its high expression in nociceptors, TRPV1 has received intense interest from a pain-management perspective, and blockers of TRPV1 have been shown to have analgesic properties. However, while capsaicin is able to produce peripheral and central sensitization associated with secondary hyperalgesia, prolonged or repetitive administration of capsaicin locally

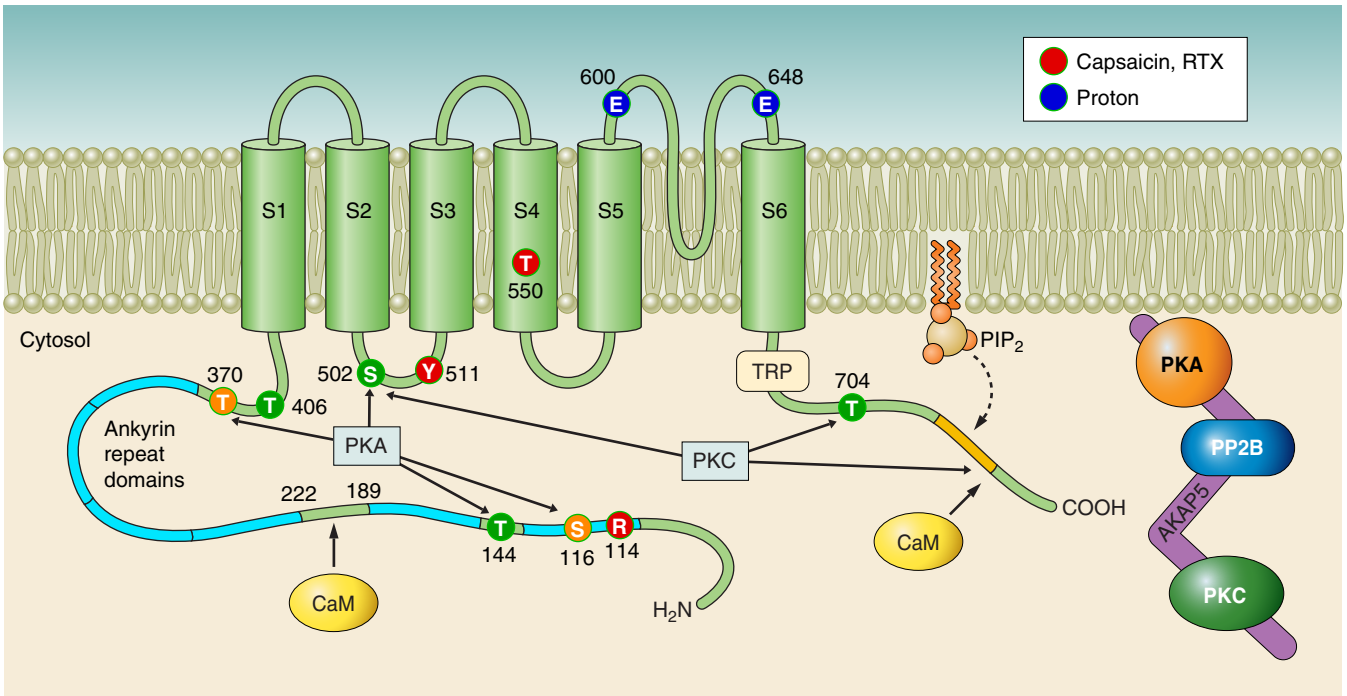


FIGURE 7. TRPV1 channel topology highlighting multiple regulatory sites for protein kinase C (PKC), protein kinase A (PKA), calmodulin (CaM), phosphatidylinositol 4,5-bisphosphate (PIP_2). TRPV1 phosphorylation and sensitization is facilitated by AKAP5 expression in sensory DRG neurons. Sites for proton binding are indicated.

on the epidermis results in channel desensitization and skin fiber removal (429, 821). Indeed, excessive TRPV1 activation leads to calcium cytotoxicity and specific ablation of TRPV1 positive nociceptors. Use of the potent agonist resiniferatoxin (RTX), which is 1,000-fold more potent than capsaicin (824), has demonstrated that removal of TRPV1-expressing nociceptors reduces experimental inflammatory hyperalgesia and neurogenic inflammation in rats as well as naturally occurring cancer pain and debilitating arthritic pain in dogs (456). Furthermore, desensitization of TRPV1-positive fibers with systemic RTX administration abolishes spinal nerve ligation (SNL) injury-induced thermal hypersensitivity and spontaneous pain, but has no effect on tactile hypersensitivity (484). In humans, a single high dose or repeated application of capsaicin (injected or topical) causes rapid desensitization (468, 509, 659, 781), whereas single low doses lead to activation of nociceptive fibers and thermal hyperalgesia (24, 843). Topical administration of capsaicin as a cream or local patch (NGX-4010, Qutenza) has been developed for chronic pain conditions that result from postherpetic neuralgia (51, 441), human immunodeficiency virus-associated distal sensory neuropathy (782), or diabetic neuropathy (664). In contrast, attempts to develop TRPV1 antagonists have been less successful. Given the role of this channel in the regulation of body temperature (314, 409), most of the antagonists tested in preclinical and human studies presented hyperthermic side effects (620). Current investigations are centered on separating the analgesic potency of TRPV1 blockers from their hyperthermic action. The solution may reside in specifically targeting one of the three modalities of channel activation (capsaicin, low pH, or heat). An alternative strategy is to generate a channel-permeant activator with the ability to desensitize the channel from within its open state during neuronal hyperactivity. This would prevent inactivation of silent (closed) channels to preserve normal nociception (620). Another strategy consists of introducing the membrane-impermeant sodium channel blocker QX-314 into primary afferent nociceptors through the TRPV1 channel pore. Using this approach, Binshtok et al. (92) were able to rapidly and specifically block the excitability of TRPV1-expressing C-fibers (92).

1. TRPV1 and inflammatory pain

A main hallmark of TRPV1 is its sensitization in response to mediators contained in the inflammatory milieu after infection and injury or in autoimmune diseases. While protons, heat, pressure, or lipids directly activate TRPV1 (136, 855), proinflammatory mediators such as serotonin, bradykinin, histamine, proteases, chemokines, or NGF (800, 1014) are able to regulate TRPV1 activity and trafficking, leading to channel sensitization and thermal hyperalgesia (453) **(FIGURE 8)**. This is of particular significance, since NGF levels are increased during nerve injury (525, 735) or in murine models of visceral hyperalgesia, including gastric ulcers (508) and colitis (175, 572, 797). Other inflammatory mediators such as prostaglandin E_2 (PGE_2) trigger sensitization of TRPV1 channels via phosphorylation, leading to development of thermal hyperalgesia (395, 623, 787). Recent studies have shown a role of TRPV1 in pain behaviors associated with the rodent monoiodoacetate (MIA)-induced osteoarthritis model (449, 714, 765). Particularly, the severity of arthritic pain (mechanical hyperalgesia) is reduced by blocking or deleting TRPV1. This fits with a number of pharmacological and null mouse studies that have shown impaired detection of heat and reduced thermal hyperalgesia during inflammation (24, 137, 843).

2. TRPV1 and visceral pain

It is now well established that TRPV1 is expressed in visceral afferent neurons that are immunoreactive for the proinflammatory neuropeptides SP and CGRP (831, 832). These neurons project from the gut to the CNS to evoke abdominal pain in gastrointestinal disorders such as inflammatory bowel disease (384). Tan et al. (831) showed that the majority (82%) of colon-innervating DRG neurons are TRPV1 positive and 60% of these neurons express neuropeptides CGRP and SP. In contrast, only 32% of TRPV1-positive neurons are vagal afferents (832). In a functional correlate of these immunohistological studies, TRPV1 appears to play a key role in visceral mechanosensation and pain (738) and was recently shown to be upregulated in experimental and human colitis (14; for review, see Refs. 17, 272). Indeed, this channel mediates pain and visceral hypersensitivity both in irritable bowel syndrome associated with subtle inflammation and in inflammatory bowel disease during remission (14, 15). Increased TRPV1 expression in the absence of inflammation has also been observed in idiopathic rectal hypersensitivity and fecal urgency (147) and Hirschsprung's disease (280). Moreover, TRPV1 activation induces hypersensitivity of pelvic afferents in response to colorectal distension, whereas channel blockers reverse the hypersensitivity observed after acute stress exposure of adult rats that have been subjected to maternal separation as neonates (895). While TRPV1 is not a mechanosensor per se, it enables sensitization of distension-responsive colon afferents that present a low frequency of firing (573). In human volunteers, instillation of capsaicin at different levels of the gut causes heartburn, cramps, pressure, nausea, and increases the sensitivity to balloon distension (40, 156, 360, 481, 522, 760). Finally, the TRPV1 G315C polymorphism was found to influence the susceptibility to pain in the upper abdomen associated with functional dyspepsia. Therefore, TRPV1-induced visceral hyperalgesia has been recognized as an important factor in the pathogenesis of functional disorders of the gastrointestinal tract (585).

3. TRPV1 in neuropathic pain

Studies of neuropathic pain conditions following nerve injury, viral infection, or metabolic disorders such as painful diabetic neuropathy revealed a pronociceptive role of TRPV1. Channel expression is enhanced in response to spinal nerve ligation or ventral root transection, promoting thermal and mechanical hyperalgesia in those neuropathic pain models (308, 965). In

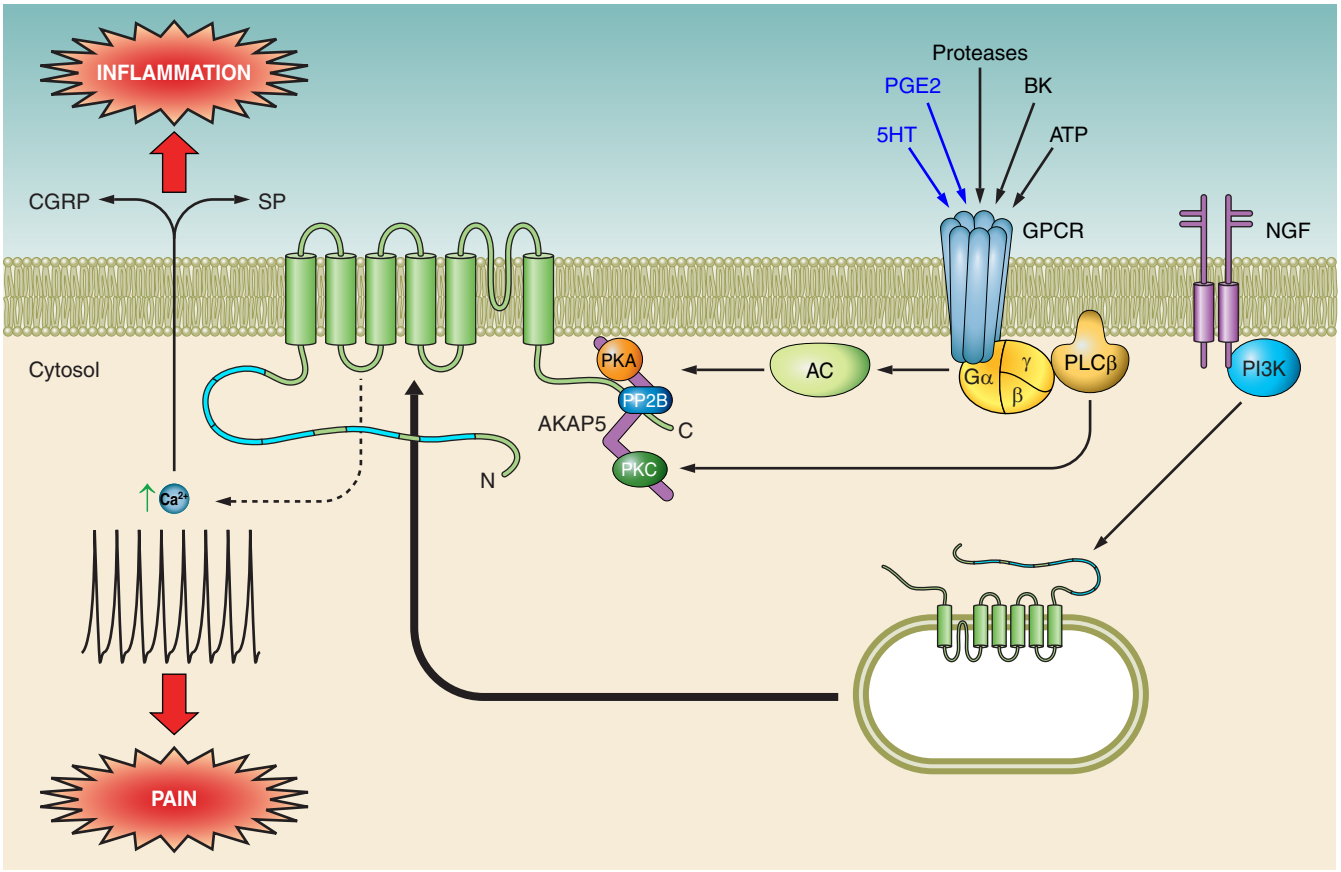


FIGURE 8. Signaling pathways involved in the sensitization of TRPV1: NGF activates the TrkA receptor to increase TRPV1 membrane trafficking via phosphatidylinositol 3-kinase (PI3K). In addition, both PKC-ε and PKA sensitize TRPV1 function. The A-kinase anchoring protein (AKAP5) is an essential scaffolding molecule in this process. TRPV1 sensitization by inflammatory mediators enhances calcium influx in response to TRPV1 activation, which leads to neurogenic inflammation and hypersensitivity.

contrast, TRPV1 silencing with injection of antisense oligodeoxynucleotides suppresses mechanical hypersensitivity (176). Cell-specific expression of TRPV1 and functional sensitization of the channel via subunit oligomerization and cell surface translocation were described in DRG neurons from diabetic rats (385), suggesting a role in diabetic neuropathy.

At the central level, a role for TRPV1 in inhibitory interneurons of the spinal cord was recently identified. Kim et al. (480) reported that mechanical hypersensitivity after peripheral nerve injury is attenuated in TRPV1 knockouts, but not in mice lacking TRPV1-expressing peripheral neurons. Cold hypersensitivity is another feature of neuropathic pain, and in this context, McCoy et al. (588) observed that CGRP-positive/TRPV1-positive primary afferent neurons encode heat and itch. These peptidergic sensory neurons tonically cross-inhibit cold-responsive neurons in the spinal cord. Ablation of this specific subset of neurons disrupts the inhibitory crosstalk, thereby eliciting cold hypersensitivity. Interestingly, the TRPV1-positive interneurons described by Kim et al. (480) appear to be responsible for masking the tonic activity of cold-responsive TRPM8 neurons described by McCoy et al. (588). These results could provide a mechanistic framework for the development of cold hypersensitivity associated with neuropathic pain. Along these lines, specific silencing of TRPV1-positive primary afferents using the sodium channel blocker QX-314 with capsaicin reduces heat, mechanical, and cold hyperalgesia in an inflammatory setting, but cannot abolish tactile and cold allodynia in response to peripheral nerve injury (111). With regard to therapeutic development, two recent TRPV1 antagonists PHE377 and DWP-05195 have entered phase I clinical trials for neuropathic pain management, and it will be interesting to learn whether these compounds affect temperature perception. Moreover, topical applications of capsaicin are used for treating neuropathic pain conditions (823); however, the tolerability of capsaicin and the maintenance of the analgesic effect still need consideration.

C. TRPA1

TRPA1 has also emerged as an important player in pain processing. The TRPA subfamily has only one characterized member (TRPA1) and is named for the high number of ankyrin domains at the NH_2 terminus of the protein (807). TRPA1, formerly referred to as ANKTM1, was originally identified and cloned by Jaquemar et al. in 1999 (430). The mammalian TRPA1 gene is orthologous to the nociception

gene *painless* in *Drosophila melanogaster*, thus suggesting a conserved role for TRPA1 in sensory functions across phyla (196, 864). In both humans and rodents, TRPA1 is expressed in a subpopulation of small-diameter peptidergic nociceptors of the dorsal root, nodose, and trigeminal ganglia, along with TRPV1 (636, 807). In humans, TRPA1 is localized in spinal cord motoneurons and nerve roots, peripheral nerves, intestinal myenteric plexus neurons, and skin basal keratinocytes (32). TRPA1 is also present in the substantia gelatinosa (SG) of the spinal cord where it facilitates spontaneous glutamatergic excitatory transmission (496). Lastly, the channel is expressed in vagal and primary afferent fibers innervating the bladder, the pancreas, the heart, the respiratory tract, and the gastrointestinal tract (653).

TRPA1 is activated by a variety of noxious stimuli, including cold temperatures, pungent natural compounds, and environmental irritants (567, 807). In vitro, covalent modification of reactive cysteines within TRPA1 activates the channel (567). Therefore, TRPA1 serves as a broad-spectrum irritant receptor for a variety of reactive chemicals, including both electrophilic and nonelectrophilic compounds (54). Dietary irritants such as isothyocyanates (mustard oil, wasabi, horseradish) and allycin (garlic) can covalently bind to and activate the channel in heterologous expression systems and in DRG neurons (73). Moreover, TRPA1 responds to endogenous inflammatory/tissue damage mediators including cyclopentane prostaglandins, by-products of oxidative stress [4-hydroxynonenal (4-HNE), 4-oxononenal] and hydrogen peroxide, making TRPA1 a key transducer of inflammatory pain (35, 53, 442, 872). Specifically, TRPA1 is a major effector of the known proinflammatory mediator bradykinin, which elicits sensory neuron excitation ex vivo and hyperalgesia in vivo (53, 72). As observed for bradykinin-induced sensitization of TRPV1, bradykinin enables disinhibition of TRPA1 via a PLC activation pathway and PIP_2 breakdown (53), as well as PKA activation (925). Further evidence of this functional relationship was obtained from TRPA1−/− mice in which intraplantar injection of bradykinin failed to produce thermal and mechanical hypersensitivity (72). Finally, the importance of TRPA1 was highlighted by the description of a gain-of-function mutation in humans suffering from episodic pain syndromes. This autosomal dominant mutation in the fourth transmembrane domain of TRPA1 generates normal pharmacological profile but increases inward current at resting potentials. Since cold temperature is a trigger of enhanced pain perception in this human cohort study, it confirms the role of the TRPA1 channel as a noxious cold sensor as well as an irritant sensor (498).

1. TRPA1 in inflammatory and neuropathic pain

In metabolic disorders linked to diabetes mellitus and renal failure, nociceptor sensitization can be induced by altered levels of specific metabolites, which triggers neuropathic pain. One of these metabolites, methylglyoxal (MG), can directly bind to TRPA1 and trigger the release of proinflammatory neuropeptides (263). This could explain why metabolic disorders exacerbate cold or pain perception and often itch. In diabetic animals, mechanical hypersensitivity is reduced by low doses of TRPA1 antagonist (932). Following spinal nerve ligation, TRPA1 upregulation has been shown to promote cold hypersensitivity (460). Thus accumulating evidence confirms that therapeutic targeting of TRPA1 may prevent neuropathic pain associated with both nerve injury and metabolic disorders.

Antagonists of TRPA1 have been tested in acute and chronic inflammatory pain models. The selective blocker HC-030031 reduces pain behaviors in response to paw inflammation. These data agree with the lack of pain hypersensitivity in TRPA1-deficient mice that received an injection of Formalin in the hindpaw (593). Using a chronic model of inflammatory pain induced by intraplantar injection of CFA, Eid et al. (268) observed that HC-030031 can reverse mechanical hyperalgesia. Identical effects were obtained with the HC-030031 compound in the spinal nerve ligation model, suggesting common mechanisms of TRPA1 sensitization in both inflammatory and neuropathic pain models. An additional novel selective TRPA1 antagonist from Abbott laboratories (A-967079) was used to demonstrate that TRPA1 contributes to normal and pathological noxious mechanosensation. Systemic injection of this potent nanomolar affinity blocker decreases spontaneous activity of spinal neurons in models of CFA-mediated inflammation and in osteoarthritic rats (589). Interestingly, this compound also shows analgesic properties in isothiocyanate- and osteoarthritis-induced pain. Whereas cold allodynia produced by nerve injury is reduced, noxious cold sensation and body temperature are not altered (157). Recently, a new series 641 of 7-substituted-1,3-dimethyl-1,5-dihydro-pyrrolo[3,2-d] pyrimidine-2,4-dione derivatives have been developed as efficient TRPA1 antagonists, yet their analgesic properties in rodent animal models of inflammatory and neuropathic pain still need to be validated (56).

2. TRPA1 and visceral pain

The role of TRPA1 in visceral inflammation and nociception has been studied most thoroughly in the gastrointestinal tract (510). Administration of the TRPA1 agonist mustard oil by enema induces characteristic symptoms of colitis including abdominal pain and release of inflammatory mediators driving leukocyte rolling. Given that TRPA1 is present at the nerve terminals of nociceptive and vagal afferents, it is ideally positioned to sense endogenous and dietary irritants from the gut. Activation of TRPA1 in these fibers stimulates the release of the pro-inflammatory neuropeptides SP and CGRP, which in turn exacerbates the inflammatory state and causes visceral hyperalgesia. A recent demonstration of the direct activation of TRPA1 channels by 2,4,6-trinitrobenzene-sulfonic-acid (TNBS), currently used as a rodent model of colitis, confirmed the role of TRPA1 in the control of intestinal inflammation. While colitis is induced and maintained through a TRPA1-dependent release of colonic SP, the inflammatory response and histological damages are significantly reduced in

TRPA1−/− mice or upon systemic administration of the TRPA1 blocker HC 030031 (273). In addition to its contribution to the inflammatory response, TRPA1 stimulation by agonists allyl isothiocyanate and *trans*-cinnamaldehyde also promotes mechanosensory responses in vagal and pelvic serosal afferents of normal mice, but not in TRPA1-deficient animals. However, in vivo recording of visceromotor responses to colorectal distension showed a significant reduction in mechanical hyperalgesia in TRPA1−/− mice, thus directly implicating TRPA1 in colonic pain (113). Interestingly, induction of colitis exacerbated the mechanical hypersensitivity of colonic fibers, in response to TRPA1 agonists. These data not only suggested a role of TRPA1 in mechanosensation, but they confirm that channel sensitization mediates mechanical hyperalgesia in a colitis setting. These observations were corroborated by results obtained with in vivo knock down of TRPA1 via antisense oligodeoxynucleotide, which reduced colonic hypersensitivity induced by TNBS-mediated colitis in mice (977). Likewise, knocking down TRPA1 using intrathecal injection of antisense oligonucleotides led to attenuation in hypersensitivity in the rat stomach (494).

D. TRPV4

Although the identification of mechanically activated Piezo channels was recently in the spotlight (200, 336, 477), TRPV4 has been the main candidate for sensing osmotic changes, pressure, and shear stress in both neurons (657) and muscle tissue (380). TRPV4 is ubiquitously expressed and regulates intracellular calcium signaling, temperature sensing, osmo- and mechano-transduction, as well as maintenance of cell volume and energy homeostasis (983). TRPV4 is present in various cell types, including endothelial cells, chondrocytes, epithelial cells, and adipocytes (657). Channel expression in DRG and trigeminal ganglia neurons has suggested a role in pain responses to mechanical stimuli in somatic tissue and visceral organs. This thermosensitive channel responds to various exogenous chemical ligands including synthetic 4α-phorbol esters and the plant extract bisandrographolide A (657). Like TRPV1 and TRPA1, TRPV4 is also activated by polyunsaturated fatty acids. Metabolites of arachidonic acid activate TRPV4 by an indirect mechanism involving the cytochrome *P*-450 epoxygenase-dependent formation of 5,6-epoxyeicosatrienoic acid (EET) and 8,9-EET (929).

Although the TRPV4 channel does not appear to transduce mechanical stimuli directly, this channel may be involved in triggering mechanical hyperalgesia. Work from Levine and colleagues has suggested a role for TRPV4 in osmotic stimulus-induced nociception. Both hypotonic and hypertonic stimuli produces mechanical hyperalgesia in the hindpaw (19, 20) which is sensitized by the inflammatory mediator PGE_2 and reduced by in vivo silencing of the TRPV4 channel, suggesting that the channel could be a molecular mechanotransducer of major relevance to human pain disorders. Functional cooperation of TRPV4 with $\alpha 2\beta 1$ integrin and Src tyrosine kinase was shown to contribute to the development of mechanical hyperalgesia in response to inflammation and in diverse models of peripheral painful neuropathy (18). In addition, TRPV4−/− mice exhibit impaired mechanical nociception along with altered sensitivity to acid and thermal selection behavior, whereas responses to noxious heat and low-threshold mechanical stimuli are conserved (537, 538, 817). While NGF-mediated sensitization of TRPV4 triggers pressure-evoked pain, the sensitization to heat and mechanical stimuli by von Frey filaments is identical in wild-type and knockout mice (21). In contrast, the contribution of TRPV4 in thermal hyperalgesia was highlighted in these TRPV4 null mice which display longer latency to escape from a hot plate following carrageenan-induced inflammation (850). Remarkably, thermal hyperalgesia is observed in mice overexpressing TRPV3 channels in keratinocytes, suggesting a similar role for TRPV3 and TRPV4 (398).

The ability of TRPV4 to promote inflammation and mechanical allodynia was reported in a rat model of joint inflammation in which administration of PAR2 agonist could sensitize channel response to 4α-phorbol 12,13-didecanoate (4αPDD) (229). Consistent with a change in pH and osmolarity during inflammation (356), these studies may collectively point to the TRPV4 channel as a mediator of pain hypersensitivity associated with inflammatory or neuropathic conditions. It is possible that this PAR2-mediated effect involves tyrosine kinase phosphorylation (931).

1. TRPV4 and visceral hypersensitivity

It is interesting to note that TRPV4 appears to be enriched in splanchnic and pelvic colonic afferents that project to the thoraco-lumbar and lumbo-sacral DRGs (114). While gut-innervating sensory neurons display low- and high-threshold mechanosensitivity, channel activation is only responsible for sensitizing high-threshold mechanosensitive fibers (114). Channel activation by the phorbol ester 4α-phorbol 12,13 dideconoate (4αPDD) induces visceral hypersensitivity in response to colorectal distension (144), and these nocifensive responses are suppressed by in vivo channel knock down (209). Hence, the TRPV4 channel is an essential mediator of visceral hypersensitivity, one of the main symptoms of IBS. Likewise, in an experimental model of acute pancreatitis, TRPV4 null mice exhibited both reduced nociceptor activation as well as pain behavior. In contrast, intraductal administration of 4αPDD to the murine pancreas enhanced c-*fos* expression in the superficial lamina of the dorsal horn, indicating activation of nociceptive neurons in the spinal cord (145).

E. TRPM8

The calcium-permeable TRPM8 channel functions as the primary mammalian sensor of cold. TRPM8 channels are present in 10% of small (<20 μm) DRG and trigeminal ganglia neu-

rons that do not express the classical markers of nociceptors such as TRPV1 and CGRP, initially suggesting that TRPM8 is a nonnoxious cool thermosensor (592, 689). TRPM8-expressing sensory neurons depolarize below ~25°C and respond to cooling agents (177) such as menthol and eucalyptol, thus explaining why these compounds evoke a psychophysical sensation of cold. Both pharmacological and genetic studies support the idea that these channels likely govern the neuronal sensing of both innocuous cool temperatures and noxious cold (74, 192, 238). Indeed, administration of 1-phenylethyl-4-(benzyloxy)-3-methoxybenzyl(2-aminoethyl) carbamate (PBMC) produces a hypothermic response independently of other thermosensitive channels, TRPV1 and TRPA1 (489). A recent study reported that inflammatory mediators inhibit TRPM8 activity through direct binding of the $G\alpha_q$ subunit (896). The $G\alpha_q$-coupled receptors for bradykinin, histamine, serotonin, ATP, and PGE_2 enhanced the heat response but reduced the cold response of sensory fibers of the cornea (896). These findings provide a framework mechanism relevant to the abnormal sensation of cold (dysesthesia) observed in response to injury and/or inflammation. Interestingly, muscarinic receptor-mediated inhibition of TRPM8 via the $G\alpha_{11}$ pathway appeared to be less effective (533), suggesting that channel activity can be fine-tuned through activation of different types of GPCRs.

TRPM8-deficient mice display impaired discrimination of mildly warm or cool temperatures and poorly avoid noxious cold down to 5°C. Apart from its role in thermosensation, acute activation or inhibition of TRPM8 can have analgesic effects either on visceral or neuropathic pain (115, 362, 713) and can also attenuate cold hypersensitivity in inflammatory and nerve-injury pain models (489). Chronic inflammatory conditions are often accompanied by cold pain that results from the long-term action of cytokines and growth factors upregulating TRPM8 in DRG neurons. Accordingly, the hypersensitivity to innocuous cold in neuropathic pain models is diminished in TRPM8-deficient mice (488, 489), and their nocifensive response to cold stimulation following an intraplantar injection of CFA is reduced (192, 489). On the other hand, in vivo studies revealed that these mice display normal responses to cold pain, suggesting the existence of additional noxious cold sensors (238). Hence, further interrogations are necessary to identify the precise contribution of TRPM8 channels to cold sensing.

F. Regulation of Nociceptive TRP Channels by Proteinase Activated Receptors

Proteinases are signaling molecules that regulate numerous biological functions. The family of serine proteinases [which activates proteinase activated receptors (PARs)] triggers both protective and proinflammatory effects, and thus both serine proteinases and PARs are potential targets for inflammatory pain management (675, 903). PAR2 receptors, a family of GPCRs that is activated by serine proteinases, are activated by the proteolytic unmasking of a tethered ligand that stimulates the receptor (724). PARs have been implicated in nociception (901) and signal to TRPV1, TRPV4, and TRPA1 channels (212; for review, see Ref. 339). After injury or trauma, released proteases from mast cells, epithelial cells, and even neurons sensitize the activities of these channels, thus leading to thermal and mechanical hyperalgesia (29, 30, 211, 340). The molecular mechanisms of the sensitization process seem to point towards a reduction of phosphatidylinositol-4,5-bisphosphate (PIP_2)-mediated inhibition of TRP channels. PAR2 triggers TRPV1 and TRPV4 phosphorylation via $G\alpha_q$ coupling and the activation of the protein kinase C (PKC-ε) pathway, leading to channel potentiation (30, 340) **(FIGURE 8)**. This enhancement of TRPV1 activation occurs via an increase in open probability of the channel, inhibition of desensitization, and/or increased cell surface expression (339). Likewise, TRPA1 channel sensitization by PAR2 activation could be mimicked through PIP_2 antibody sequestration or PLC-mediated hydrolysis (212). Finally, the sensitization of TRP channels contributes to neurogenic inflammation in the context of diseases such as asthma, irritable bowel syndrome, or pruritus. TRPV1 channel activation by capsaicin triggers SP and CGRP release at the nerve terminal and at central synapses in the dorsal horn. Consequently, PAR2-induced sensitization of TRPV1 exacerbates neurogenic inflammation at the periphery and amplifies pain transmission in the spinal cord, exemplified by mechanical and thermal hyperalgesia (339, 390, 802). A similar regulation by PAR2 has been described for TRPV4 (144, 340, 902).

TRPA1 and PAR2 have been observed in a colocalization pattern in rat DRG neurons (212). PAR2 activation mediates TRPA1 sensitization through a mechanism similar to bradykinin-evoked sensitization, and this involves the cleavage of PIP_2 by PLC-β, which relieves the inhibition of TRPA1. PAR2 has also been shown to activate PKA, which modulates TRPA1 activity via channel phosphorylation as well as enhanced trafficking of TRPA1 to the cell surface (161, 761). The regulation of TRPA1 by PAR2 might play a central role in different experimental models of gastrointestinal disorders. Cattaruza et al. (140) have reported that TRPA1 deletion significantly reduces mechanical colonic hyperalgesia induced by PAR2 activating peptide. Furthermore, mast cell-dependent mechanical hyperalgesia in the inflamed esophagus involves PAR2-mediated sensitization of TRPA1 (991).

Finally, TRPV4 sensitization by PAR2 produces mechanical hyperalgesia following intraplantar injection of PAR2 activating peptides in the paw (340). Likewise, PAR2 agonist-induced visceral hypersensitivity in response to colorectal distension is dependent on TRPV4 expression in colon-innervating DRG neurons (144). The functional coupling between PAR2 and the channel is thought to involve tyrosine phosphorylation (705). Specifically, the activation of PAR2 induces the production of arachidonic acid-derived signaling mediators that activated TRPV4. PAR2-TRPV4 coupling is blocked

by using the Src inhibitor-1 or the TRPV4 tyrosine mutant Y110F. This mechanism of PAR2-TRPV4 cooperativity may be required for sustained inflammation leading to somatic and visceral nociceptive hypersensitivity.

In summary, the thermosensitive TRP channels are primary sensors of various noxious stimuli and molecules released in trauma or in an immune response. These stimuli encompass membrane stretch and direct channel activation by protons or reactive oxygen species, among many others. Acute regulation by signaling molecules and long-term changes in TRP channel expression are key contributors to persistent pain, and are promising targets for the development of nonopioid and NSAID analgesics. Our understanding of the role of thermosensitive TRP channels will also rely on determining their endogenous activators and regulators. Lipid mediators that belong to the lipoxin and resolvin families have emerged as crucial candidates. Future investigation will be warranted to determine what type of metabolites are released in pathological pain states and how they impact on TRP channel function and pain signaling. Finally, apart from their role as noxious sensors and their electrogenic action in primary afferent neurons, TRP channels generate intracellular calcium changes. Thereby, TRP channels may serve as signaling molecules involved in transcriptional regulation and neuroanatomical changes of primary afferent neurons, two major regulatory mechanisms of chronic pain establishment.

VIII. ACID SENSING ION CHANNELS

A. Subtypes and Molecular Structure

The acid sensing ion channels belong to the superfamily of ion channels comprising the epithelial sodium channel and degenerin in *Caenorhabditis elegans* (superfamily ENaC/DEG; Ref. 466). Originally named MDEG, BNC1, and BNaC1–2 after their initial identification, they have been characterized as proton-activated channels and therefore named "acid sensing ion channels" or ASICs (919). In rodents, at least six ASIC subunits (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4) encoded by four genes (ACCN1–4) have been described. The functional diversity of ASIC channels arises from these four genes and their alternative splice variants: ASIC1a and 1b (68, 316, 919), or ASIC2a and 2b (316, 711). Genome sequencing information of several species confirms the presence of ASIC genes across evolution. However, the profile of alternative splicing may differ. In humans for example, ASIC1b has not been described yet, while ASIC3 and ASIC4 have three and two variants, respectively (16, 48, 345), with still unknown physiological impact. For human ASIC3, it should be noted that in addition to acidic pH, alkalinization is also able to activate the channel, thus opening new conceptual views of the pathophysiological impact of this ASIC isoform (228).

Structurally, each ASIC subunit has the same topology as P2X proteins with two hydrophobic transmembrane domains linked by a large extracellular loop. Crystal structure resolution of chicken ASIC1a showed that the functional channel is a trimeric assembly (334, 434). Channels formed of homomeric and heteromeric composition of ASIC subunits exist, thus further multiplying their functional diversity with regards to their current kinetics, ionic selectivity, and pH sensitivity (47, 542). ASIC2b and ASIC4 do not form functional channels on their own (16, 345, 542). ASIC2b, however, modulates the properties of other ASIC subunits and contributes to the unique properties of heteromeric channels (235, 542). The functional role of ASIC4 is not yet understood, but it may negatively modulate surface expression of the other isoforms (253).

In rodents, ASIC1a, ASIC2a, and ASIC2b are expressed in the CNS (63, 64, 316, 711, 919, 936, 961), whereas ASIC1b appears to be restricted to peripheral neurons (153, 574, 703, 909, 918). In humans, this tissue distribution seems to be significantly different since ASIC3 is present in the PNS as well as in the CNS (228).

ASIC channels are cationic channels whose activation leads to membrane potential depolarization. Although they are largely permeable to sodium ions, ASIC channels can be permeable to calcium ions (919), with relative calcium to sodium permeability varying between studies (P_{Na}/P_{Ca} from 2.5 to 50) (68, 937, 986). However, it has been proposed that calcium permeation through human and chicken ASIC1a is limited when extracellular calcium and sodium are maintained in physiological conditions (751). Nevertheless, ASIC activity is also indirectly linked to intracellular calcium elevation due to their depolarizing effect and subsequent activation of voltage-gated calcium channels (373, 751, 969, 970, 986), and/or due to the release of calcium from intracellular stores (1009). Interestingly, ASIC1a-mediated activation of N-type calcium channels has been demonstrated, and this could be of great importance in the context of synapses in the dorsal horn of the spinal cord (969).

ASIC currents, gated by a drop in extracellular pH, are generally of a transient nature. Their activation threshold and kinetics differ as a function of channel subunit composition. This heterogeneity confers onto ASIC channels an array of functional properties that can optimally correspond to distinct extracellular acidification stimuli. ASIC1a and ASIC3 are the most sensitive to protons since they have a threshold of activation as low as a −0.4 or −0.2 pH drop from 7.4, and a half activation ($pH_{1/2}$) around ~6.5 (234, 973). In contrast, ASIC2a channels are activated by a more substantial drop in extracellular pH, with thresholds at pH ~6.0 and $pH_{1/2}$ between 5 and 4 (62, 64, 542). Of the distinct ASIC-mediated currents, ASIC3 differs by generating biphasic components: a transient, and a sustained phase that is maintained as long as the pH stays acidic (231, 234, 749, 973). This property confers onto ASIC3 a role in sustained neuronal activity, and this can

become of particular physiological importance in conditions in which the sustained phase is upregulated (234, 438, 953).

As with many other types of channels, ASIC subunits can be part of macromolecular signaling complexes that regulate their correct subcellular localization and their functional properties (37, 61, 232, 235, 253, 260, 313, 393, 394, 699, 712, 763, 943, 1009). Among the interactors of ASIC channels are other ion channels (such as BK potassium channels, NMDARs), as well as adaptor proteins involved in coupling with kinases, the cytoskeleton, and neurotransmitter receptors (such as PSD95, lin7b, NHERF, CIPP, AKAP150, and PIKC1 that associate via PDZ ligand/PDZ domain interactions). They also interact with stomatin and thereby may play a role in mechanoreceptor complexes in sensory neurons. Therefore, ASIC channels may support a multitude of cellular functions depending on their subunit composition and interacting partners.

Given the ASIC subtype-specific physiological roles, the identification of specific ASIC pharmacology has been crucial. A number of nonspecific molecules (i.e., amiloride) or NSAIDs (i.e., diclophenac, aspirin, or ibuprofen) inhibit ASIC1a and ASIC3 (909). New specific drugs that have been discovered over the last few years have emerged as key tools to elucidate ASIC functions in particular in the pathophysiology of pain. Among these are specific toxin antagonists such as PCTx1, APETx2, and Manbalgine-1 isolated from tarantula, sea anemone, and black Mamba snake venom, respectively (243, 244, 277). Conversely, the ASIC agonist MitTx isolated from Texas Coral snake venom has provided new insights into ASIC function (94, 99). Small organic molecules would be interesting as clinical means to treat disorders linked to ASIC overexcitability. To this end, chemical screens identified A-317567, a compound that has a more potent efficacy than amiloride (257). Moreover, 2-guanidine-4-methylquinazoline (GMQ) is able to activate ASIC3 at neutral pH but at a rather high concentration (1 mM). At lower doses, it potentiates acidic stimulation by binding to an extracellular site distinct from the proton site (535, 993, 994). Altogether, there is a slowly growing arsenal of pharmacological tools that allows in depth analysis of ASIC function at the physiological and pathophysiological level.

B. ASIC Channels and Pain

The first experimental proof of ion channels gated by extracellular medium acidification (that are known now to be ASICs) was obtained from primary sensory neurons from dorsal root and/or trigeminal ganglia (500, 501). ASIC channels are expressed in almost all subtypes of primary sensory neurons including in peripherin positive small DRG neurons comprising all the C-fibers (68, 153, 676, 703, 909). ASIC channels are expressed in both the DRG cell soma and in axonal fibers where they are targeted to peripheral sites (256, 317). The impact of ASIC channels to cutaneous somatic pain has been initially difficult to decipher due to the lack of altered nociceptive phenotype in mice deficient of ASIC1a, ASIC2, and ASIC3 (155, 676, 709, 710). In these animals, the responses to acid injection are unaffected. However, ASIC3 null mice do present altered responses to strong mechanical stimuli (155), but other studies have reported contradictory results (676). In contrast to mice, evidence of an involvement of ASIC channels in somatic pain in rats and humans has been obtained using intrathecal RNAi delivery and pharmacological studies (234, 438). In rats, A-317576 has analgesic effects toward thermal inflammatory and postoperative pain (257, 736). More selective approaches with RNAi and APETx2 demonstrated the impact of ASIC3 in cutaneous peripheral terminals, an effect that is increased in inflammatory conditions due to elevated ASIC3 level (234, 575). In contrast, the use of the ASIC3 agonist GMQ showed a painful phenotype upon peripheral intraplantar injection, an effect that is absent in ASIC3 null mice (993). The use of PCTx1 was instrumental for demonstrating the minimal role of ASIC1a in the periphery (256, 587).

In contrast to somatic cutaneous pain, ASIC channels seem to have a more prominent effect in deep muscle pain. ASIC3 is largely expressed in nociceptors innervating tissues such as the plantar muscle (233). In a model of postoperative pain targeting this muscle, the use of APETx2 has a clear analgesic effect. This is also true for the model of gastrocnemius muscle acidification or in the knee osteoarthritis model (424, 457). The use of ASIC1 and/or ASIC3 null mice also revealed a role in muscular pain in naive and inflammatory conditions (122, 413, 414, 786, 917). Overall, these data provide evidence for a role of ASIC1a and ASIC3 in nociceptors that innervate muscles.

A separate series of studies explored the role of ASICs in primary afferents during visceral pain. The pH changes inside the lumen of the gastrointestinal tract are highly significant, and this is particularly the case in the stomach in the context of pathological conditions such as ulcer. Indeed, stomach sensory afferents densely express ASIC like currents (812). Further down in the gastrointestinal tract, ASIC1, -2, and -3 channels have been identified in myenteric plexus neurons, with ASIC3 showing the largest expression. Retrogradely labeled colonic DRGs also revealed that ASIC3 is the most abundant isoform expressed (403). This is consistent with the impact of this channel in colon-nerve teased fiber excitability induced by stretch and mechanical stimulation of the receptive field (439, 677). Altogether, the contribution of ASIC channels to the perception of mechanical stimuli in visceral tissues appears to be more important than their role in cutaneous afferents.

Finally, the contribution of ASIC channels has been investigated in primary afferent neurons innervating the heart in the context of ischemia generated by angor. Ed McCleskey's group showed that ASIC currents are prominently expressed in these neurons and proposed that they are key sensors of pain associated with cardiac ischemia (86). They demon-

strated that this current likely originates from an ASIC3/ASIC2b assembly (815). These channels are particularly sensitive to acidification, and a drop in pH to 7.0 that is reached rapidly during the first minutes of ischemia is sufficient to activate them. Interestingly, lactic acid produced in this pathological situation can lead to ASIC3 sensitization (417). Therefore, ASIC3 channels may be key sensors of ischemia-induced cardiac pain.

What remains to be determined is whether ASICs contribute to pain signaling by virtue of their effect on membrane potential, or instead by their role as calcium entry pathways. To date, it remains unclear whether ASIC-mediated calcium entry activates cell signaling processes and plasticity that lead to long-term sensitization of pain pathways in tissues such as the gut.

IX. SUMMARY

A plethora of ion channels and receptors contribute to afferent pain signaling. Among the numerous ion channels that are involved in this process, there are a number of channel subtypes that permit the influx of calcium ions. Hence, in addition to regulating membrane potential and excitability, these channels mediate a chemical cell signaling function. This includes the release of neurotransmitters (i.e., N-type and T-type calcium channels), the activation of calcium-dependent enzymes (e.g., NMDARs, P2X receptors, TRP channels), and calcium-dependent changes in plasticity and gene transcription (e.g., NMDARs). Unlike electrical signaling, these downstream signaling events are immensely complex and likely vary with the cellular and subcellular localization of source channels. Indeed, it is intriguing to note that although all of the ion channel types discussed in this review allow the entry of calcium into the cytosol, these channels fulfill unique roles and physiological functions (often within the same type of afferent neuron), and are in turn regulated by specific cell signaling molecules such as kinases, phosphatases, and G proteins. In the context of pain signaling, these channels often become hyperactive, whether at the functional level, or at the membrane expression level. While ion channel blocking drugs may be effective against both of these mechanisms, specifically targeting the mechanisms that cause aberrant upregulation of channel density in the plasma membrane may offer opportunities for selective interference with dysregulated channels, while sparing the essential calcium signaling functions that are inherent to the channel subtypes discussed herein.

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Bourinet, Emmanuel, Christophe Altier, Michael E. Hildebrand, Tuan Trang, Michael W. Salter, and Gerald W. Zamponi. Calcium-Permeable Ion Channels in Pain Signaling. *Physiol Rev* 94: 81–140, 2014; doi:10.1152/physrev.00023.2013.—The correct version of **FIGURE 1** is shown below.

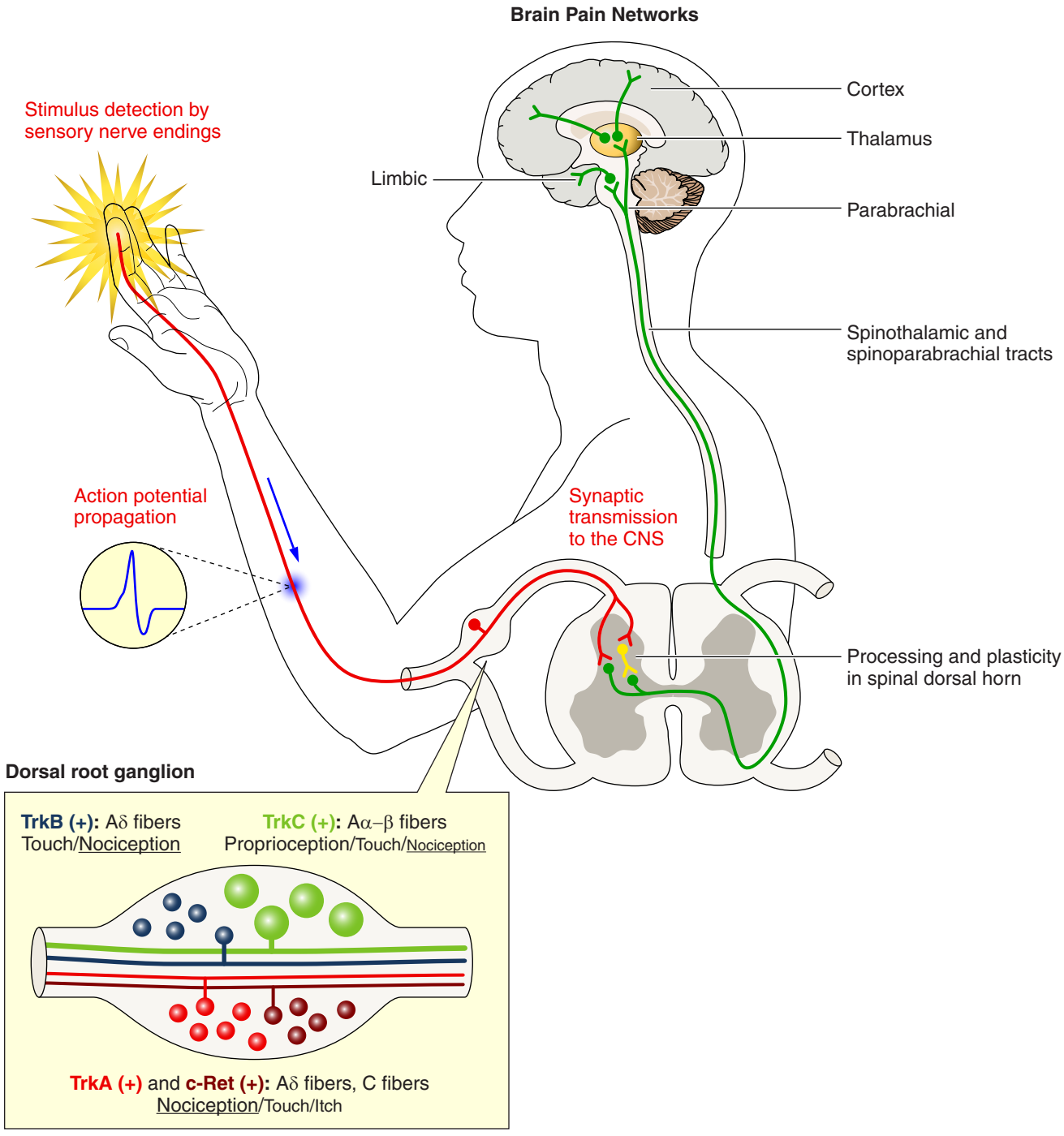

FIGURE 1. Ascending pain neuraxis. Pain-sensing neurons in the peripheral nervous system have their soma located in the dorsal root ganglia (DRG). These neurons have a peripheral axon innervating the distal territories (skin, viscera, etc.) where they detect painful stimuli leading to an action potential that travels along the fibers up to the DRG and then to the first relay in the dorsal spinal cord. Sensory neurons within the DRGs are diverse and can be separated based on the expression of neurotrophin receptors. The majority are TrkA- and c-Ret-positive small-diameter sensory afferents that correspond to unmyelinated C fibers mainly involved in nociception. TrkB- and TrkC-positive myelinated larger diameter afferents correspond to Aδ and Aα-β fibers, respectively. They convey touch and proprioception signals, although both of these subclasses contain nociceptive neurons. The sensory information is processed locally in neuronal circuitry within the dorsal horn of the spinal cord before being sent to the thalamus to convey nociceptive information. Following thalamic filtering, the information is sent to the cortical structures of the pain matrix.

Calcium-Permeable Ion Channels in Pain Signaling

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