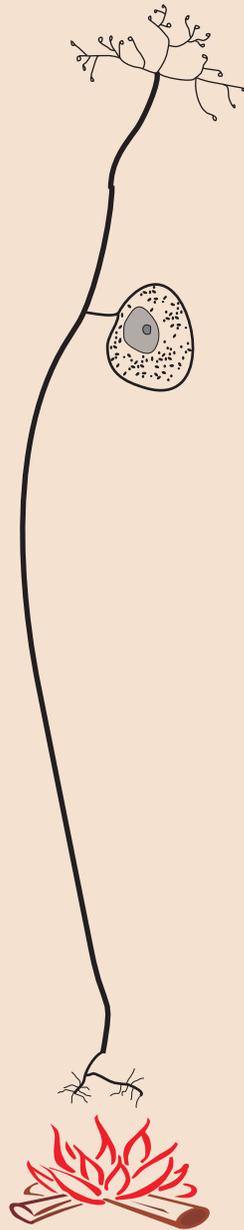# NOCICEPTORS AND THE PERCEPTION OF PAIN

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Revised May 2014

NOCICEPTORS AND THE PERCEPTION OF PAIN

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NOCICEPTORS AND THE PERCEPTION OF PAIN

CONTENTS

Chapter 1 **INTRODUCTION**

Pain is an unpleasant feeling that is an essential component of the body's defense system. It provides a rapid warning to the nervous system to initiate a motor response to minimize physical harm. Lack of the ability to experience pain, as in the rare condition congenital insensitivity to pain with anhidrosis (Axelrod and Hilz 2003), can cause very serious health problems such as self-mutilation, auto-amputation, and corneal scarring.

Up until the twentieth century there was a vigorous and heated debate about the nature of pain. One side held that sensory stimuli, which activate ordinary sense organs, such as those for warmth or touch, would initiate pain through the same sense organs if the stimuli were strong enough. The other held that there was a separate set of specialized sense organs that were specific for pain. It was not until the twentieth century that the debate was settled when it was shown conclusively that there were specialized sensory organs that signaled pain.

Perception is the process that allows us to interpret sensory information. For example when we hear music we may think it is beautiful or we may eat a food and think it has a horrible taste. One can make a distinction between the sensory information we receive and how we perceive that information. This distinction also applies to pain. Pain is a **perception** that is a process that allows us to interpret a certain type of sensory information. Sometimes the link between the sensory information and the perception is suppressed, for example, during battle soldiers have reported a lack of pain despite severe injuries.

The word "pain" comes from the Greek: poinē, meaning penalty. Physiologists distinguish between pain and nociception; where nociception refers to signals arriving in the central nervous system resulting from activation of specialized sensory receptors called nociceptors that provide information about tissue damage. Pain then is the unpleasant emotional experience that usually accompanies nociception.

Two types of nociceptive pain are usually distinguished: pain emanating from the skin and deeper tissues (e.g. joints and muscle) is referred to as somatic pain while pain emanating from the internal organs is referred to as visceral pain. Somatic pain is usually well localized whereas visceral pain is harder to pinpoint.

In contrast to **nociceptive pain** *neuropathic pain* results from damage to the nervous system and two types of **neuropathic pain** have been distinguished.

Peripheral Neuropathic pain is pain resulting from a wound or damage to a primary nociceptor. While **central pain** is caused by damage to the central nervous system.

Historically, to learn something about the stimuli that activate nociceptors large numbers of randomly selected nerve fibers that innervate the skin were typically studied. Large peripheral nerves in mammals are actually compound nerves composed of bundles of thousands of individual nerve fibers enclosed in a loose connective tissue sheath. The

conduction velocity with which the individual nerve fibers within a bundle transmit action potentials to and from the nervous system can vary more than 100-fold, making it of interest to know the conduction velocity of the fibers that carry the signal from nociceptors to the brain. The electrical activity of an individual nerve fiber from a nerve bundle can be isolated and recorded from using a variety of methods, one of which is shown in Figure 1-1. In the example given, an intracellular electrode was used to impale the cell body of a sensory neuron in the dorsal root ganglion (DRG) and thereby record its electrical activity. The DRG are comprised of the cell bodies of sensory neurons, and are located lateral to the spinal cord in the vertebral column. These sensory neurons have an axon that projects to peripheral tissues, such as the skin, and are responsible for our sensation of our bodies. The trigeminal ganglion is analogous to the dorsal root ganglia of the spinal cord and is responsible for sensation in the face. The conduction velocity of the impaled neuron in Figure 1-1 was measured by using a brief voltage pulse applied to the extracellular stimulating electrodes to evoke action potentials in the nerve fibers composing the nerve bundle. By knowing the distance from the stimulating electrodes to the recording site, and the time it takes the action potential to reach the recording site following application of the voltage pulse, the conduction velocity can easily be calculated. Many of the afferent (sensory) neurons isolated in this way respond to low-intensity mechanical or thermal stimulation, that is, stimuli that in individuals evoke an innocuous or non-painful sensation. In addition, these fibers exhibit the full range of conduction velocities exhibited by the nerve. Relatively high thresholds for activation distinguish some of the neurons recorded this way, i.e. they can only be activated by intense (mechanical, thermal or chemical irritant) stimuli that are potentially damaging to tissues. These high threshold neurons are thought to be the primary afferent nociceptors.

We have all probably experienced that pain can be caused by thermal, mechanical and chemical stimuli that produce tissue injury. Several possibilities might explain how these different stimuli could result in the sensation of pain. One possibility is that individual nociceptors are sensitive to all of these different stimuli. Another is that there are several different types of nociceptors with each being sensitive to a specific stimulus. As we shall see below it turns out that both possibilities are found in nature: some nociceptors are sensitive to a specific stimulus while others are sensitive to multiple types of stimuli.

Classification of nociceptors by the conduction velocity of their axons

The nerve fibers (axons) within a compound nerve include both afferent nerves and efferent (motor and autonomic) nerves. The speed at which an individual nerve fiber conducts action potentials is related to the diameter of the fiber. In the larger myelinated fibers, the conduction velocity in meters per second is to a first approximation six times the axon diameter given in microns (see Figure 1-2). The histogram of the distribution of conduction velocities has four peaks: the slowest conducting fibers are unmyelinated and designated C; the faster conducting myelinated fibers are designated Aδ, Aβ and Aα. The widely held view that is presented in most present day textbooks is that only the smallest diameter and slowest conducting nerve fibers the C- and Aδ-fibers carry the afferent signal from nociceptors that is perceived as pain. Never the less the available evidence, which has been thoroughly reviewed (Lawson 2002, Djouhri and Lawson 2004),
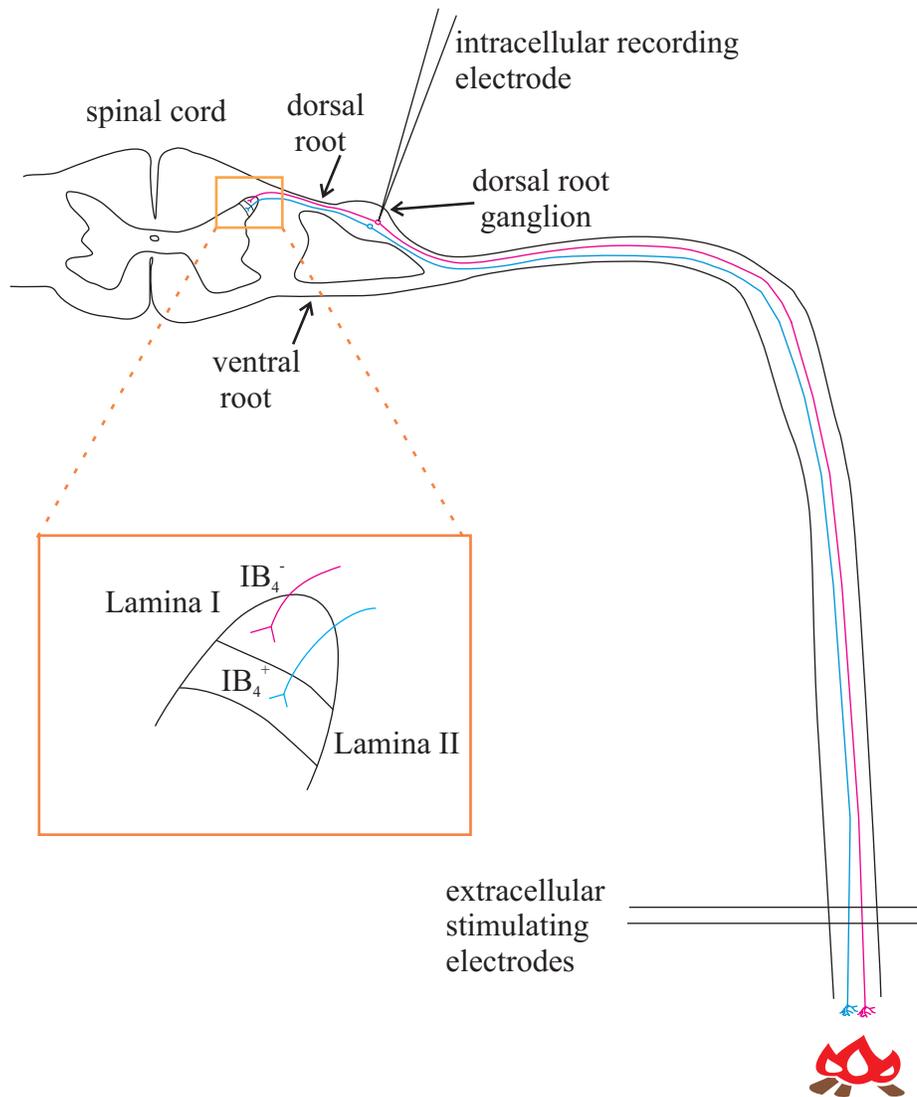
Figure 1-1. Intense heat from a fire activates the terminals of two nociceptors.
Action potentials are propagated along the axons of the nociceptors into
the spinal cord and the activity of one of the nociceptors is monitored by an
intracellular electrode which impales its cell body which is located in the
dorsal root ganglion (DRG). The central terminal of a fiber staining positive
for the plant lectin isolectin B4 (IB_4^+) is shown terminating in lamina II and
that of an IB_4^- fiber is shown terminating in lamina I. The extracellular
stimulating electrodes are connected to a pulse stimulator (not shown) and
are used to initiate action potentials in the nerve fibers.
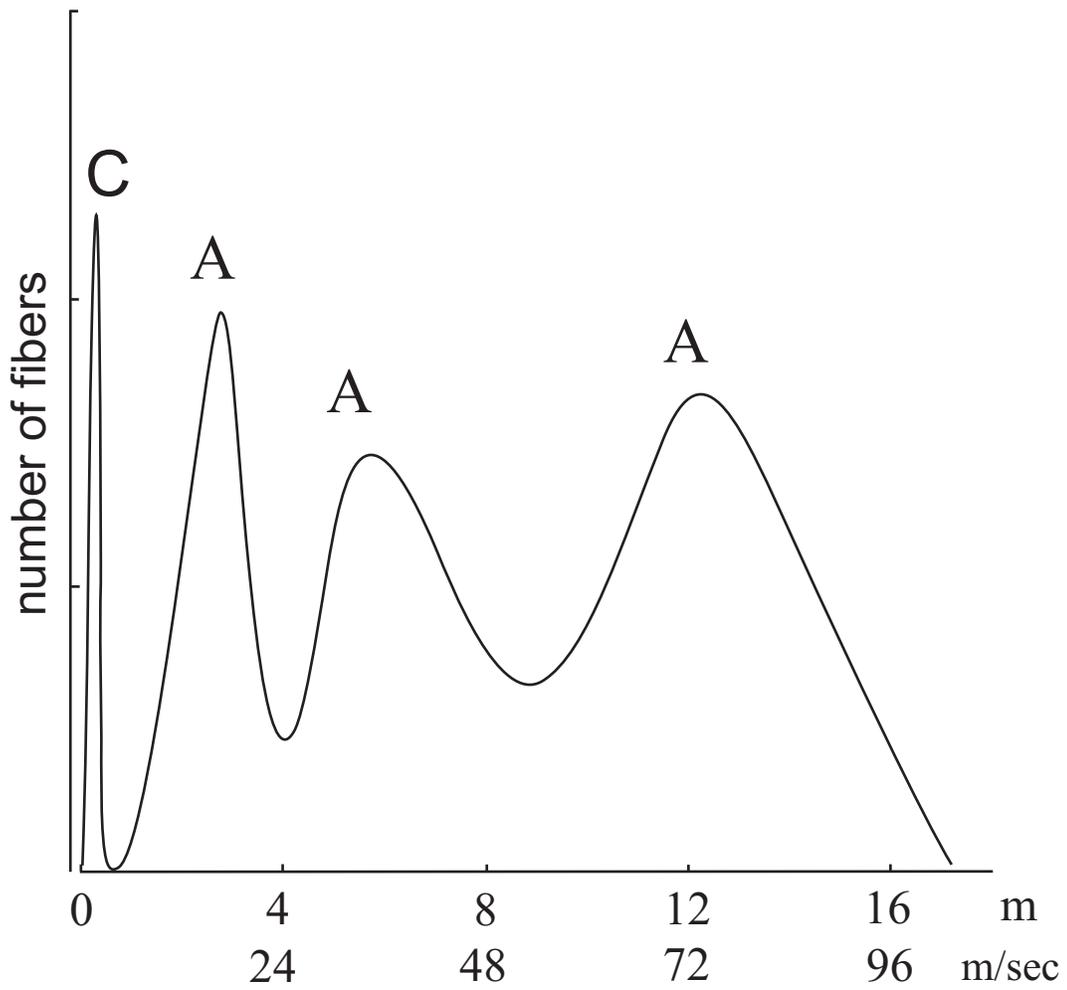
Figure 1-2. Axon diameters and conduction velocities in a peripheral nerve. Axon diameters are given in micrometers and conduction velocities are given in meters per second. The fibers designated with a C are unmyelinated and those with an A have a myelin coat.

suggests that a substantial fraction of the A-fiber nociceptors may conduct in the Aβ conduction velocity range. Hence, to allow for this possibility, the designation used here is that the signal from nociceptors is carried by unmyelinated C-fibers and myelinated A-fibers conducting in the A(δ-β) conduction velocity range. It should be kept in mind that the reverse is not true, not all C-fibers and A(δ-β) fibers are nociceptors. The C and A(δ-β) fibers also carry signals for non-noxious innocuous mechanical, warm and cold stimuli.

Because of the difference in conduction velocity between the C and the A(δ-β) fibers, the signal from the A(δ-β) fibers arrives at the spinal cord before that from the C-fibers. This raises the possibility that painful stimuli evoke two successive and possibly distinct painful sensations. The evidence supporting the view that C and A(δ-β) fibers signal distinct painful sensations comes from experimental conditions (electrical stimulation and nerve block) where the activity of the A- and C- fibers are studied in isolation. When this is done stimulation of the A-fibers is described as causing a sharp pricking pain sensation and that of the C-fibers a dull, aching burning pain. It is usually stated that for painful stimuli there is a biphasic subjective response: a short-latency pricking pain followed by a second long latency pain of a burning and less bearable quality. However, the evidence for two successive painful sensations is much less compelling than it is for two distinct painful sensations. In the original report showing that C and A(δ-β) fibers signal distinct painful sensations, it was stated that such a biphasic subjective response to a single transient painful stimulus is often absent in normal subjects (Bishop, Landau et al. 1958). The inability of many normal subjects to experience a first and second pain from one stimulus to the skin surface should not be taken to imply that these two types of pain are artifacts of the experimental conditions under which they were observed. Rather when both are activated simultaneously under normal conditions it is difficult for each to be identified by the observer.

When an observer can distinguish a first pain from a second pain, the first pain is usually felt within about several hundred milliseconds after stimulus application. Whereas the slower second pain typically begins after about 1 second and increases slowly over time.

If a noxious thermal stimulus consisting of a rapid step in temperature, using a laser thermal stimulator, is applied to the volar surface of the forearm a double pain sensation is perceived. First there is a sharp pricking sensation followed after a lull by a second burning feeling. For this stimulus the first pain sensation must be signaled by A(δ-β) fibers because for the highest temperatures the sensation is perceived within 400 ms which implies a conduction velocity of greater than 6 meters per second (Campbell and LaMotte 1983). Interestingly when the same stimulus was applied more distally to the thenar eminence there was no first and second pain but only a longer latency burning pain. Two classes of A fiber nociceptors have been characterized in monkeys. Type I fibers are responsive to mechanical and chemical stimuli and also heat stimuli with thresholds greater than 50°C. For brief short duration heat stimuli thresholds can be greater than 53°C which may account for the reason that the responsiveness of type I fibers to heat had been overlooked. Type II fibers on the other hand are either mechanically insensitive or have a very high mechanical threshold which may explain

why they were rarely encountered when mechanical stimuli were used to search for nociceptors. They have a lower heat threshold, below 50°C, than type I fibers and have an early peak response to noxious thermal stimuli. **Activity of type II fibers is thought to mediate the first pain to thermal stimuli in humans.**

Classification of nociceptors by the noxious stimulus

Nociceptors respond to noxious **cold**, noxious **heat** and high threshold **mechanical** stimuli as well as a variety of **chemical mediators.** However, not every nociceptor responds to each of the noxious stimuli. The apparent lack of a response to a noxious stimulus may result because the stimulus intensity is insufficient. Additionally, application of a high intensity stimulus of one modality may alter the response properties of the nociceptor to other modalities. Consequently it is not possible to generate a comprehensive list of all the different types of nociceptors and the noxious stimuli and chemicals each one responds to.

Several classes of nociceptors: mechanical, thermal, mechano-thermal, polymodal, and silent, have been described. Mechanical nociceptors respond to intense pressure while thermal nociceptors respond to extreme hot or cold temperatures (>45°C or <5°C) and mechano-thermal nociceptors respond to both thermal and mechanical stimuli. Typically these three types of nociceptors have myelinated A fibers that conduct impulses at a velocity of 3 to 40 m/s. Collectively, these 3 types of nociceptors are called A(δ- β)-nociceptors. Polymodal nociceptors respond to noxious mechanical, thermal, and chemical stimuli and typically have small, unmyelinated C fibers that conduct impulses at a velocity of less than 3 m/s. Remember that the small, myelinated A(δ- β) fibers carry the nociceptive input responsible for the sharp pricking pain and the small, unmyelinated C fibers carry the nociceptive input responsible for the dull burning pain. Silent nociceptors are activated by chemical stimuli (inflammatory mediators) and respond to mechanical and thermal stimuli only after they have been activated. These nociceptors also have small, unmyelinated C fibers that conduct impulses at a velocity of less than 3 m/s.

The basic function of nociceptors is to transmit information to higher-order neurons about tissue damage. Individual receptors can be regarded as an engineers "black-box", which transforms tissue damage into an appropriate signal for successive nerve cells. The ultimate function of a nociceptor could be fully described if its input-output relationship alone were given. Here input, of course refers to tissue damage. What about output? One of the central concepts of neurobiology holds that neurons communicate with each other via synapses. The most commonly encountered synapses release chemicals, known as synaptic transmitters. It is by releasing these transmitters that one cell is able to communicate with its postsynaptic neighbors. Because nociceptors are neurons with chemical synapses, their output is encoded in the release of their neurotransmitters: the input-output relationship is simply a conversion of tissue damage into transmitter release.

Direct measurement of synaptic transmitter release under physiological conditions is very difficult and has not been accomplished for any nociceptor. It would thus seem that a

derivation of the input-output relationship is beyond reach. However, another nearly universal neural property is of assistance: transmitter release is directly controlled by synaptic membrane potential. Therefore, by recording the variation of the membrane potential at the synapse, the nociceptor output could be indirectly surmised. Unfortunately, in most cases, it is technically difficult, if not impossible to record intracellularly from a synaptic terminal. The vast majority of electrophysiological recordings have been carried out on other regions of the cell because these regions are more accessible. Electrical activity in nociceptors as in most neurons is associated with propagating action potentials, which occur on a time scale of milliseconds. These action potentials propagate to the synaptic terminal and thereby regulate transmitter release. Two recording techniques are typically used to record nociceptor action potentials: either extracellular electrodes record their occurrence somewhere along the nociceptor axon or they are recorded intracellularly from the nociceptor cell body as illustrated in Figure 1-1. Thus, sensory transduction for nociceptors is typically measured as the conversion of tissue damage into the patterned firing of action potentials.

During the past century, the basic framework of sensory transduction for different senses and for many species of vertebrates and invertebrates was established. The typical sensory cell was shown to have a specialized region where sensory receptor molecules detect the stimulus, which for nociceptors is tissue damage. The sensory stimulus causes a conformational change in the receptor molecule, which triggers the transduction process that brings about a change in the membrane potential of the receptor cell. The resulting change in membrane potential is called the receptor potential. In the typical sensory neuron, the part of the cell where sensory transduction takes place is often distant from the synaptic terminal. Therefore, the receptor potential needs to be converted into a series of propagating action potentials, which in turn carry the signal along the axon to the synapse. Unstimulated nociceptors typically fire few or no action potentials, and their response to tissue damage is an increase in the rate of firing of propagating action potentials along the cells' axon. These findings are summarized in Figure 1-3, which shows the four most significant regions of an idealized nociceptor, the sensory transduction region, the axon, the cell body, and the synaptic terminal.

In parallel with the anatomic regions of a nociceptor shown in Figure 1-3, one can describe a functional scheme for the mechanism of operation of a nociceptor as in Figure 1-4. The cell body, axon and presynaptic terminal of nociceptors should function more or less as they do in other cells. The arrows going from the cell body to the other regions of the nociceptor are meant to indicate that the cell body is necessary to maintain the other regions of the cell, without the cell body the cell would eventually die. The axon conducts the action potential to the synaptic terminal where the transmitter(s) are released. The release of transmitter at the synaptic terminal is subject to modulation by agents released by other neurons and possibly glial cells. Stimuli that cause tissue injury may activate the sensory transduction region of nociceptors either directly or indirectly. For example, a nociceptor may contain heat sensitive receptor molecules in the plasma membrane that respond directly to a damaging heat stimulus or conversely tissue damage by the stimulus might result in the production of a factor that in turn activates the nociceptor. A third possibility is that, because of the injury the tissue becomes inflamed

receptor
potential

cell
body

stimulus

presynaptic
terminal

sensory
transduction
region

axon
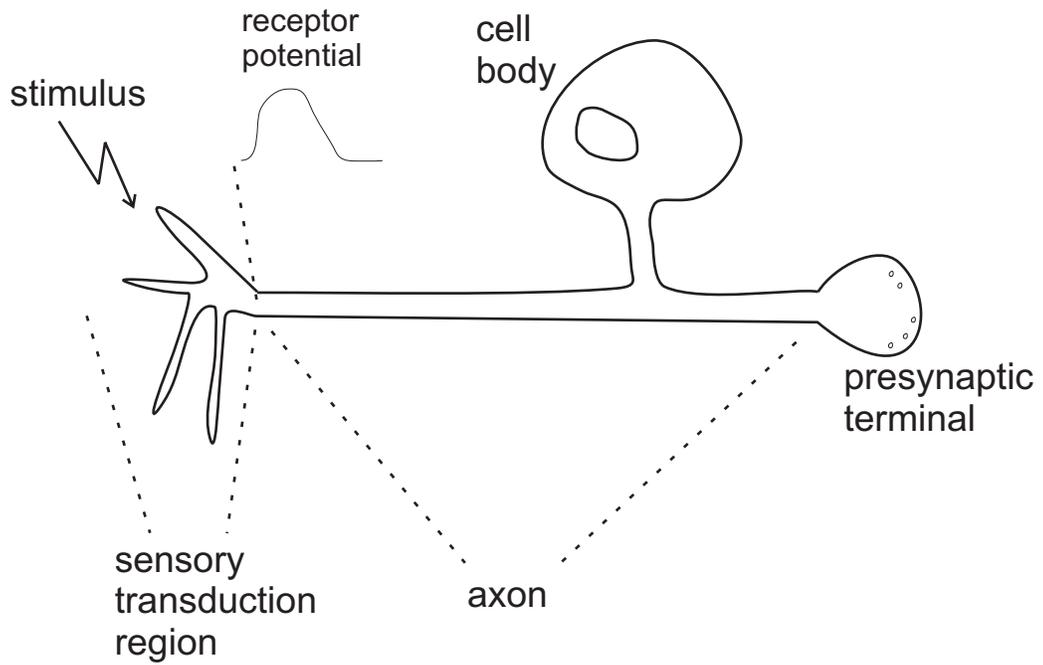
Figure 1-3. Schematic drawing of a nociceptor showing the
four regions of the cell.

and an extrinsic factor, that activates the nociceptor, enters the damaged region. The box labeled perireceptor events in Figure 1-4 allows for the production, during tissue injury, of factors, which might activate or modulate the nociceptor. Intense noxious stimuli often lead to an increase in the response to subsequent painful stimuli (gain control in Figure 1-4). After the noxious stimulus is transduced into a receptor potential the response must be transformed or encoded into a series of action potentials, which carry the signal to the synaptic terminal. The current prevailing view is that free nerve endings of A(δ- β) and C fibers constitute the sensory region of nociceptors. There are no specialized structures associated with the nerve endings as there are for other sensory receptors such as mechanoreceptors. Many other sensory receptors can be isolated functionally intact, and the study of isolated photoreceptors, auditory receptors, olfactory receptors, etc. has told us a great deal about their mechanisms of operation. Ideally, to study the pathways involved in the transduction process of nociceptors, a preparation of isolated nociceptors is needed. The free nerve endings of nociceptors are extremely fine and are embedded in a tissue matrix, which if it were to be dissected, to isolate the nociceptors, would release the very molecules that the nociceptor nerve terminal is meant to detect. Because of this lack of accessibility, it is not possible to directly study the nociceptor transduction machinery both in an unstimulated state and in its' normal native environment. It should be kept in mind that not all free nerve endings represent the sensory transduction region of nociceptors. Free nerve endings are also responsible for detecting temperature, mechanical stimuli (such as pressure), and information about touch.

Because it is not possible to isolate the nociceptors sensory nerve endings in an unstimulated state, studies on isolated nociceptors are often carried out on the cell bodies of nociceptors. For example, the neuronal cell bodies of a dorsal root ganglion are isolated by enzymatic treatment and are cultured before use. The sensory endings are completely removed during the isolation procedure, and it is hoped or assumed that the properties of those terminals are recreated in the cultured cell bodies. In as much as the original ganglion contained more than just nociceptors, only a fraction of the cultured cell bodies will actually be the cell bodies of nociceptors. This preparation of cultured cell bodies is often used for experiments investigating the cellular and molecular basis of detection of painful stimuli. The uncertainties and assumptions associated with these procedures make it essential that the findings be checked very carefully and shown to resemble what actually occurs *in vivo.*

As mentioned above unstimulated nociceptors typically fire few or no action potentials, and their response to tissue damage is an increase in the rate of firing of action potentials. Since it takes a membrane depolarization to cause an increase in the rate of firing this finding implies that the receptor potential of nociceptors is a membrane depolarization, as shown in Figure 1-3. Thus, the transduction machinery in Figure 1-3, by necessity, must somehow gate ion channels or carriers that can depolarize the plasma membrane of the transduction region. The encoding region (see Figure 1-4) in turn converts the membrane depolarization into an increase in the rate of firing of action potentials.
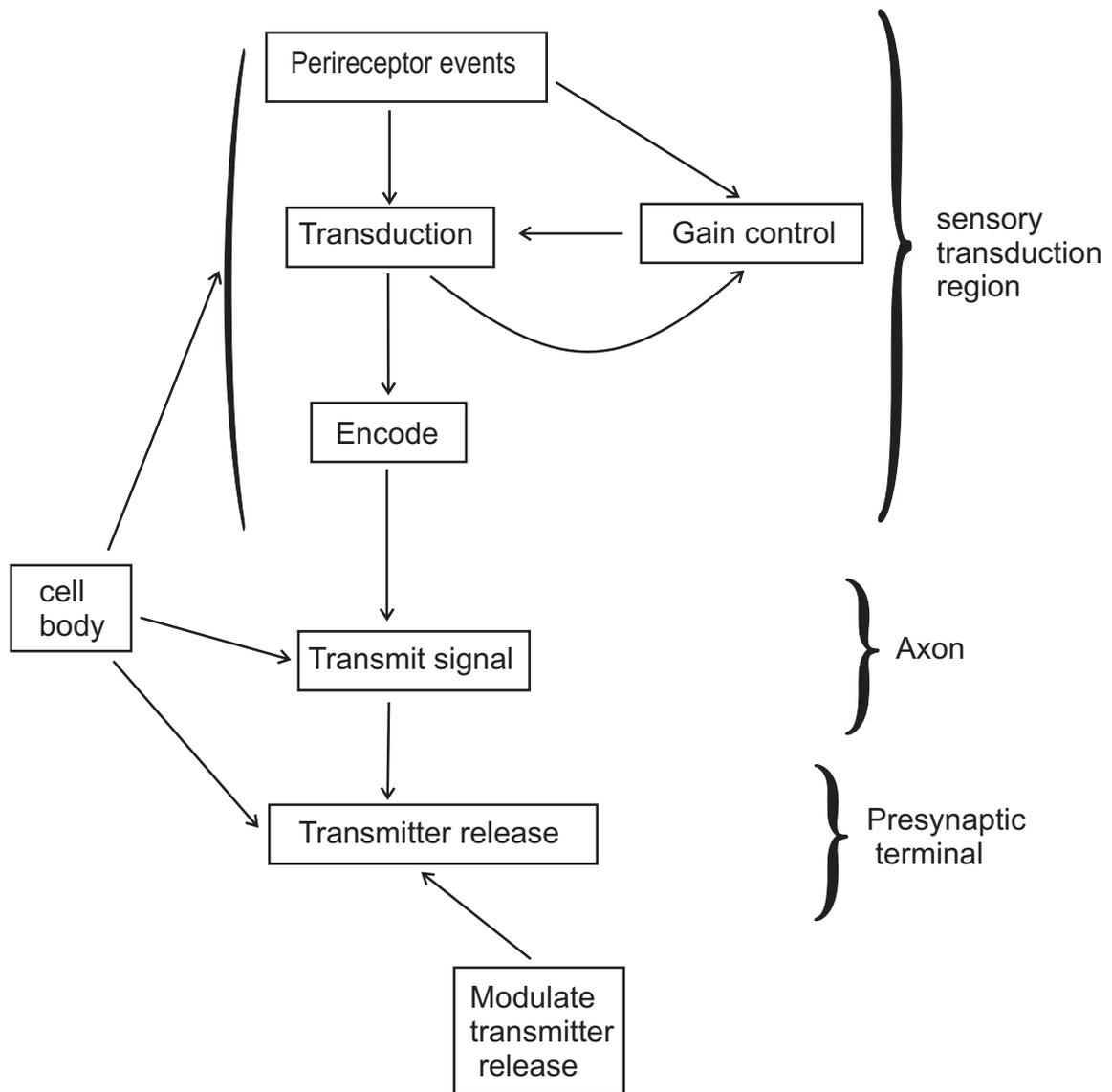
Figure 1-4 Functional schematic of nociceptor operation
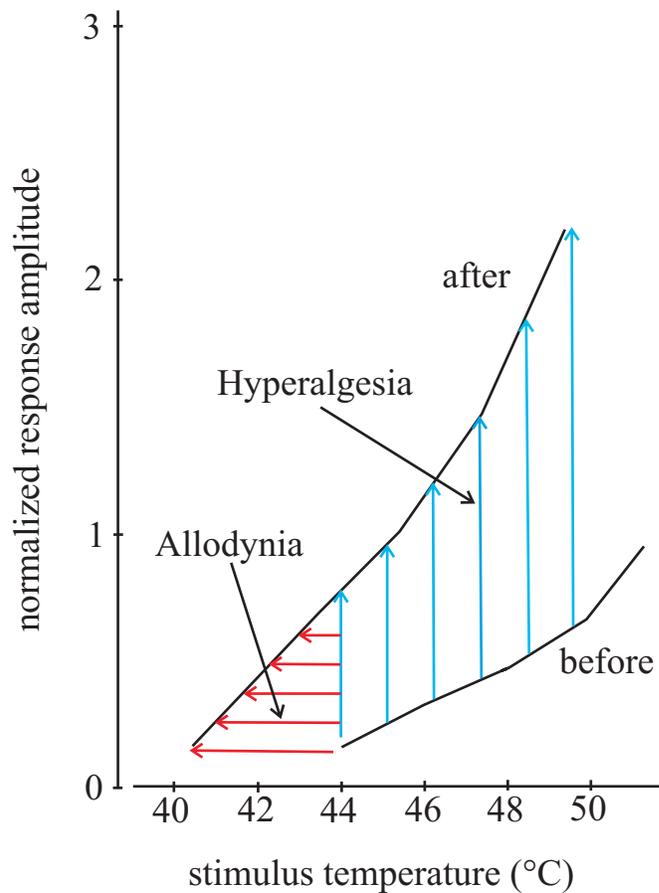
Figure 1-5. Sensitization of a thermal nociceptor to stimuli that heated an area of the skin to the temperature indicated. ( )Individual responses to thermal stimuli obtained before the area of skin was burnded. ( ) Sensitized responses obtained from the same area after the burn injury.

Hypersensitivity: hyperalgesia and allodynia

The properties of nociceptors considered so far were elucidated primarily from studies of uninjured tissue. However, intense noxious stimuli resulting in tissue damage often lead to an increase in the response to subsequent painful stimuli, called hypersensitivity, that is, an excessive sensitiveness or sensibility to pain. Hypersensitivity comprises both primary hypersensitivity, an increased sensitivity within the injured area predominantly due to peripheral nociceptor sensitization, and secondary hypersensitivity, an increased sensitivity in the surrounding uninjured area mediated centrally (see Chapter 7). Those of us that have been injured can probably remember having experienced this hypersensitivity to pain at the site of injury and the surrounding region. For example, the inflammation due to a sore throat can be so bad that the mere act of swallowing is painful. No description of the properties of nociceptors would be complete without a consideration of nociceptor sensitization resulting from tissue injury. Hyperalgesia and allodynia (Figure 1-5) can be thought of as useful adaptations for better protection of injured tissues while healing. However, pain hypersensitivity may persist long after the initial cause has disappeared, then pain is no longer a symptom of the injury but rather a disease in its own right.

Sensitization is a leftward shift (that is toward lower intensities) of the stimulus-response curve, which relates the magnitude of the neural response to the stimulus intensity. As shown in Figure 1-5, sensitization of a nociceptor is characterized as a decrease in threshold and an increase in the magnitude the response to suprathreshold stimuli. Pain scientists distinguish two aspects of sensitization: allodynia (pain resulting from a normally innocuous stimulus) and hyperalgesia (an enhanced response to a normally painful stimulus). Keep in mind that some but not all nociceptors exhibit sensitization.

In contrast to nociceptors, which **sensitize** when exposed to intense noxious stimuli, other types of sensory receptors **desensitize** when exposed to intense stimuli. We have all probably experienced the desensitization of the photoreceptors in our eyes when we are exposed to bright lights. If we leave a brightly lit street and enter a darkened theater, we are suddenly "blind," we find our way by among the rows by touch and feel. With time in the dark our vision improves so that what was previously invisible can eventually be seen. The loss of sensitivity when exposed to increased ambient light intensities is referred to as light adaptation.

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Chapter 2   **IONIC PERMEABILITY AND SENSORY TRANSDUCTION**

In trying to understand nociceptor sensory transduction, it has proven to be instructive to consider the molecular mechanisms utilized for signaling elsewhere in the nervous system. Mechanisms used by the other senses as well as those used for synaptic transmission have turned out to be most useful. Numerous studies have shown that both the sensory receptor potential and the synaptic potential are the result of changes in the ionic permeability of the plasma membrane. That is, they are the result of ions flowing through integral membrane proteins called ion channels. Before considering how the opening and closing of ion channels is regulated during nociceptor signal transduction, it is important to understand the forces that cause ions to flow through these channels. Basically there are two types of forces that drive ionic movement across cell membranes. There is the electric field across the cell membrane, which is manifest as an electrical potential across the membrane and there is the concentration gradient for each ionic species.

Most cells of the body, including neurons, maintain their cytoplasm at a negative potential with respect to the outside of the cell. For a cell that is in an unstimulated resting state, scientists typically use the term *resting potential* in referring to this negative potential across the cell's plasma membrane. Generally speaking, the resting potential depends on the concentration of ions on the two sides of the plasma membrane and the resting permeability of the cell to these ions. Neglecting the small but not unimportant contribution of other ions we focus our attention on sodium (Na) and potassium (K). If the membrane were exclusively permeable to K, the membrane potential (V_M) would be given by

$$V_M = E_K = (RT/F)\ln[(K)_o/(K)_i] \qquad \text{(Equation 2-1)}$$

Where V_M is the membrane potential, inside minus outside, E_K is the potassium equilibrium potential, $(K)_o$ the extracellular potassium activity, $(K)_i$ the intracellular potassium activity, R the universal gas constant, T the absolute temperature and F the Faraday constant. Similarly, if the membrane were exclusively permeable to Na, the membrane potential would be given by

$$V_M = E_{Na} = (RT/F)\ln[(Na)_o/(Na)_i] \qquad \text{(Equation 2-2)}$$

Where E_{Na} is the sodium equilibrium potential, $(Na)_o$ the extracellular sodium activity, $(Na)_i$ the intracellular sodium activity. As shown in Figure 2-1, for most cells including neurons $(Na)_o$ is much higher than $(Na)_i$, and $(K)_i$ is much higher than $(K)_o$.

In general, at rest, biological membranes and neuronal membranes in particular are permeable to both Na and K and therefore their *resting potential* lies somewhere between E_{Na} and E_K, the proximity to either of these equilibrium potentials depending on the relative permeability to Na and K. For the typical cell shown in Figure 2-1, V_M must therefore lie some where between $E_{Na} \approx 65$ mV and $E_K \approx -85$ mV. Cells are typically much more permeable to K at rest than Na; therefore the resting potential is always inside

$Na^+ = 140$ mM

$K^+ = 5$ mM

$Cl^- = 105$ mM

outside

inside

$Na^+ = 10$ mM

$K^+ = 150$ mM

$Cl^- = 10$ mM

E_{Na} 65 mV

E_K -85 mV

Figure 2-1. Extracellular and intracellular concentrations of K^+, Na^+ and Cl^- for a typical cell.

negative and can vary from about -30 mV up to about -80 mV, depending on the degree of permeability to Na.

Whenever the membrane potential lies between E_{Na} and E_K, Na will tend to leak into and K will tend to leak out of the cell. Unless the cell compensates for the constant loss of K and gain of Na, the ionic concentration gradients will run down, E_{Na} and E_K will decrease toward zero, and the membrane potential will disappear. Cells have metabolically dependent enzymes, called pumps, which compensate for this passive leakage by pumping K into and Na out of the cell. This molecule (Na^+/K^+ ATPase) is a Na-K pump, which uses ATP to catalyze the movement of 3 Na ions out of the cell for every two K ions moving inward. There is a net extrusion of one positive charge out of the cell for each cycle of the pump, thus the pump is electrogenic. It is generally agreed that the pump does not directly participate in the generation of electrical signals but rather has its primary effect by maintaining the ionic concentration gradients for Na and K across the cell membrane. Calcium ions have also been found to play an important role in synaptic transmission and sensory transduction and cells have both calcium pumps and exchangers which keep calcium inside the cell, at a much lower concentration, than outside.

ION CHANNELS

Molecular biology has provided us with a basic understanding of the relationship between the structure and function of ion channels in general. Channel proteins have amino acid sequences that extend across the lipid bilayer of the plasma membrane from the inside to the outside of the cell. They contain a specialized region called the P- or pore region, which forms a channel or pore that provides a path through which ions such as Na^+, K^+, Ca^{2+}, and Cl^- can pass through the membrane. The salient feature of the ion channels that underlie the receptor potential and the synaptic potential is that they undergo a transition from a closed to an open state (see Figure 2-2) that is regulated or gated by changes to the channel that result from the sensory stimulus or the synaptic transmitter. Two well understood mechanisms used to gate these channels are shown in Figures 2-3 A & B. For synaptic transmission (Figure 2-3A) the synaptic transmitter (i.e. ligand) binds to extracellular sites on the ion channel in the postsynaptic membrane, and gates it open. These ligand-gated channels are also sometimes referred to as *ionotropic channels*. For many sensory stimuli an intracellular second messenger, generated by the sensory transduction process (Figure 2-3B) gates the channel open. For the purposes of simplicity, the channel shown in Figure 2-3 A is shown with two external binding sites and that in Figure 2-3B with two internal binding sites, although in nature channels often have more than two binding sites.

Channels are typically not the property of a single protein molecule, but rather are the result of the non-covalent binding of several subunits facing one another to form the pore region. Channels can be either *homomeric*, in which all the subunits are identical, or *heteromeric*, that is having non-identical subunits with different properties. The pore region can be selective for either: Na^+, K^+, Ca^{2+}, and Cl^-. Additionally some channels are
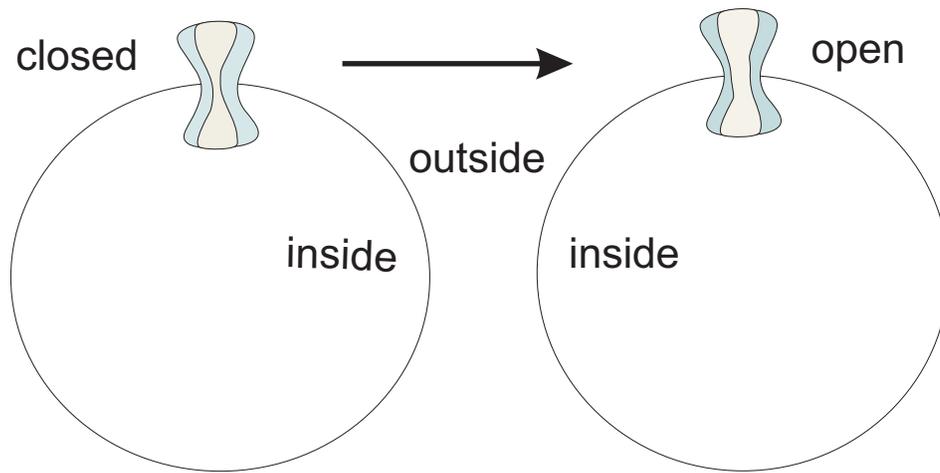
Figure 2-2. Transition of a membrane channel pore region from the closed to the open state.
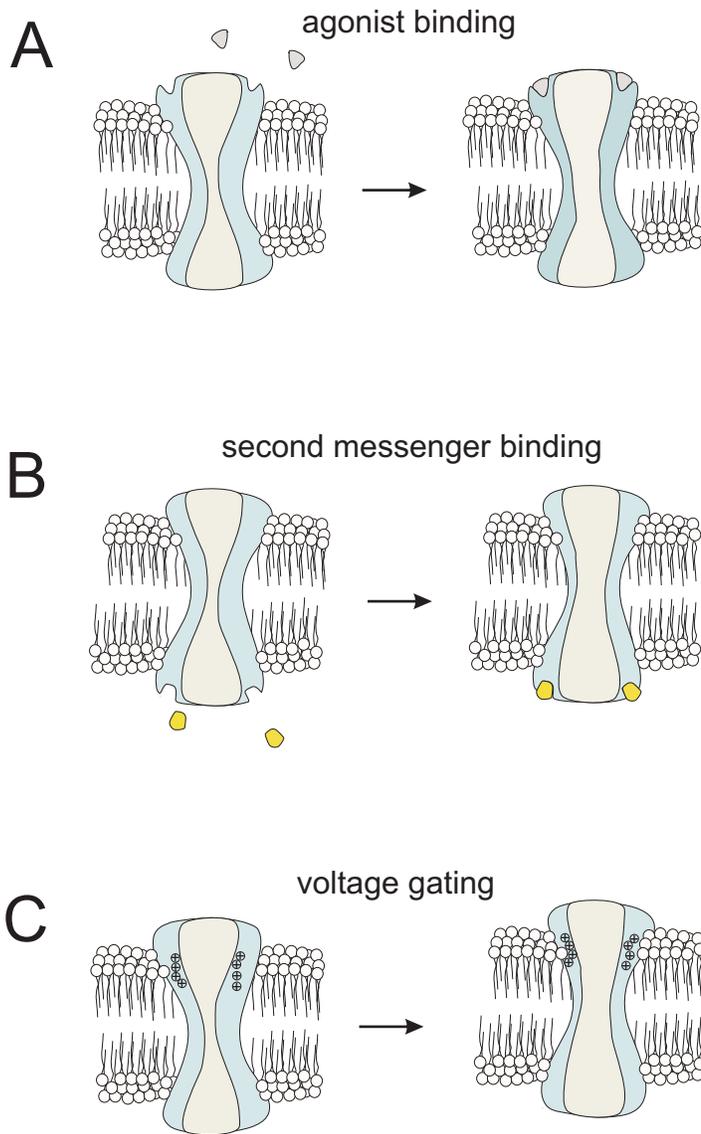
Figure 2-3. Several mechanisms of ion channel gating, (A) Binding of and extracellular agonist gates the channel open. (B) Binding of an intracellular second messenger gates the channel open. (C) Charge movement within the channel protein due to membrane depolarization gates the channel open.

found to allow all three cations (Na^+, K^+ and Ca^{2+}) to pass through their pore region, such channels are referred to nonselective cation channels.

The majority of ion channels have three, four, or five subunits, arranged in circular symmetry, forming a single aqueous pore at the axial intersection (see Figure 2-4A for an example with four subunits). And as shown in the figure each subunit has only a single pore domain. In contrast, as shown in Figure 2-4B, K^+ leak channels are composed of two pore domain K^+ channels (K_{2P} channels). Potassium leak channels are essential to neuromuscular function because they are responsible for cells being more permeable to K^+ at rest than to Na^+, they typically stabilize the cells membrane potential at hyperpolarized voltages below the firing threshold of nerves and muscles. In these channels, as shown in Figure 2-4B, each subunit has two pore domains arranged in tandem.

Except for those cases where binding of the ligand to the ionotropic channel actually decreases the permeability of the channel, and decreases membrane conductance, the transmitter usually opens the channel, allowing ions to flow through it, thus increasing the conductance of the cells membrane for ions. The response to the ligand turns off when the ligand unbinds and diffuses away (or is broken down), the channel then shifts back to its closed conformation. Surprisingly, molecular biology has revealed a multiplicity of genes for ionotropic receptors that appear to have essentially identical functions. For example, the nicotinic acetylcholine receptor found in neurons which typically has five subunits (it is pentameric), consisting of only two types of subunits, alpha and beta (2 alphas and 3 betas). It turns out that there are at least 8 genes that encode alpha subunits and 4 that encode beta. Thus there are a large number of different possible combinations of alpha and beta subunits in one animal, the function of which is not understood. The tacit assumption is that these different genes evolved because they sub serve different functions. Two obvious possibilities are that they have different affinities for acetylcholine and therefore open at different concentrations or they have slightly different ionic permeability properties.

 Unlike the ***ionotropic receptors***, where the receptor and the channel are the same molecule, the receptor molecule for the ***metabotropic receptor*** gates the channel indirectly, that is the receptor is a separate molecule from the ion channel that underlies the receptor potential. The metabotropic receptors can be classified into two types: the G-protein-coupled receptors (GPCRs) and receptor tyrosine kinases. The family of GPCRs are coupled to an effector molecule via a guanosine nucleotide-binding protein (a G-protein), hence their name. Activation of the effector component typically requires the participation of several other proteins in addition to the G-protein. Usually the effector molecule is an enzyme that produces a diffusible second messenger, for example, cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), arachidonic acid, diacylglycerol, or an inositol polyphosphate. These second messengers can either directly gate the ion channel (see Figure 2-3B) or can trigger a further biochemical cascade. For example the second messenger might mobilize calcium ions from intracellular stores and the elevated intracellular calcium might directly gate an ion channel. Another possibility is that the second messenger activates specific protein
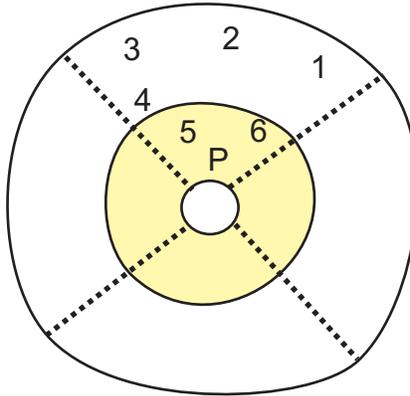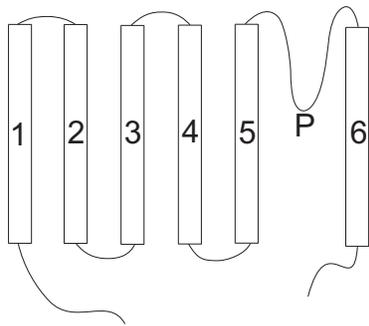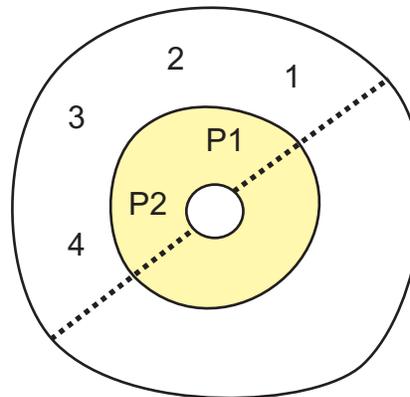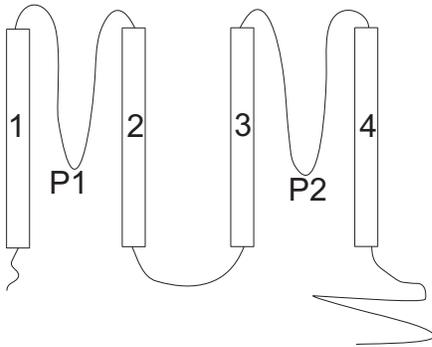
Figure 2-4. Schematic illustration of the structure of ion channels with one pore domain (A) or two pore domains (B).

kinases (phosphate transferring proteins) that phosphorylate the ion channel or other cellular proteins (thereby altering their activities) and either initiating or modulating the receptor potential. In some instances, the G protein of the second messenger can act directly on an ion channel. The receptor tyrosine kinases might gate the ion channel directly or indirectly via phosphorylation that is they transfer a phosphate group to the channel or other cellular proteins.

The channels found in both the encoding region (see Figure 1-4) and the axon, which convert the receptor potential into a train of propagating action potentials are gated by membrane depolarization (see Figure 2-3C). Both voltage gated Na^+ and K^+ channels play an important role in the generation and propagation of action potentials. The voltage gated Ca^{2+} channels play an important role at the presynaptic terminal where they function in the release of the synaptic transmitter.

SENSORY STIMULI

Before delving into the molecular mechanisms underlying nociceptor signal transduction, it is helpful to briefly consider the types of stimuli that occur during tissue damage, with an emphasis on stimuli that one might reasonably assume to be involved in signal transduction. First there are the stimuli themselves, such as mechanical tissue deformation, and either increases or decreases in tissue temperature. These stimuli might directly regulate ion channels (see Figure 2-2) in the nociceptor plasma membrane thereby giving rise to the receptor potential. Next there are the local changes in the extracellular milieu resulting from release and or exposure of molecules from the damaged tissue. That is, molecules normally found either inside cells or in the cell membrane might now be found in or exposed to the extracellular space where they can bind to receptors in the plasma of the nociceptor. Finally there are the molecules that enter the damaged region, as part of the bodies' inflammatory response to injury, where they can bind to receptors in the nociceptor plasma membrane. Any of these three possibilities might reasonably be expected to participate in nociceptor signal transduction.

Ideally one would like to identify the specific role in nociceptor signal transduction if any, of all the substances that appear in damaged tissue during painful stimuli. However as pointed out in Chapter 1, it is not possible to isolate the nociceptors sensory nerve endings in an unstimulated state and study how they respond to painful stimuli. Rather, as we shall see one is forced to use indirect methods. For example, the neuronal cell bodies of a dorsal root ganglion are often used after being isolated and cultured.

Chapter 3. **THERMAL RECEPTORS AND MECHANICAL RECEPTORS**

The TRP channel family is of interest because several members have been implicated in the signal transduction of nociceptors. I have strived to limit the consideration of TRP channels to their role in nociception resulting in a superficial consideration of all their known properties.

MAMMALIAN TRP CHANNELS

The *trp* mutant was originally isolated from *Drosophila* photoreceptors in which the light response decayed to baseline during prolonged illumination, hence the name *t*ransient *r*eceptor *p*otential, *trp*. The gene was found to encode a Ca^{2+}-selective ion channel responsible for the major component of the light response. Based on sequence homology numerous members of the TRP channel family have been identified in vertebrates; the mammalian members of this family have been classified into 6 subfamilies: TRPC (Canonical), TRPV (Vanilloid), TRPM (Melastatin), TRPP (Polycystin), TRPML (Mucolipin) and TRPA (Ankyrin). Mammalian TRP channels are permeable to cations, and have 6 transmembrane domains flanked by intracellular N and C-terminal regions. Four subunits are thought to assemble as homo-and/or heterotetramers to form functional channels. Although TRP channels may be weakly voltage-dependent they lack the voltage sensor of voltage-gated channels (see Figure 2-3C).

CHEMESTHESIS

The sense of taste (gustation) is the ability to perceive the flavor of substances such as food. Taste sensations include sweet, salty, sour, bitter and umami (savory). Umami is the taste that occurs when foods with the amino acid glutamate are eaten. In contrast to the sense of taste, the trigeminal nerve conveys information about irritating and noxious molecules that come into contact with the mouth. **Chemesthetic** sensations are defined as those that occur anywhere in the body when chemicals activate receptors for other senses. Thus the sensations transmitted to the brain when noxious molecules activate pain fibers of the trigeminal nerve would be described as chemesthetic sensations. The burn from **chili pepper** and the cooling from the menthol in mouthwash are examples of chemesthesis.

MEDIATORS OF NOXIOUS HEAT

TRPV1

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) the pungent ingredient of hot **chili peppers** that gives them their burning sensation or piquancy was first isolated in the nineteenth century. Interest in the sensory effects of capsaicin has a very long history. Christopher Columbus described the eating of chili peppers by natives in the New World more than 500 years ago and Wilbur Scoville developed a test and a scale in 1912 to measure the hotness or piquancy of chili peppers, and the Scoville scale is still in use today.

The effects of capsaicin are best understood in terms of its excitatory and desensitizing, actions on polymodal nociceptors. Electrophysiological studies reveal that capsaicin depolarizes DRG neurons (see Figure 1-1) and decreases their input resistance in a concentration dependent manner, suggesting that the specific excitatory effect of capsaicin on nociceptive neurons involves an increase in membrane permeability to ions such as sodium and/or calcium. Subsequent studies showed that the ionic permeability pathway discriminated poorly between cations, with divalent cations being relatively more permeable than monovalent cations. The discovery of resiniferatoxin an ultra potent capsaicin analog that mimics the cellular actions of capsaicin, and of the potent capsaicin antagonist, capsazepine, strongly suggested the existence of a specific capsaicin receptor.

The capsaicin receptor (TRPV1) was cloned using a calcium influx assay of non-neuronal cells transfected with cDNA constructed from dorsal root ganglia RNA (Caterina, Schumacher et al. 1997). Electrophysiological analysis proved that the cloned receptor was similar to the native capsaicin-receptor of sensory neurons in several ways. Capsaicin-evoked currents were reversible upon ligand removal and lower concentrations of resiniferatoxin evoked maximal responses that persisted after ligand removal. The activation curves for capsaicin-currents from both native channels and the cloned receptor showed Hill coefficients of 2 suggesting the existence of more than one capsaicin-binding site. TRPV1 channels are not only activated by capsaicin but also by elevated temperatures and by protons at pH below 6.5, confirming earlier studies showing that currents evoked by heat, low pH and capsaicin were commonly found in the same sensory neuron. Interestingly, TRPV1 is the only member of the TRPV channel family that is activated by capsaicin: knockout of TRPV1 in man and mouse result in capsaicin insensitivity (Caterina, Leffler et al. 2000, Park, Lee et al. 2007). We will consider the effects of low pH on TRPV1 along with its effect on other ion channels elsewhere.

Earlier studies showed that heat-evoked and capsaicin-evoked currents were commonly found in the same sensory neuron. This raised the possibility that TRPV1 was a temperature detector that enabled thermal nociceptors to respond to a range of hot temperatures. If this were so then in animals in which TRPV1 was knocked out one would expect a deficit in responding to those hot temperatures that activate TRPV1. Paradoxically mice in which TRPV1 is knocked out exhibited deficits in their response to hot temperatures above 50°C whereas TRPV1 is activated at temperatures at or above 42°C. Although these animals were impaired in the detection of painful heat it is clear that either they normally use an alternative mechanism, other than TRPV1, to detect noxious heat at temperatures below 50°C, or they have a backup mechanism.

Remember that intense noxious stimuli resulting in tissue damage often lead to an increase in the response to subsequent painful stimuli, referred to as hyperalgesia, and that primary hyperalgesia is due to peripheral nociceptor sensitization or hypersensitivity. Interestingly, TRPV1 knockout mice exhibited little thermal hypersensitivity in the setting of tissue inflammation whereas wild type mice exhibited normal hypersensitivity. We will return to this finding elsewhere when we consider the mechanisms of hyperalgesia.

We are all probably familiar with the burning pain produced by the application of alcoholic tinctures, such as tincture of iodine, to skin wounds. The burning sensation, raises the possibility that ethanol might be activating TRPV1. To test this idea the effect of ethanol on isolated neurons from the trigeminal or dorsal root ganglia as well as TRPV1-expressing HEK293 cells was investigated (Trevisani, Smart et al. 2002). It was found that ethanol activated TRPV1 and potentiated responses to capsaicin and other activators of TRPV1; supporting the notion that alcohol causes a burning sensation by activating TRPV1. The uncertainties and assumptions associated with using isolated and cultured trigeminal and DRG neurons as well as cells made to express TRPV1 require that these findings be checked very carefully and shown to reflect what actually happens *in vivo*.

TRPV1 AS A THERAPEUTIC TARGET

In contrast to the hyperalgesia (excessive sensitiveness or sensibility to pain) following intense noxious stimuli, exposure to capsaicin can result in a subsequent desensitization. Whereas desensitization to comparatively low doses of capsaicin may be specific for capsaicin and its congeners desensitization to higher doses is associated with a loss of responsiveness to other chemicals, heat and noxious (high threshold) mechanical stimuli. This cross desensitization of noxious stimuli by capsaicin suggests the use of capsaicin or an analog of it as an analgesic. Of course the ultimate goal, not yet achieved, is to find an analog of capsaicin that induces analgesia without first causing pain.

Capsaicin desensitization is well documented, with the extent of desensitization depending on the capsaicin concentration, how frequently it is applied and for how long. Capsaicin induced desensitization has been observed both by recording the activity of DRG neurons as well as by monitoring behavioral (pain) reactions. With low doses of capsaicin given at appropriate time intervals, desensitization does not necessarily take place so that painful excitation can be reproduced with each capsaicin application. With higher doses or prolonged exposure desensitization ensues and consecutive applications of capsaicin become less effective or fail to produce any effect.

A novel method for producing analgesia using capsaicin in combination with a membrane impermeable local anesthetic (QX-314) has been described (Binshtok, Bean et al. 2007). QX-314 is a positively charged blocker of voltage gated sodium channels, which inhibits action potentials when applied intracellularly but fails to block when applied extracellularly. The idea was to introduce QX-314 intracellularly to pain sensing neurons through the open TRPV1 channel, thereby avoiding the motor and tactile effects that occur with the extracellular application of local anesthetics such as lidocaine. We have all probably experienced the inhibition of motor control and tactile senses with the use of local anesthetics during dental procedures. One limitation of the combination treatment is the same as with the use of capsaicin alone and that is the capsaicin itself causes a painful sensation, which with the combination treatment is expected to last until the QX-314 takes effect.

Inhibition of TRPV1 would seem to be a simple approach for producing analgesia. However the situation is not that simple; following the identification of TRPV1 in nociceptors a variety of cell types including keratinocytes, pancreatic β cells, endothelial cells, lymphocytes, macrophages and cells from different regions of the brain were shown to also express TRPV1. Its presence in all these cell types in different parts of the body suggests that TRPV1 is normally stimulated by an endogenous ligand (endovanilloid) and not by thermal stimulation. In this context it is important to point out that there is accumulating evidence suggesting that activation of TRPV1 by its endogenous ligand is essential for the maintenance of internal body temperature. Capsaicin in addition to eliciting the sensation of burning also causes hypothermia in a variety of animals and introduction of TRPV1 antagonists leads to hyperthermia in rats, mice, monkeys and man. One suggestion arising from these studies is that the TRPV1 channels, which function in the regulation of body temperature, are tonically activated via an endogenous ligand. Because TRPV1 antagonists cause hyperthermia it is unlikely that they can be developed for use systemically as standalone agents for the treatment of pain.

One possibility that needs to be considered is that an endovanilloid is produced during tissue damage and thereby mediates nociceptor activation. For example, the putative endovanilloid *N*-Arachidonoyldopamine (NADA) was identified as an endogenous molecule in the mammalian nervous system occurring in several brain nuclei and the dorsal root ganglion. It was originally studied because it activated cannabinoid receptors and it was subsequently found to potently activate the TRPV1 receptor. As would be expected for an endogenous ligand of the TRPV1 receptor NADA was found to increase the frequency of action potential firing of spinal nociceptive neurons and enhance the response to thermal stimuli. Further work is needed to determine whether NADA or another endovanilloid is in fact the normal activator of TRPV1 channels.

In order to ablate neurons expressing TRPV1 the human diphtheria toxin receptor was inserted into all cells that express TRPV1 (TRPV1-DTA mice). Mice in which diphtheria toxin A was then used to kill neurons expressing TRPV1, were unresponsive to painful and non painful hot and cold thermal stimuli and exhibited defective body temperature control but retained normal touch and mechanical pain sensation (Mishra, Tisel et al. 2011). Furthermore, nociceptive responses to ATP injection are also lost in TRPV1-DTA mice. **These results are puzzling as capsaicin-sensitive nociceptors are polymodal, they respond to noxious mechanical stimuli.**

These findings indicate that distinct groups of sensory neurons respond to thermal and mechanical stimuli even where the stimulation results in pain. Moreover, TRPV1 expression (TRPV1$^+$ neurons) marks those neurons that are essential for painful temperature detection; however, the findings from TRPV1 knockout mice discussed above indicate that, other thermo sensors, which are expressed in the TRPV1$^+$ neurons are necessary for the detection of noxious heat.

TRPV2

TRPV2 is closely related to the capsaicin receptor TRPV1, with which it shares 49% sequence identity, however TRPV1 is activated by capsaicin and responds to temperatures above 43°C whereas TRPV2 does not respond to capsaicin and responds to temperatures at or above 52°C. TRPV2 is expressed in a variety of tissues including various regions of the brain, spinal cord and sensory ganglia. Its expression in tissues that are never exposed to temperatures as high as 52°C suggests that TRPV2 is normally activated by stimuli other than noxious heat in these regions of the body. Nevertheless, based on its similarity to TRPV1 and its ability to detect high heat stimuli at or above 52°C TRPV2 would appear to be a likely candidate for sensing noxious heat at or above 52°C in nociceptors. However, TRPV2 knock-out mice showed normal behavioral responses to noxious heat over a broad range of temperatures and normal thermal hypersensitivity in the setting of tissue inflammation (Park, Vastani et al. 2011).

TRPV3

TRPV3 is closely related to TRPV1 and TRPV2 with which it shares 43% and 41% sequence identity respectively. TRPV3 has a unique threshold: It is activated at innocuous temperatures with an activation threshold around 33° to 35°C and exhibits increasing responses at higher noxious temperatures. However, in contrast with TRPV1 knockout mice, TRPV3 knock-out mice showed normal behavioral responses to noxious heat over a broad range of temperatures and deficits in heat hyperalgesia were not observed (Huang, Li et al. 2011).

TRPV4

TRPV4 is a calcium permeable nonselective cation channel that shares 40% amino acid identity with TRPV1. It exhibits remarkable gating properties being activated by hypotonic solutions, by certain phorbol ester derivatives and by innocuous temperatures in the range of 27°C to 34°C. Activation by hypotonic solutions suggests that it serves as a sensor for osmolarity and/or mechanical stretch associated with cellular swelling. Additionally TRPV4 is activated by a process involving the cytochrome P450 epoxygenase dependent formation of epoxyeicosatrienoic acids: submicromolar concentrations of 5',6' -epoxyeicosatrienoic acid activates TRPV4. These findings indicate that, a range of physical and chemical stimuli, which may or may not share a common mechanism with each other, can activate TRPV4.

TRPV4 knockout mice exhibited several abnormalities in physiological functions that were commensurate with the known gating properties of the channel. TRPV4 knockout mice exhibited abnormalities in osmotic regulation and a marked reduction in the sensitivity of the tail to pressure. . However, TRPV4 knock-out mice showed normal behavioral responses to noxious heat over a broad range of temperatures and normal thermal hypersensitivity in the setting of tissue inflammation (Huang, Li et al. 2011). We shall consider the role of TRPV4 in detecting noxious mechanical stimuli in chapter 4

when we consider the role of protease-activated receptor-2 (PAR2) activation in mechanical hyperalgesia.

Table 3-1 Effects of knocking out TRPV channels in Mice

	Deficits in response to noxious temperatures in knockout mice	Deficits in hypersensitivity in knockout mice
TRPV1	>50°C	Exhibited little thermal hypersensitivity
TRPV2	Not observed	Exhibited normal thermal hypersensitivity
TRPV3	Not observed	Exhibited normal thermal hypersensitivity
TRPV4	Not observed	Exhibited normal thermal hypersensitivity PAR2 mediated mechanical hyperalgesia was not observed in TRPV4 knockout mice

In summary, of the TRPV channels studied with genetic knockout studies only TRPV1 has been shown to play a role in thermal nociception and thermal hypersensitivity.

TRPM3

TRPM3 (transient receptor potential melastatin-3) is a Ca^{2+} permeable nonselective cation channel that is activated by the neuroactive steroid pregnenolone sulfate (PS). A large subset of heat-sensitive neurons responds to both PS and capsaicin, indicating that TRPV1 and TRPM3 are co expressed in the same neurons. TRPM3 knockout mice exhibit deficits in their avoidance of noxious heat and in the development of inflammatory heat hyperalgesia. However, blocking both TRPM3 and TRPV1 only partially blocks noxious heat sensation, indicating the existence of a TRPV1 and TRPM3 independent noxious heat sensor (Vriens, Owsianik et al. 2011).

ANO1

Anoctamin 1 (ANO1) is a Ca^{2+}-activated chloride channel that is primarily expressed in TRPV1^{+} neurons and is activated by temperatures above 44 °C. Because ANO1 knockout mice die early in the neonatal period it was necessary to study animals in which ANO1 was deleted in DRG neurons. ANO1 deletion in DRG neurons substantially reduced nociceptive behavior in thermal pain models. ANO1 is therefore a sensor that detects nociceptive thermal stimuli in DRG neurons and potentially plays a role in the detection of noxious heat (Cho, Yang et al. 2012).

Taken together, the above findings suggest that **TRPV1, ANO1 and TRPM3** are co expressed in heat sensitive nociceptors where they play a role in the detection of noxious

heat and the development of heat hyperalgesia. It is not clear whether additional, as yet undiscovered, heat sensitive ion channels are necessary for the detection noxious thermal stimuli.

TRPA1

Heterologously expressed TRPA1 ion channels are activated by irritant compounds from mustard seed, wasabi, horseradish, winter green, cinnamon, garlic, vehicle exhaust fumes and tear gas, all of which elicit a painful burning or pricking sensation. TRPA1 is expressed in DRG neurons and in the inner ear; however, TRPA1 is apparently not essential for the initial detection of sound by hair cells. The role of TRPA1 as a sensor of noxious cold has been controversial; mouse TRPA1 when expressed in CHO cells is activated at temperatures starting near 17°C, which is close to the threshold of noxious cold for humans (15°C) (Story, Peier et al. 2003). The controversy arose when the rat and human orthologues of TRPA1 expressed in either a human embryonic kidney (HEK293) cell-line or *Xenopus* oocytes were not activated by cold (Jordt, Bautista et al. 2004). Subsequently, another group was unable to elicit cold activation of heterologously expressed mouse TRPA1 channels in HEK293 cells (Nagata, Duggan et al. 2005). Yet a fourth study (Sawada, Hosokawa et al. 2007) found that mouse TRPA1 expressed in HEK293 cells is a cold-activated channel, which supports the previous findings that TRPA1 responds to noxious cold. The controversy also extended to TRPA1 knockout mice. Nociceptive behavioral responses to contact with a cold surface or to acetone-evoked evaporative cooling were evaluated by two different groups (Bautista, Jordt et al. 2006, Kwan, Allchorne et al. 2006); with the former finding a lack of involvement of TRPA1 in the acute detection of cold and the latter finding a reduced sensitivity to cooling. These contradictory findings regarding the cold activation of TRPA1 appear to have been resolved by subsequent work. A study in mice in which all sensory neurons expressing the tetrodotoxin resistant voltage activated sodium channel ($Na_v1.8$, see Chapter 5) were eliminated, showed resistance to noxious cold, assayed using a cold plate kept at 0°C (Abrahamsen, Zhao et al. 2008). This finding was similar to what was observed in the TRPA1 knockout mice using a cold plate at 0°C (Kwan, Allchorne et al. 2006). Significantly, the $Na_v1.8$ knockout mice also exhibited a significant reduction in the expression of TRPA1 in DRG neurons and a lack a TRPA1-mediated nociceptive response to formalin (see below). It has been suggested that the findings from the experiments at 0°C were the result of cold evoked tissue damage which was then detected by TRPA1(Knowlton, Bifolck-Fisher et al. 2010). Thus, TRPA1 appears to be the sensor for noxious cold at 0°C, although the mechanism may be indirect through tissue damage, rather than TRPA1 responding directly to 0°C.

A gain-of-function point mutation in TRPA1 is the cause of the rare Familial Episodic Pain Syndrome (FEPS) first identified in a family from Colombia, South America (Kremeyer, Lopera et al. 2010). FEPS patients experience episodes of pain as a consequence of altered functional properties of mutated TRPA1. The mutation causes an increase in current flow through the activated TRPA1 channel.

There is considerable evidence that the analgesic acetaminophen has a central site of action. Metabolites of acetaminophen have been demonstrated to activate human TRPA1 receptors and intrathecal (within the sheath of the spinal cord) administration of these metabolites in mice produced analgesia that was lost in TRPA1 knockout mice (Andersson, Gentry et al. 2011). These findings suggest that metabolites of acetaminophen produce a TRPA1 mediated spinal analgesia in mice and by extension in humans also.

In contrast to the debate over the role of TRPA1 as a sensor of noxious cold its role in bradykinin evoked nociceptor excitation and pain hypersensitivity was not controversial. Bradykinin (BK) is a peptide containing nine amino acid residues (nonapeptide) that is released into inflamed tissues where it induces pain and mechanical and thermal hypersensitivity. Bradykinin injections in TRPA1 knockout mice were much less painful and showed little or no evidence of thermal hypersensitivity following the injections. Both consequences are expected if TRPA1 mediates the actions of bradykinin. We will more thoroughly consider the effects of bradykinin on TRPA1 along with its effect on TRPV1 and other ion channels in chapter 4.

TRPA1 knockout mice exhibit a significant reduction in all phases of formalin-induced pain behavior (McNamara, Mandel-Brehm et al. 2007). Formaldehyde, the active ingredient in formalin, is a fixative that covalently cross-links proteins in a nonspecific manner. This cross-linking leads to a variety of effects including general tissue damage, which was thought to release intracellular compounds that activate nociceptors (see Chapter 4). However, this finding with TRPA1 suggests that there is a direct effect of formalin on TRPA1, which may be similar to the irritant compounds described above that are thought to induce covalent modification of TRPA1.

TRPM8

The ability of recombinant TRPM8 to be activated by cold is widely accepted. TRPM8 is activated by cooling agents such as menthol or at temperatures below 26°C. Additionally, three independent studies using TRPM8 knockout mice (Bautista, Siemens et al. 2007, Colburn, Lubin et al. 2007, Dhaka, Murray et al. 2007) indicate that TRPM8 is involved in sensing noxious cold. Pain-induced by evaporative cooling of the paw was measured by observing licking and flinching responses of the stimulated paw in normal and TRPM8 knockout mice (Bautista, Siemens et al. 2007): the knockout mice displayed significantly reduced behavior compared to the normal mice. A similar result was found by others (Dhaka, Murray et al. 2007) who additionally found that " … injection of icilin, a synthetic compound that activates TRPM8 and, to a much lesser extent, TRPA1, into the hind paw of wild-type mice causes the rapid induction of hind paw withdrawal when the mice are placed on a 1°C cold plate and that this behavior is completely ablated in TRPM8 knockout mice, suggesting that TRPM8 activation can elicit a nociceptive-like response." The third group (Colburn, Lubin et al. 2007) also found a reduced nociceptive response to evaporative cooling of the paw in TRPM8 knockout mice. Furthermore, these

authors also found that following constriction injury caused by ligation of the sciatic nerve (see Chapter 6) normal mice exhibited an enhanced sensitivity to acetone with protracted licking and shaking of the paw whereas TRPM8 knockout mice exhibited no significant increase in the response to evaporative cooling of the paw. These data plainly indicate that TRPM8 is involved in sensing noxious cold. TRPM8 knockout mice retain a number of cold sensitive neurons indicating that TRPM8 is not the only receptor activated by cold.

The above findings were extended using TRPM8/TRPA1 double-knockout mice which showed similar behavior to TRPM8 knockout mice at noxious cold temperatures above 0° C (Knowlton, Bifolck-Fisher et al. 2010). In summary, both TRPA1 and TRPM8 play a role in detecting noxious cold but at different temperatures.

As was done with neurons expressing TRPV1 the human diphtheria toxin receptor was inserted into all cells that expressed TRPM8 (TRPM8-DTA mice). Ablation of TRPM8 neurons with diphtheria toxin A in adult mice resulted in a more profound deficit in cold behaviors compared with TRPM8 knockout mice but retained normal touch and mechanical pain sensation (Knowlton, Palkar et al. 2013). This finding indicates that in TRPM8 neurons molecules other than TRPM8 are involved in noxious cold transduction.

As mentioned above expression of TRPV1 in neurons indicates that those neurons are essential for painful temperature detection. Likewise expression of TRPM8 indicates a population of neurons that are involved in cold transduction. Thus it would appear that at the level of the afferent nerve there are neuronal populations carrying information for specific noxious modalities. **This result is puzzling as many cold-sensitive neurons are also sensitive to noxious mechanical stimuli and as pointed out above capsaicin-sensitive nociceptors are polymodal, they respond to noxious mechanical stimuli.**

MECHANICAL NOCICEPTORS

It was only in 2010 that the first mechanically activated cation channels Piezo1 and Piezo2 were cloned. Of the two Piezo2 was strongly expressed in DRG neurons indicating a possible role in sensing of mechanical pain.

When viable Piezo2 knockout mice become available the role of Piezo2 in mediating mechanical pain should become clearer.

Remember that mice in which diphtheria toxin A was used to kill neurons expressing TRPV1 appeared to retain normal mechanical pain sensation and that this result is *puzzling* since capsaicin-sensitive nociceptors are polymodal, they respond to noxious mechanical stimuli. However, the finding is consistent with the idea that mechanical nociception involves the utilization of a mechanically activated ion channel such as Piezo2.

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Chapter 4   CHEMICAL MEDIATORS OF PAIN AND THEIR RECEPTORS

One of the long-standing goals of pain research is to identify the chemical mediators released into injured or diseased tissues that are responsible for the activation and sensitization of nociceptors. Pain scientists distinguish two aspects of sensitization: allodynia (pain resulting from a normally innocuous stimulus) and hyperalgesia (an enhanced response to a normally painful stimulus). As mentioned in chapter 2 these mediators interact with ion channels in the plasma membrane of the nociceptor utilizing mechanisms used for signaling elsewhere in the nervous system. These mediators either act directly on ligand-gated ion channels (ionotropic channels) or indirectly via metabotropic receptors that are either G-protein-coupled receptors (GPCRs) or receptor tyrosine kinases.

SEROTONIN

Injection of serotonin (5-Hydroxytryptamine, 5-HT) produces pain and hyperalgesia in humans. In-vivo the source of this 5-HT in humans is platelets, which are known to play an important role in inflammation. In support of this idea is the finding that cutaneous injection of platelets causes acute pain and hyperalgesia (Schmelz, Osiander et al. 1997). Unlike the nerve terminal of 5-HT containing neurons, the platelet cannot synthesize 5-HT, rather it relies upon 5-HT uptake. Interestingly, the protein responsible for the human platelet 5-HT uptake is identical to that for the brain 5-HT transporter, and the selective serotonin reuptake inhibitors, used to treat depression, significantly reduce the 5-HT concentration in the platelets of depressed patients (Maurer-Spurej, Pittendreigh et al. 2004). One might reasonably assume that in these patients there will be a significant reduction in the amount of 5-HT released from platelets into damaged inflamed tissue resulting in reduced 5-HT induced pain.

The mammalian family of serotonin receptors is very large consisting of fourteen different receptor subtypes, grouped into seven families. Although 5-HT is known to play an important role in nociception, there is only a limited appreciation of the 5-HT receptor subtypes involved in this process, and how they interact with each other and other chemical mediators of nociception. Intraplantar injection, in the rat, of either the 5-HT or the 5-HT$_{2A}$ receptor agonist (α-methyl 5-HT), significantly reduced the paw-withdrawal latency to radiant heat stimulation. Furthermore, pretreatment with the 5-HT$_{2A}$ receptor antagonist (ketanserin), attenuated the behavioral response following the injection of 5-HT (Tokunaga, Saika et al. 1998). These findings strongly suggest that the 5-HT$_{2A}$-receptor subtype is involved in 5-HT-induced hyperalgesia in acute injury and inflammation. These findings were extended by showing that 5-HT$_{2A}$ receptor inhibition in rats: by local injection (intra plantar) of sarpogrelate blocked primary thermal hyperalgesia (Sasaki, Obata et al. 2006), and systemic injection of sarpogrelate blocked complete Freund's adjuvant (CFA) induced thermal hyperalgesia (Okamoto, Imbe et al. 2002), or by local injection of ketanserin, produced dose-dependent inhibition of carrageenan-evoked hyperalgesia (Wei, Chen et al. 2005). Taken together these results suggest that 5-HT has a role in hyperalgesia resulting from tissue injury by activating 5-HT$_{2A}$ receptors at nociceptor nerve terminals.

The 5-HT$_{2A}$-receptor is a G-protein coupled receptor, and its activation leads to depolarization of the resting membrane potential of acutely isolated rat DRG neurons. In cells exhibiting a 5-HT$_{2A}$-mediated response, 5-HT and α-methyl 5-HT depolarized the resting membrane potential and decreased the membrane permeability (measured electrically as an *increase* of the slope of the current voltage relationship of the membrane) (Todorovic, Scroggs et al. 1997). In order to understand the different mechanisms by which different ion channels depolarize the plasma membrane we will consider the idealized situation illustrated in Figure 4-1. The two situations illustrated in Figure 4-1 are for a cell that has a resting permeability to both Na$^+$ and K$^+$. In both the situations illustrated in Figure 4-1 the membrane depolarization results from an increase in the ratio P_{Na}/P_K (i.e. the permeability to sodium increases relative to that for potassium). Ideally, this can happen either when P_{Na} increases and P_K remains the same as illustrated on the left of Figure 4-1 (increased permeability causes a decreased slope resistance) or when P_{Na} remains the same and P_K decreases (decreased permeability causes an increased slope resistance), as illustrated on the right of the Figure 4-1. In the acutely isolated cells that showed a 5-HT$_{2A}$-mediated depolarization the reversal potential (E_{rev}) for the depolarization was linearly related to the logarithm of the extracellular potassium concentration $[K^+]_{out}$, indicating the depolarization resulted from a decrease in the resting K$^+$ permeability (Todorovic, Scroggs et al. 1997).

Please remember from chapter 2 that K$_{2P}$ channels are thought to be responsible for the cells' resting K$^+$ permeability. Therefore it seems reasonable to *speculate* that the decrease in K$^+$ permeability results from the closing of K$_{2P}$ channels in the DRG cells studied. In an heterologous expression system the excitatory effects of a G-protein coupled receptor have been shown to occur via inhibition of some K$_{2P}$ channels (Chemin, Girard et al. 2003). The TREK-1 channel is a member of the K$_{2P}$ channel family and is extensively colocalized with TRPV1 (Alloui, Zimmermann et al. 2006) making it a candidate for one of the channels responsible for the resting K$^+$ permeability of nociceptors. Moreover, in animals in which TREK-1 is knocked out "animals are more sensitive to low threshold mechanical stimuli and display an increased thermal and mechanical hyperalgesia in conditions of inflammation"(Alloui, Zimmermann et al. 2006). It would be interesting to determine whether TREK-1 or some other K$_{2P}$ channel is involved in the 5-HT$_{2A}$-mediated response. If the closing of K$^+$ channels can cause pain as these findings indicate then another conclusion to be drawn from this work is that the opening of K$^+$ channels in nociceptors is potentially an important mechanism in antinociception. We will return to this idea when we consider the role of K$^+$ channels in the antinociception induced by opioid receptor agonists.

In contrast to all the other serotonin receptors, which are G-protein coupled receptors, the 5-HT$_3$-receptor is a ligand-gated cation channel consisting of five monomers forming a central pore region (see Figure 2-4A). Five monomer subtypes, the 5-HT$_{3A-E}$ subunits, have been identified and functional homomeric 5-HT$_{3A}$ receptors and heteromeric 5-HT$_{3A/B}$ receptors were found to be expressed in neurons. In DRG neurons exhibiting a 5-HT$_3$ receptor response, 5-HT and 2-methyl-5-HT produced depolarization with decreased input resistance. Moreover, the reversal potential (E_{rev}) for the depolarizing response

65 — $[E_{Na}]$

membrane potential (mV)

0 —

-85 — $[E_K]$

measured electrically
as a decreased
slope resistance

P_{Na} increased
P_K unchanged

measured electrically
as an increased
slope resistance

P_{Na} unchanged
P_K decreased
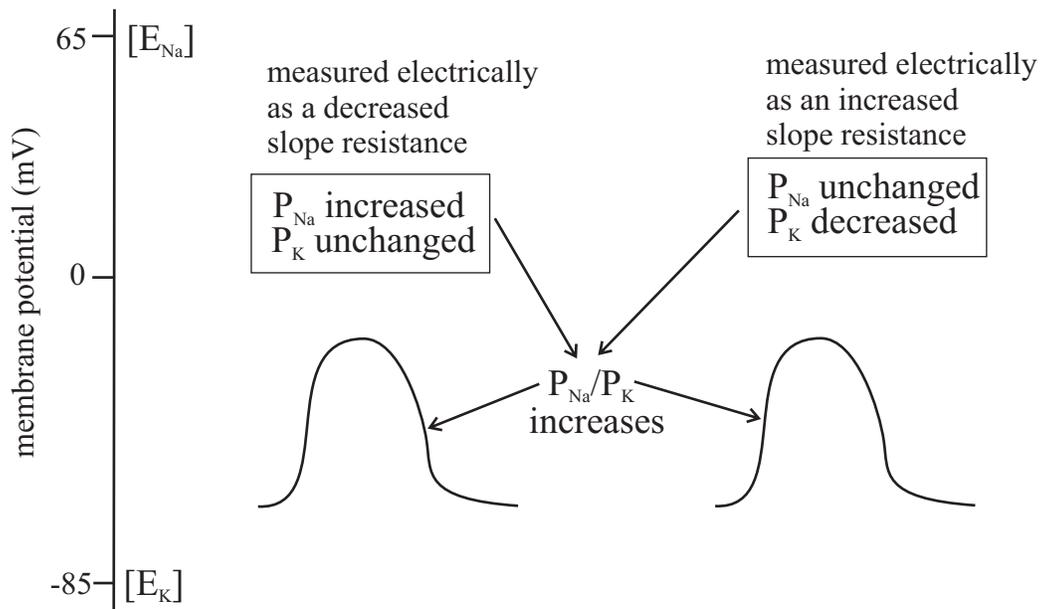
P_{Na}/P_K
increases

Figure 4-1. The same depolarizing receptor potential
can be generated by either an increase in sodium
permeability or a decrease in potassium permeability.

became less negative when the extracellular K^+ concentration was raised to 10 mM and the depolarization was converted to hyperpolarization in a Na^+ free solution, indicating that the $5\text{-}HT_3$-response resulted from an increased permeability to Na^+ and K^+.

Studies in $5\text{-}HT_{3A}$-knockout mice lead the authors to the interpretation that $5\text{-}HT_3$ receptors are not involved in acute pain but are required for persistent pain. The formalin test is widely used for evaluating the effects of analgesic compounds in laboratory animals. The noxious stimulus in the formalin test in mice is an injection of a dilute formalin solution under the skin of the dorsal surface of a hind paw. The response is the amount of time the animals spend licking the injected paw. There are two distinct periods of licking, an early phase lasting the first 5 min and a late phase lasting from 20 to 30 min after the injection. It is generally thought that the early phase is due to a direct effect on nociceptors. Using the formalin test they found that the first-phase of pain behavior did not differ in wild type and mutant mice. In contrast, the second phase of pain behavior was significantly reduced in the mutant animals, indicating that $5\text{-}HT_3$ receptors are important for persistent pain (Zeitz, Guy et al. 2002). Moreover, they also observed a significant reduction of the second-phase behavior in the formalin test following intrathecal (inside the spinal canal) administration of a $5\text{-}HT_3$-receptor antagonist, suggesting that the $5\text{-}HT_3$ receptors affected are in the spinal cord (see Chapter 7).

BRADYKININ

Intradermal injection of bradykinin in humans produces a dose-dependent pain and a heat hyperalgesia, indicating that bradykinin both excites and sensitizes nociceptors (Manning, Raja et al. 1991). Bradykinin is a polypeptide formed in the blood; it causes contraction of non-vascular smooth muscle, is a potent vasodilator of certain blood vessels, increases vascular permeability and most importantly for our purposes is involved in the mechanism of pain. Local inflammation following tissue damage triggers the release of bradykinin (the nonapeptide H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) produced by kallikrein mediated enzymatic cleavage of kininogen at the site of tissue injury and inflammation. Kallikreins are serine proteases that liberate kinins (bradykinin and kallidin) from the kininogens (High-molecular weight kininogen and low-molecular weight kininogen). Human tissue kallikrein preferentially releases the decapeptide Lys- bradykinin (kallidin) from kininogens. Prekallikrein is the precursor of kallikrein and it can only activate kinins after being activated during tissue injury and inflammation.

Once formed bradykinin is degraded by two enzymes carboxypeptidase-N, also known as kininase-1, and angiotensin converting enzyme (ACE), also called kininase-2. Kininase-1 transforms bradykinin and kallidin into their active metabolites, des-Arg^9-bradykinin and Lys-des-Arg^9- bradykinin (i.e. bradykinin and kallidin without their C-terminal arginine residues). ACE removes the C-terminal dipeptide from bradykinin or Lys- bradykinin, which leads to their inactivation. ACE inhibitors lead to an increase in bradykinin due to decreased degradation and also to a decrease in angiotensin (a vasoconstrictor), for which they are used in the treatment of hypertension. It has been suggested that some of the

blood pressure lowering effects of ACE inhibitors may be due to their effects on bradykinin.

The actions of bradykinin are mediated through two G-protein-coupled receptors, denoted B_1 and B_2. Bradykinin activates B_2 receptors while B_1 receptors exhibit higher affinity for des-Arg[9]-bradykinin (i.e. the B_1 receptor is selective for kinin metabolites without the C-terminal arginine residue). B_2 receptors are constitutively expressed in DRG neurons and are thought to be the predominant functional bradykinin receptor subtype in non-traumatized tissues. On the other hand B_1 receptors are not normally constitutively expressed to a significant extent, but are up regulated during chronic inflammation. Since the B_1 receptor is not present in non-inflamed tissues it is an appealing target for the development of antagonists, as they would be expected to cause few unwanted side effects.

Since the B_2 receptor is a G-protein-coupled receptor there needs to be an ion channel that is activated downstream from B_2. Also given that B_2 receptor activation causes thermal hyperalgesia and that TRPV1 knockout mice exhibit little thermal hypersensitivity in the setting of tissue inflammation, it is plausible that TRPV1 might be the ion channel that is acted upon downstream from bradykinin. In two studies (Kollarik and Undem 2004, Rong, Hillsley et al. 2004) with TRPV1 knockout mice, bradykinin elicited an action potential discharge in C-fibers, and in both studies there was no difference in the initial response in the knockout mice compared to the normal mice. In one of the studies (Kollarik and Undem 2004) the response in the knockout mice was less persistent than in normal mice, while in the other there was no significant difference. These findings suggest that TRPV1 contributes to, but is not required for, B_2 receptor mediated nociceptor excitation. On the other hand, intraplantar injection of bradykinin produced substantial thermal hypersensitivity in wild-type mice but not in TRPV1 knockout mice, demonstrating that TRPV1 is necessary for the development of bradykinin-induced thermal hypersensitivity in vivo (Chuang, Prescott et al. 2001).

PHOSPHOLIPASE-C AND PHOSPHOLIPASE-A$_2$

Activation of most cells by bradykinin is mediated by phospholipase C and/or phospholipase A_2; therefore these are the biochemical pathways that are likely to mediate between B_2 and TRPV1. The model in Figures 4-2A summarizes how phospholipase C and phospholipase A_2, respectively, are thought to couple B_2 to TRPV1. Much of the evidence supporting this model comes from experiments using isolated neuronal cell bodies of a dorsal root ganglion or from heterologous expression systems. It is important to keep in mind that the uncertainties and assumptions associated with these procedures make it essential that the findings be checked very carefully and shown to resemble what actually occurs *in vivo.*

PHOSPHOLIPASE-C

As illustrated in Figure 4-2A & B, B_2 is coupled to the enzyme phospholipase C-β via the guanosine nucleotide-binding protein Gq/11. Heterotrimeric G proteins (Gαβγ) of the $G_{q/11}$ family stimulate phospholipase C-β via Gq/11α family members (Gqα, G11α, G14α
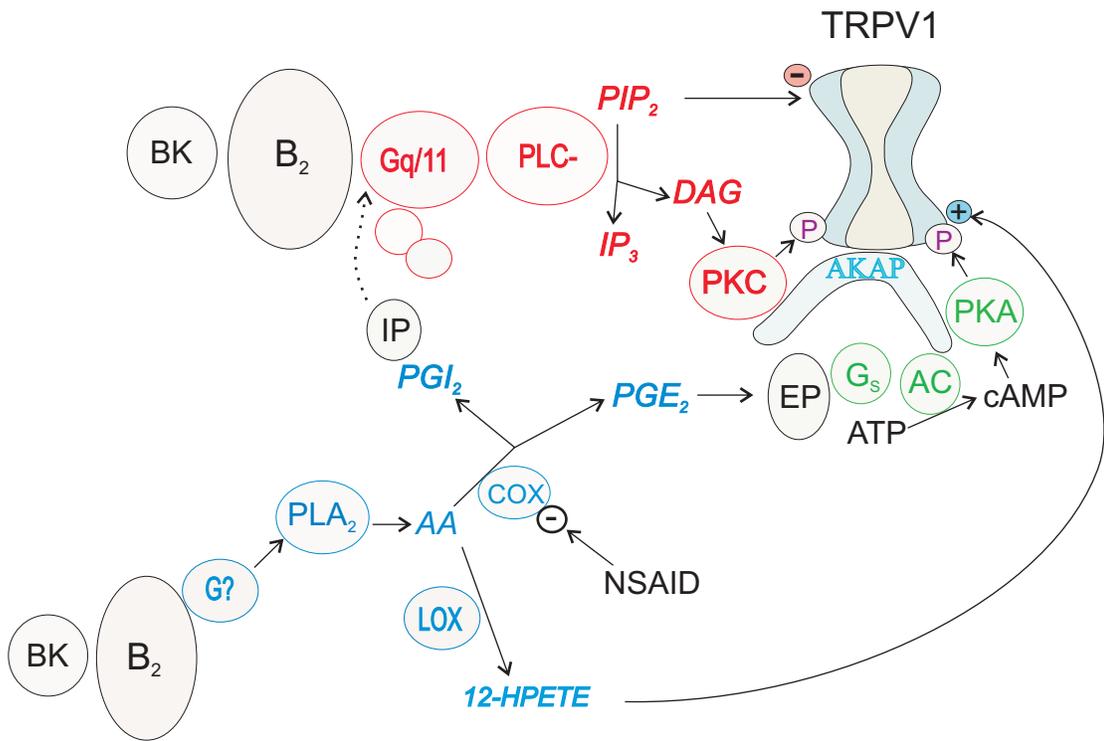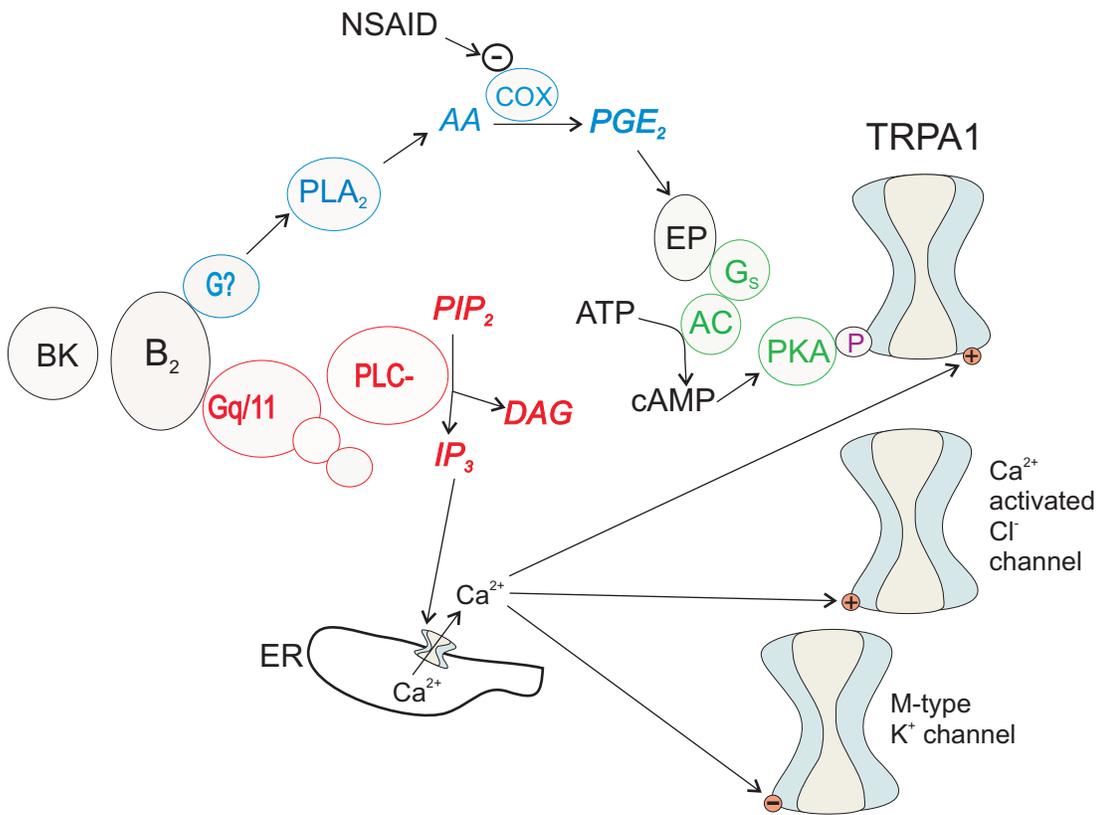
Figure 4-2. Scheme showing the biochemical pathways that have been implicated in the modulation of TRPV1 (A&B) and TRPA1, M-typeK$^+$ channels and Ca^{2+} activated Cl$^-$ channels (C) by bradykinin (BK). Slee text for futher details

and G15/16α). Five different *β*- and 12 *γ*-subunits have been described allowing for numerous coexpression possibilities for α,β and γ subunits. The exact subunit composition for Gq/11 coupled to B_2 is unknown and is indicated as Gq/11αβγ in Figure 4-2A & B. Binding of bradykinin to B_2 leads to the activation of Gq/11α via exchange of GTP for GDP in the nucleotide-binding pocket and dissociation of the βγ subunits. In turn the activated Gq/11α with bound GTP activates the enzyme PLC-β that hydrolyzes PIP_2 (phosphatidylinositol (4,5)-bisphosphate) to form IP_3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). The Gq/11α -subunit is an intrinsic GTPase, hydrolyzing the terminal phosphate of GTP to restore GDP to the nucleotide-binding pocket leading to the reassociation of the Gq/11α with the βγ subunits thereby returning Gq/11α to the inactive state.

As illustrated in Figure 4-2A the available evidence has so far suggested two possible mechanisms by which hydrolysis of PIP_2 modulates the activity of TRPV1: either by phosphorylation of TRPV1 through the activation of PKC (protein kinase C) via DAG, and/or through depletion of PIP_2 which normally acts to inhibit TRPV1. Briefly, evidence from isolated DRG neurons and HEK293 cells expressing TRPV1 and B_2 has shown that stimulation with bradykinin, or activation of PKC, lowers the threshold temperature for activation of TRPV1 currents (Cesare and McNaughton 1996, Sugiura, Tominaga et al. 2002). Moreover, inhibition of PKCε results in a 70% decrease in the sensitization of TRPV1 by bradykinin (Cesare, Dekker et al. 1999). This finding does not rule out a role for other isoforms of PKC from playing a role in TRPV1 sensitization by bradykinin. Since DAG normally activates PKCε it is reasonable to assume that this is the mechanism of PKCε activation in nociceptors (see Figure 4-2A).

Turning to the other mechanism: in a study using heterologous expression systems, it was shown that decreasing the level of PIP_2 in the plasma membrane mimics the potentiating effects of bradykinin on TRPV1 (Chuang, Prescott et al. 2001). It has been suggested that the regulation of TRPV1 by PIP_2 is dependent on the membrane protein Pirt (phosphoinositide interacting regulator of TRP) which binds both TRPV1 and PIP_2 (Kim, Tang et al. 2008). However, this suggestion has been questioned and the PIP_2 sensor was proposed to be directly on TRPV1(Ufret-Vincenty, Klein et al. 2011).The role of PIP_2 in regulating TRPV1 may be more complicated than shown in Figure 4-2A: the evidence suggests a model in which PIP_2 has both an inhibitory and activating effects on TRPV1 (Lukacs, Thyagarajan et al. 2007). A concomitant activating and inhibitory effect might be the result of a bell-shaped dependence of TRPV1 channel activity on PIP_2 levels. If resting PIP_2 levels are high, that is to the right of the peak of the bell-shaped dose response curve, a moderate decrease in PIP_2 levels will result in increased TRPV1 channel activity, whereas further decreases to PIP_2 levels to the left of the peak will result in channel inhibition. Remember from chapter 3 that exposure of TRPV1 containing nociceptors to high doses of capsaicin is associated with a loss of responsiveness to capsaicin as well as other chemicals, heat and noxious mechanical stimuli. It has been suggested that high doses of capsaicin maximally activate TRPV1 causing a large influx of calcium, which activates PLC thereby depleting PIP_2 causing a profound inhibition of TRPV1, which is responsible for the loss of responsiveness to capsaicin and other stimuli.

PHOSPHOLIPASE-A$_2$

Cumulative evidence from a number of cell types has shown that GPCRs can couple to PLA$_2$, however which G-protein is used to couple B$_2$ to PLA$_2$ in nociceptors has not yet been determined, hence the question mark between B$_2$ and PLA$_2$ in Figure 4-2A & B. Activated PLA$_2$ catalytically hydrolyzes phospholipids releasing arachidonic acid. Two important pathways for arachidonic acid metabolism are the cyclooxygenase (COX) and 12-lipoxygenase (LOX) pathways. The COX pathway forms intermediate compounds, which are then converted into biologically active compounds, which include the prostaglandins PGE$_2$ and PGI$_2$, while the LOX pathway produces 12- HPETE. Thus the lipid mediators PGE$_2$, PGI$_2$ and 12-HPETE are hypothesized to be produced in the nociceptor in response to bradykinin. Although we are considering the role of PGE$_2$, PGI$_2$ and 12-HPETE in mediating the effects of bradykinin, it should be kept in mind that these lipid mediators do not have to be produced in the nociceptor where they act; they can also be produced by other nearby cells during tissue injury (not necessarily in response to bradykinin), and then diffuse to the nociceptor; this is referred to as paracrine signaling.

12-LIPOXYGENASE (LOX) PATHWAY

First consider the evidence supporting the AA→ LOX→ 12-HPETE pathway in Figure 4-2A. Experiments with cultured DRG neurons and cutaneous nerve fibers in the in vitro skin-nerve preparation demonstrated that bradykinin, acting via B$_2$ receptors, excite sensory nerve endings by activating TRPV1 via the production of LOX metabolites of arachidonic acid (Shin, Cho et al. 2002). Moreover bradykinin directly stimulated the production of 12-HPETE which was shown in an expression system to directly activate TRPV1 (Hwang, Cho et al. 2000). It is unclear to what extent this pathway normally contributes to B$_2$ receptor mediated nociceptor excitation given the evidence, discussed above, from TRPV1 knockout mice, that TRPV1 activation is not required for nociceptor activation by bradykinin (Kollarik and Undem 2004, Rong, Hillsley et al. 2004).

CYCLOOXYGENASE (COX) PATHWAY

We need to consider whether PGE$_2$ by itself causes pain and thermal hyperalgesia. Intraplantar injection of PGE$_2$ into the hind paw of mice produced a dose dependent short duration paw licking (nociceptive) behavior when compared with control animals (Kassuya, Ferreira et al. 2007). Additionally there was a reduction of paw withdrawal latency (thermal hyperalgesia) following intraplantar PGE$_2$ injection which was significantly diminished in TRPV1 knockout mice (Moriyama, Higashi et al. 2005). Furthermore, in an isolated skin nerve preparation the bradykinin induced thermal hyperalgesia was mediated by cyclooxygenase activation (Petho, Derow et al. 2001).

The diversity of actions of PGE$_2$ is believed to result from its interaction with a family of G-protein-coupled (prostanoid E receptors), EP receptors, designated EP$_1$ – EP$_4$, all of which are found in DRG neurons (Southall and Vasko 2001). The identity of the EP receptor(s) that couple PGE$_2$ to thermal hyperalgesia is still controversial. The activation

of EP receptors by PGE_2 can stimulate PKA, PKC and mitogen-activated protein kinases (MAPKs). In cells transfected with TRPV1 heat activated currents were greatly potentiated by activation of PKA and the potentiation was greatly reduced in cells transfected with TRPV1 mutants having mutations at the PKA phosphorylation sites (Rathee, Distler et al. 2002). Furthermore, disruption of the PKA anchoring protein AKAP150 in mice diminishes the decrease in paw withdrawal latency, to thermal stimuli (Schnizler, Shutov et al. 2008). This is because the A-kinase anchoring protein AKAP (see Figure 4-2A) organizes a protein complex between PKC, PKA and adenylate cyclase (AC) with TRPV1 to enhance the phosphorylation efficiency of TRPV1 (Efendiev, Bavencoffe et al. 2012). These findings strongly suggest that PGE_2-induced thermal hyperalgesia is mediated in part by PKA. The above findings are summarized in Figure 4-2A where EP is coupled to PKA via the G protein (G_S) which activates AC. AC catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) which in turn activates PKA. In a separate study thermal hyperalgesia induced by intraplantar injection of PGE_2 was unaffected by knockout of PKCε (Khasar, Lin et al. 1999). Most recently it was shown that disruption of the interaction between TRPV1 and AKAP disrupts the sensitization of TRPV1 via PKA and PKC (Fischer, Btesh et al. 2013).

In contrast to the family of EP receptors for PGE_2, there is only a single IP receptor for PGI_2. Using the acetic acid-induced writhing test, where injection of a dilute acetic acid solution intraperitoneally causes writhing responses in wild-type mice, IP knockout mice showed a markedly reduced writhing response compared to the wild-type mice (Murata, Ushikubi et al. 1997). Furthermore, thermal hyperalgesia induced by intraplantar PGI_2 injection was significantly diminished in TRPV1 knockout mice and IP knockout mice, indicating that PGI2 sensitizes TRPV1 via IP receptors (Moriyama, Higashi et al. 2005). Moreover, in DRG neurons PGI_2 -induced potentiation of capsaicin activation of TRPV1 was suppressed by a PLC inhibitor and also by a PKCε inhibitor suggesting the involvement of a PLC-PKCε dependent pathway (Moriyama, Higashi et al. 2005), this is indicated by a dotted line going from IP to Gq/11α in Figure 4-2A.

It is important to check that the biochemical pathways activated by the B_2 receptor in Figure 4-2A are actually involved in nociceptor excitation given that in TRPV1 knockout mice TRPV1 activation is not required for nociceptor activation by bradykinin. The mechanism of bradykinin-induced nociception (paw licking) was tested by injecting bradykinin into the paw of mice in the presence of different enzyme inhibitors. Selective inhibitors of phospholipase-C, PKC, PLA_2 or LOX markedly decreased the nociception caused by BK but not that of capsaicin (Ferreira, da Silva et al. 2004). Of these inhibitors the one for phospholipase-C caused the greatest inhibition. These findings implicate the biochemical pathways presented in Figure 4-2A, but they don't indicate the ion channel(s) targeted.

TRPV1 is not the only TRP channel that plays a role in the activation and sensitization of nociceptors by bradykinin; TRPA1 has also been implicated. In TRPA1 knockout mice the bradykinin-induced response of DRG neurons was significantly reduced but not absent and comparable to that of TRPV1 knockout mice (Bautista, Jordt et al. 2006). In a

behavioral study it was found that following subcutaneous intraplantar bradykinin injection in mice, wild-type mice spent almost three times as long tending to the affected paw as TRPA1 knockout mice (Kwan, Allchorne et al. 2006). These findings suggest that TRPA1 activation plays a role in the acute pain caused by bradykinin. With respect to pain hypersensitivity there was no evidence of bradykinin-induced thermal hypersensitivity with intraplantar bradykinin injection in TRPA1 knockout mice (Bautista, Jordt et al. 2006). Thus both TRPV1 and TRPA1 are necessary for the development of bradykinin-induced thermal hyperalgesia. Colocalization studies using antibodies to TRPA1 and TRPV1 showed that all TRPA1-positive neurons also expressed TRPV1 (Bautista, Movahed et al. 2005), indicating that the biochemical pathways shown in Figure 4-2A are available to modulate TRPA1. Although the experimental evidence is not as extensive as for TRPV1, we can suggest (see Figure 4-2B) that to some extent the same pathways that modulate TRPV1 also affect TRPA1. Using a heterologous expression system and dorsal root ganglia neurons both the PLC and PKA pathways were shown to potentiate currents carried by TRPA1 (Wang, Dai et al. 2008). Therefore in Figure 4-2B the pathways for activation of PLC and PKA are the same as in Figure 4-2A.

However, the PKC inhibitor did not prevent the potentiation by bradykinin of the currents carried by TRPA1 and the PKC activator did not potentiate the TRPA1 response (Wang, Dai et al. 2008). These findings indicate that PKC activation does not contribute to the sensitization of TRPA1, which is different from the mechanism for TRPV1 sensitization; hence PKCε is not included in Figure 4-2B. Intracellular calcium is an important intracellular messenger and the release of calcium from intracellular stores has been shown to directly activate TRPA1 (Jordt, Bautista et al. 2004, Zurborg, Yurgionas et al. 2007). Hence, the inositol 1,4,5-trisphosphate (IP_3) induced calcium release pathway is included in Figure 4-2B. Further studies will be required to elucidate the exact nature of the functional interaction between TRPV1 and TRPA1. One possibility is that TRPV1 and TRPA1 combine to form hetero multimeric channels.

The analgesic action of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, is produced through their inhibition of cyclooxygenase (COX), the enzyme that makes prostaglandins. Based on the molecular model in Figure 4-2 we can conclude that one of the NSAID sites of action is the COX activated by bradykinin in nociceptors.

There must be other channels beside TRPV1 and TRPA1 that play a role in the acute pain caused by bradykinin, since knockout of either channel fails to block bradykinin induced acute pain. Furthermore these other channels need to be activated via the phospholipase-C pathway. It has been shown that bradykinin acting through its B_2 receptor, phospholipase-C and release of calcium from intracellular stores inhibits M-type K^+ channels and opens Ca^{2+} activated Cl^- channels (see Figure 4-2B) (Liu, Linley et al. 2010). Furthermore, inhibitors of Ca^{2+} activated Cl^- channels and specific M-type K^+ channel openers were found to attenuate pain induced by intradermal bradykinin injection.

ATP

It was mentioned in chapter 1 that during tissue damage the release of components of the cell cytoplasm are likely candidates to act on nociceptors to cause pain. ATP (Adenosine-5'-triphosphate) is an important source of intracellular energy where it is produced during cellular respiration and consumed by many cellular processes. Human experiments have shown that delivery of ATP into skin causes pain in a dose dependent manner (Hamilton, Warburton et al. 2000). Since ATP is membrane impermeable, receptors for ATP located in the nociceptor plasma membrane are needed to detect the ATP released from damaged cells into the extracellular space. ATP targets two distinct receptor subtypes of the P2-receptor family: ATP activates ionotropic P2X receptors and metabotropic P2Y receptors. Currently, seven different P2X receptor subtypes and eight P2Y subtypes have been identified.

P2X RECEPTORS

Normally, $P2X_2$ and $P2X_3$ are expressed by small sensory neurons of the DRG. The experimental findings suggest that ATP-induced currents in DRG neurons are mediated largely by homomeric $P2X_3$ receptors and heteromeric $P2X_2$/ $P2X_3$ receptors. In $P2X_3$ knockout mice there was a significant loss of approximately 90% of DRG neurons responsive to ATP (Cockayne, Hamilton et al. 2000). A small residual sustained response to ATP was seen in some DRG neurons from $P2X_2$/$P2X_3$ double knockout mice indicating the presence of low levels of other P2X subunits or P2Y receptors in some neurons (Cockayne, Dunn et al. 2005).

The early phase of formalin-induced pain behavior was significantly reduced in $P2X_3$ knockout mice, although responses to other noxious stimuli were normal (Cockayne, Hamilton et al. 2000, Souslova, Cesare et al. 2000). In contrast, the early phase of formalin-induced pain behavior was not attenuated in $P2X_2$ knockout mice (Cockayne, Dunn et al. 2005). These in-vivo findings taken together with the in-vitro findings discussed above support the notion that the ionotropic $P2X_3$ receptor signals acute pain from damaged tissue.

A-317491 (Jarvis, Burgard et al. 2002) is a non-nucleotide antagonist that has high affinity and selectivity for blocking $P2X_3$ and $P2X_2$/ $P2X_3$ channels and was found to produce a similar reduction of formalin-induced pain as was produced in the knockout mice. Moreover, as in the knockout mice responses to noxious mechanical and thermal stimuli were normal. Taken together, the in-vitro findings, the findings from the knockout mice and those with A-317491 have provided strong evidence that $P2X_3$-containing channels contribute to nociception.

A-317491 also inhibited chronic neuropathic pain (see Chapter 6), being most potent against the mechanical allodynia and thermal hyperalgesia induced by chronic constriction of the sciatic nerve, both of which it abolished. The effect had a rapid onset and lasted for 5 hours. A-317491 was also effective against tactile allodynia induced by L5–L6 spinal nerve ligation, but was less potent than against sciatic nerve ligation.

As described in chapter 3 TRPA1 knockout mice exhibit a significant reduction in all phases of formalin-induced pain behavior including the early phase (McNamara, Mandel-Brehm et al. 2007). The attenuation of the early phase of formalin-induced pain behavior in TRPA1 knockout mice is much greater than in $P2X_3$ knockout mice. At the present time it is unclear what relationship if any exists between $P2X_3$ and TRPA1 receptors. Formaldehyde, the active ingredient in formalin, is a fixative that covalently cross-links proteins in a nonspecific manner. This cross-linking leads to a variety of effects, including general tissue damage. The thinking has been that the tissue damage releases ATP from cells and the released ATP activates $P2X_3$ receptors. In contrast the finding with TRPA1 indicates that there is a direct effect of formalin on TRPA1 rather than an indirect effect through nonspecific tissue damage (McNamara, Mandel-Brehm et al. 2007). Remember from chapter 3 that TRPA1 channels are activated by a variety of irritant compounds; the mechanism of formalin activation is likely to be similar to these compounds, which are thought to induce covalent modification of TRPA1.

VISCERAL PAIN

It has been proposed that distension of visceral organs leads to release of ATP from the epithelium lining the organ, the released ATP then acts upon P2X receptors in subepithelial nociceptors to elicit visceral pain (Burnstock 1999). However, A-317491 (Jarvis, Burgard et al. 2002) was ineffective in reducing nociception in animal models of visceral pain.

P2Y RECEPTORS

DRG neurons also express P2Y receptors and these receptors have been implicated in the potentiation of pain: extracellular ATP injection has been shown to induce thermal hyperalgesia in mice and the ATP induced thermal hypersensitivity was lost in TRPV1 knockout mice (Moriyama, Iida et al. 2003). ATP-induced thermal hyperalgesia was preserved in $P2Y_1$ knock out mice (Moriyama, Iida et al. 2003) while $P2Y_2$ knockout mice did not show significant ATP-induced thermal hyperalgesia (Malin, Davis et al. 2008). Additionally, $P2Y_2$ knockout mice show deficits in noxious heat (but not cold) sensation compared with wild type mice. The deficit in noxious heat sensation suggests that the pathway is constitutively active in the unstimulated animal.

The obvious next question is how activation of $P2Y_2$ leads to thermal hypersensitivity that is lost in TRPV1 knockout mice. A simple explanation would be that activation of $P2Y_2$ causes TRPV1 to be modified in such a way that its thermal sensitivity is increased. The best available evidence suggests that $P2Y_2$ is a GPCR that is coupled to the enzyme phospholipase C-β via the guanosine nucleotide-binding protein Gq/11. As shown in Figure 4-3 the available evidence has so far suggested two possible mechanisms by which hydrolysis of PIP_2 modulates the activity of TRPV1: either modulation of TRPV1 occurs by phosphorylation through the activation of PKC (protein kinase C) via DAG or through depletion of PIP_2 which normally acts to inhibit TRPV1.
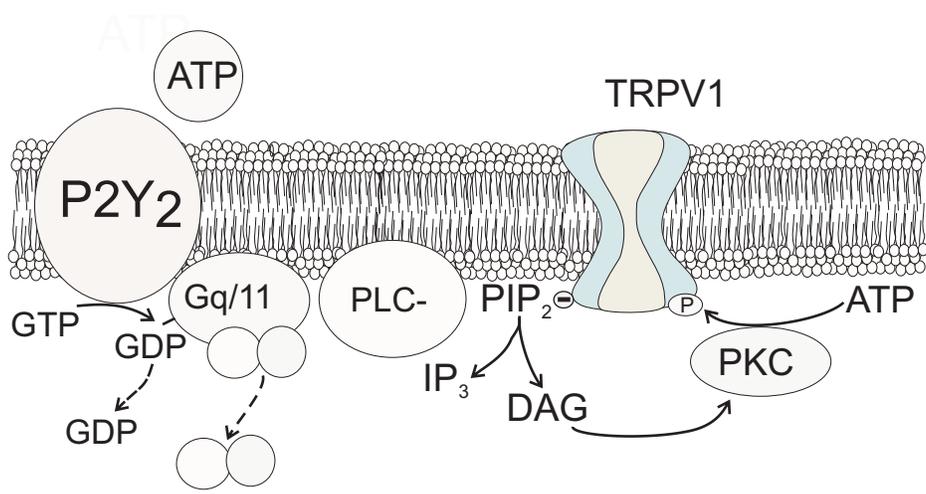
Figure 4-3. Activation of $P2Y_2$ by ATP causes
thermal hypersensitiviy via phosphorylation of
TRPV1 or release from inhibition by PIP_2.
See text for further details.

We will consider P2 receptors again when we consider the role of spinal microglia in pain.

PROTEINASE-ACTIVATED RECEPTORS

Proteases in the circulation which are generated during tissue injury have been shown to activate a family of G-protein coupled, proteinase-activated receptors (PARs). These PARs play a role in hemostasis, inflammation, and pain. The proteases cleave extracellular N-terminal domains of the PARs to expose tethered ligands that bind to and activate the cleaved receptors (see Figure 4-5). Four PARs have been identified by molecular cloning: PAR1, PAR2, PAR3 and PAR4. Of these, PAR1, PAR2 and PAR4 are present on DRG neurons and have been shown to play a role in neurogenic inflammation, that is, inflammatory symptoms that result from the release of substances from primary sensory nerve terminals (see below). Before considering the role of PAR1, PAR2 and PAR4 in neurogenic inflammation and pain we need to consider which proteases activate them and where the proteases come from. PAR2 is activated by the serine proteases tryptase and trypsin, although trypsin is able to activate PAR2, trypsin itself is not present in most tissues, thus, it is probably not the endogenous enzyme that activates PAR2. Conversely, tryptase is released during human mast cell degranulation and is able to cleave PAR2 in cells normally expressing PAR2 or in cells transfected with the receptor. Therefore tryptase is a likely candidate for the enzyme that activates PAR2 (see Figure 4-5). Thrombin appears to be the most likely agonist to activate neuronal PAR1 and PAR4.

NEUROGENIC INFLAMMATION

Over 100 years ago it was documented that antidromic stimulation of afferent sensory nerve fibers, resulting in conduction of action potentials opposite to the normal direction, results in erythema (reddening of the skin). The finding that peripheral activation of afferent sensory neurons is able to produce manifestations of an inflammatory response is referred to as "neurogenic inflammation". Destruction of capsaicin sensitive neurons greatly decreases neurogenic inflammation produced by antidromic stimulation of afferent sensory fibers, implicating the capsaicin sensitive nociceptors in neurogenic inflammation. As described in chapter 1 painful stimuli result in the generation of a train of action potentials in nociceptors that are conducted by their axons to the spinal cord, and after processing by the brain a sensation of pain occurs. On the other hand, retrograde (opposite to the usual direction) action potentials will invade the arborizations (branching terminal processes of the nociceptor) as illustrated in Figure 4-4. The resulting depolarization of the terminal causes the release of the neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP), which in turn act on target cells in the periphery such as mast cells and vascular smooth muscle producing inflammation, which is characterized by redness, warmth and swelling (Schmelz and Petersen 2001, Richardson and Vasko 2002). CGRP only induces heat hyperalgesia of nociceptors, in some mouse strains (Mogil, Miermeister et al. 2005) and presumably there is a similar variability in the ability of CGRP to induce heat hyperalgesia in the nociceptors of
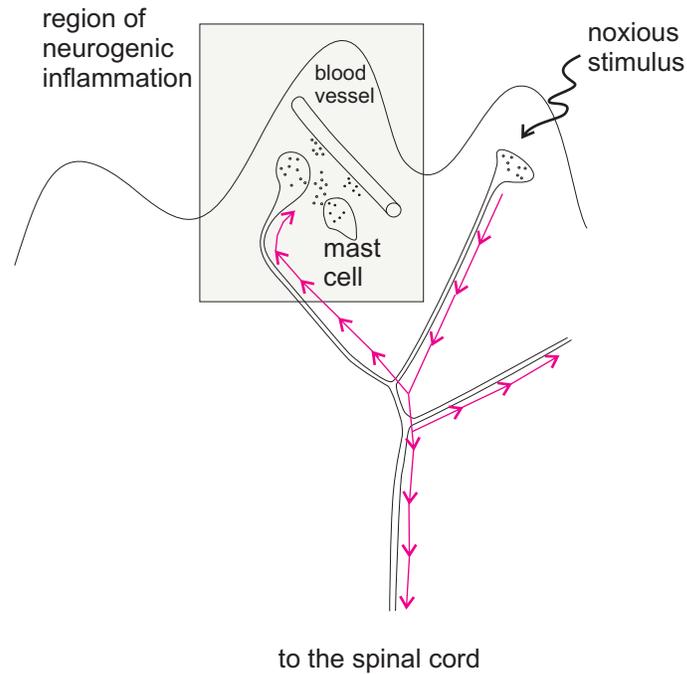
Figure 4-4. Schematic drawing of the process of neurogenic inflammation. A noxious stimulus causes the depolarization of the terminal of a nociceptor thereby initiating propagating action potentials in the axon. The direction of propagation is shown by the red arrows. Action potentials propagate along the axon towards the spinal cord and also invade the nearby branching terminal processes (arborizations) of the nociceptor. See text for further details.
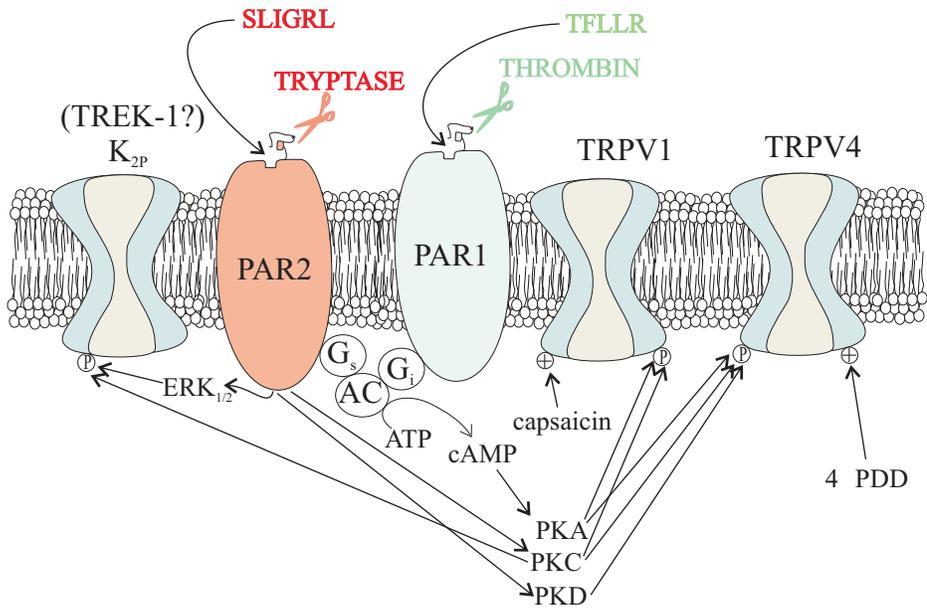
Figure 4-5. Mechanisms of nociceptor activation and inhibition by the protease receptors 2 and 1 respectively. See text for further explanation.

humans. As mentioned above PAR2 is activated by the serine proteases tryptase and trypsin, additionally a short synthetic peptide, (SLIGRL, seryl-leucyl-isoleucyl-glycyl--arginyl-leucinamide) based on the tethered ligand sequence, has been shown to activate the receptor and thereby mimic the effects of the proteases (see Figure 4-5). Injection of the synthetic SLIGRL in the rat paw has been shown to induce neurogenic inflammation that depends on the release of CGRP and SP (Steinhoff, Vergnolle et al. 2000).

Several studies have attempted to ***exclude*** the possibility that the nociceptive behavior and hyperalgesic effects of PAR2 activation might be secondary to the neurogenic inflammatory response. Intraplantar injection of the PAR2 agonist SLIGRL, at sub inflammatory doses, was found to induce thermal hyperalgesia and biting and licking (nociceptive) behavior. Remember that TRPV1 is necessary for the hyperalgesia resulting from intense noxious stimuli that result in tissue damage (see Chapter 3) and for the development of bradykinin-induced thermal hypersensitivity. Likewise, it was found that PAR2-induced hyperalgesia results from sensitization of TRPV1 (Amadesi, Nie et al. 2004) and was abolished by the capsaicin receptor antagonist capsazepine (Kawao, Shimada et al. 2002). Although capsazepine inhibited the thermal hyperalgesia it did not inhibit the PAR2-induced nociception (time spent in licking and biting the injected paw) (Kawao, Shimada et al. 2002). Further studies in DRG neurons and transfected HEK293 cells indicated that PAR2 activates PKCε and PKA in DRG neurons (see Figure 4-5), and thereby sensitizes TRPV1 (Amadesi, Cottrell et al. 2006). This finding bears similarity to the sensitization of TRPV1 by bradykinin acting via PKCε and PKA (see Figure 4-2).

Remember that although capsazepine inhibited PAR2-induced thermal hyperalgesia it did not inhibit the immediate pain, implying the existence of another pathway that excites the nociceptor. In a study of nociceptive neurons innervating the mouse colon, SLIGRL super fusion for 3 min caused a sustained depolarization, which was associated with an increased input resistance that lasted up to 60 min. and was blocked by the PKC inhibitor, calphostin, and the $ERK_{1/2}$ (extracellular signal-regulated kinase ½) inhibitor PD98059 (Kayssi, Amadesi et al. 2007). The membrane depolarization and increase in input resistance following PAR2 activation probably result from the closing of K^+ channels open at the resting membrane potential. As discussed earlier, K_{2P} channels are thought to be responsible for the cells' resting K^+ permeability and are therefore likely to be the K^+ channels that are closed following PAR2 activation (see Figure 4-5). Remember that TREK-1 is a K_{2P} channel expressed in nociceptors and TREK-1 knockout animals are hypersensitive to mechanical stimuli (Alloui, Zimmermann et al. 2006). Perhaps TREK-1 or some other K_{2P} channel is involved in the PAR2-mediated depolarization. In addition to blocking the resting K^+ permeability, SLIGRL markedly suppressed (55%) the delayed rectifier K^+ currents (Kayssi, Amadesi et al. 2007). Membrane depolarization opens the delayed rectifier K^+ channel which tends to bring the membrane potential back to the resting potential, therefore suppression of the delayed rectifier would be expected to enhance and prolong membrane depolarization caused by other ion channels.

Intraplantar injection in the rat of a sub inflammatory dose of 10 µg of SLIGRL induced a prolonged thermal and **mechanical** hyperalgesia (Vergnolle, Bunnett et al. 2001) while injection of a much lower dose produced only thermal hyperalgesia (Kawabata, Kawao et

al. 2001). Because PAR2 mediated mechanical hyperalgesia was not observed in TRPV4 knockout mice, it was "hypothesized that PAR2-mediated mechanical hyperalgesia requires sensitization of TRPV4" (Grant, Cottrell et al. 2007). Moreover, intraplantar injection of the TRPV4 agonist 4α-Phorbol 12,13-didecanoate (4α-PDD) (Watanabe, Davis et al. 2002) produced mechanical hyperalgesia in normal mice which was not observed in TRPV4 knockout mice (Grant, Cottrell et al. 2007). Lastly, it was found that "antagonists of phospholipase Cβ and protein kinases A, C and D inhibited PAR2-induced sensitization of TRPV4 Ca^{2+} signals and currents". In Figure 4-5 I have attempted to summarize the different mechanisms, just described, by which PAR2 activation is thought to cause pain and both thermal and mechanical hyperalgesia.

As mentioned above thrombin appears to be the most likely agonist to activate PAR1. Just as for PAR2, which is activated by a short synthetic peptide based on the tethered ligand sequence of PAR2, PAR1 can be selectively activated by the synthetic peptide (TFLLR) corresponding to the PAR1 receptor's tethered ligand (see Figure 4-5). Intraplantar injection of the PAR1 agonist TFLLR, at sub inflammatory doses, increased the threshold and withdrawal latency for both mechanical and thermal stimuli (Asfaha, Brussee et al. 2002). The available evidence from other systems suggests that PAR1 is negatively coupled to adenylate cyclase (AC) via the inhibitory G-protein (G_i), thereby inhibiting the activation of PKA. Thus, the PAR-1 induced analgesia might possibly be explained by inhibition of adenyl cyclase as shown in Figure 4-5.

In addition to PAR1, PAR4 has been identified as another protease activated receptor important for analgesia (Asfaha, Cenac et al. 2007). Intraplantar injection of a short synthetic peptide (GYPGKF) based on the tethered ligand sequence of PAR4 increased nociceptive threshold in response to thermal and mechanical noxious stimuli. Moreover, co-injection of GYPGKF with carrageenan significantly reduced the resulting hyperalgesia and allodynia. For the sake of simplicity PAR4 has been omitted from Figure 4-5.

The role of PAR1 and PAR4 in nociception is more complex than just described. While low doses of thrombin are anti nociceptive higher levels cause inflammation and pain (Vergnolle, Hollenberg et al. 1999, Vellani, Kinsey et al. 2010).

We will return to neurogenic inflammation and protease-activated receptors again when we consider migraine pain.

LOW pH

Acidosis is a common feature of ischemia and is *assumed* to play a critical role in ischemic pain. Injection of acidic solutions (pH 5.0 – 6.0) cause intense burning pain (Issberner, Reeh et al. 1996) and a substantial decrease in synovial fluid pH (6.6-7.4) is found in inflamed joints (Treuhaft and McCarty 1971). There is no consensus concerning the identity of the specific molecular receptor that is activated by low pH in nociceptors. A decrease in extracellular pH has two effects on TRPV1: first extracellular protons

lower the threshold for TRPV1 activation by capsaicin and heat, and second, acidification below pH 6.0 directly opens the channel. It is unlikely that TRPV1 is the sole sensor for extracellular protons because individual nerve fibers in rat skin which fire action potentials in response to protons do not always fire in response to capsaicin. Moreover, in DRG neurons from TRPV1 knockout mice the response to protons was reduced but not eliminated (Caterina, Leffler et al. 2000). With the discovery and subsequent cloning of acid sensing ion channels (ASICs), they became candidates for the sensor of extracellular protons. Normally a significant fraction of DRG neurons from mice exhibit ASIC-like transient currents in response to protons (pH 5.0) in contrast, for transgenic mice expressing a dominant-negative form of ASIC3, none of the neurons exhibited ASIC-like transient currents (Mogil, Breese et al. 2005). Surprisingly, in behavioral tests the transgenic mice were found to be **more** sensitive to a number of pain modalities than the wild type mice, making it unlikely that they are direct transducers of nociceptive stimuli. On the other hand the cardiac afferents that mediate pain during cardiac ischemia (angina), display large acid evoked depolarizing currents and they fire action potentials in response to the extracellular acidification that accompanies myocardial ischemia. Moreover it was proposed that ASIC3 is the sensor of myocardial acidity that triggers cardiac pain (Sutherland, Benson et al. 2001).

The intense pain produced by a heteromeric complex of two proteins purified from Texas coral snake venom was shown to function as a selective and potent agonist for ASIC channels. The complex was found to be selective for ASIC1 at neutral pH and at lower pH (<6.5) the complex greatly potentiated proton evoked activation of ASIC2 (Bohlen, Chesler et al. 2011). These findings suggest that ASIC channels play some as yet unidentified role in nociception.

When studying mambalgins, peptides found in the venom of the deadly African snake the Black Mamba it came as a complete surprise to the researchers that mambalgins, rather than causing pain, have a potent analgesic effect. They found that blockade of heteromeric acid sensing ion channels made of ASIC1a and ASIC2a subunits in central neurons and of ASIC1b containing channels in nociceptors is involved in the analgesic effect of mambalgins (Diochot, Baron et al. 2012). These pioneering findings introduce the possibility of using natural peptides as potent analgesics and identify specific ASIC subunits as new therapeutic targets for the treatment of pain.

The sensitivity to protons of human dorsal root ganglion (hDRG) neurons was examined by rapid acidification of the extracellular fluid from pH 7.35 to 6.0 causing a prolonged depolarization of the membrane potential in all 40 cells tested (Baumann, Burchiel et al. 1996). Surprisingly, the membrane depolarization was associated with a decrease in membrane conductance in 27 of the 40 cells tested rather than the increase in conductance that would be expected with activation of TRPV1 or ASICs (see Figure 4-1). Subsequent ion substitution experiments indicated that the decrease in conductance upon acidification was due to a decrease of the background K^+ permeability (Baumann, Chaudhary et al. 2004). In as much as K_{2P} channels are thought to be responsible for the cells' resting K^+ permeability, it is possible that an acid sensitive K_{2P} channel is involved. Two K_{2P} channels (TASK-1 and TASK-2) and an inward rectifying K^+ channel (Kir2.3)

that are blocked by protons have been identified in DRG neurons (Baumann, Chaudhary et al. 2004). Inward rectifying K^+ channels are not perfect rectifiers; they can pass some outward current in the voltage range above the resting potential, therefore their inhibition would be expected to enhance and prolong the membrane depolarization caused by other ion channels.

The effects of protons considered above might involve the direct effect of protons on several different ion channels considered above (TRPV1, ASICs, TASK-1, TASK-2, Kir2.3). However, it is possible that a receptor molecule that is actually separate from the ion channel that is being modulated mediates the effects of protons. Recently, proton-sensing G-protein coupled receptors have been identified (Ludwig, Vanek et al. 2003), and subsequently shown to be present in the small-diameter DRG neurons responsible for nociception (Huang, Tzeng et al. 2007). Using a Xenopus oocyte expression system, external acidosis was shown to profoundly down-modulate human TREK-1 activity (Cohen, Sagron et al. 2009). The authors were able to distinguish a rapid and slow component of the decrease in TREK-1 currents resulting from exposure to low external pH. The fast component resulted from protonation of extracellular residues on TREK-1. While the slow component, of TREK-1 desensitization was mediated by a proton-sensitive GPCR that appeared to activate phospholipase-C. There are a number of ways in which activation of phospholipase-C could potentially modulate TREK-1 activity (refer to Figure 4-2 for several examples). The original paper describing proton-sensing G-protein coupled receptors showed that the receptor was inactive at pH 7.8, and fully activated at pH 6.8 (Ludwig, Vanek et al. 2003). Thus it only takes weak-to moderate extracellular acidification to fully activate the proton-sensitive GPCR, making them ideal receptors for the detection of extracellular acidification.

LYSOPHOSPHATIDIC ACID

Lysophosphatidic acid (LPA, 1-acyl-*sn*-glycerol-3-phosphate), the simplest glycerol-phospholipid, has one mole of fatty acid per mole of lipid: where the fatty acid can be either saturated or unsaturated, depending on the tissue. The pathways, which might give rise to the production of LPA, are illustrated in Figure 4-6. LPA is rapidly produced and released from activated platelets and is more abundant in serum (1-5 μM) than in plasma. Where plasma is the liquid portion of the blood that is separated from the blood cells and serum is the leftover fluid after agitating the plasma to precipitate a clot. Six G protein-coupled receptors (LPA1 …… LPA6) specific for lysophosphatidic acid have so far been identified.

Autotaxin, also known as ectonucleotide pyrophosphatase/phosphodiesterase 2 (NPP2 or ENPP2), is a secreted enzyme important for generating LPA. It was discovered that autotaxin also has lysophospholipase D activity that converts lysophosphatidylcholine into LPA. This enzyme probably provides most of the extracellular lysophosphatidic acid from lysophosphatidylcholine.

Intraplantar injection of LPA in mice provokes painful responses (Renback, Inoue et al. 1999), that are substantially reduced in TRPV1 knockout mice (Nieto-Posadas, Picazo-
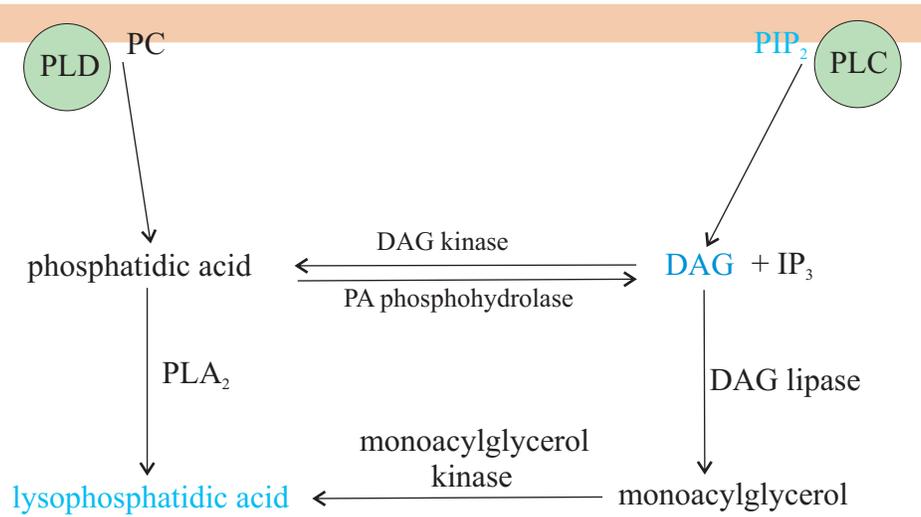
PLD phospholipase D
PC   phosphatidyl choline
PLC phospholipase C
PIP$_2$ phosphatidylinositol (4,5)-bisphosphate
DAG diacylglycerol
PA   phosphatidic acid
IP$_3$ inositol 1,4,5-trisphosphate

Figure 4-6. Pathways for the production of lysophosphatidic acid.

Juarez et al. 2011). Moreover, LPA appears to interact directly with the C terminus of TRPV1 to activate it.

The effects of LPA on TREK-1 ion channels were investigated using a Xenopus oocyte expression system (Cohen, Sagron et al. 2009). At a physiological concentration of 1 μM, LPA dramatically decreased TREK-1 currents; the effect persisted for minutes and was reversible upon washout. Pre-treatment with U73122, an inhibitor of phospholipase-C completely blocked the LPA-induced decrease in TREK-1 currents. Further experiments indicated that phospholipase-C was probably being activated by endogenous Xenopus LPA receptors.

The effects of LPA on voltage gated sodium currents in rat dorsal root ganglion (DRG) neurons were also investigated (Seung Lee, Hong et al. 2005). LPA suppressed tetrodotoxin sensitive sodium currents while increasing tetrodotoxin insensitive sodium currents. The voltage gated sodium currents will be considered more fully in chapter 5 and the role of lysophosphatidic acid signaling in the development of neuropathic pain will be considered in chapter 6.

Epac (EXCHANGE PROTEIN DIRECTLY ACTIVATED BY cAMP)

Until the discovery of Epac (exchange protein directly activated by cAMP) the effects of cAMP were originally attributed solely to the activation PKA. Epac proteins function as guanine nucleotide exchange factors (GEFs) for Rap1 and Rap2 members of the Ras family of small G proteins, which cycle between an inactive GDP-bound state and an active GTP-bound state. GEFs catalyze the exchange of GDP for GTP and thereby the activation of the G protein. It has been shown that in dorsal root ganglia both Epac1 mRNA and Epac1 protein expression levels increase during neuropathic pain and nerve damage induced allodynia is reduced in Epac1 knockout mice (Eijkelkamp, Linley et al. 2013). Furthermore the same authors provided evidence indicating that the target for Epac1 is Piezo2.

NERVE GROWTH FACTOR

Nerve growth factor (NGF) is a trophic factor that promotes the survival of nociceptors during development. For our purposes though, the important findings are that the high-affinity NGF receptor (trkA) is expressed on nociceptors in adult animals and that NGF levels are elevated in inflamed skin. Using a synthetic trkA-IgG fusion molecule to sequester NGF it was found that administration of trkA-IgG together with carrageenan blocked the hyperalgesia resulting from carrageenan-induced inflammation (McMahon, Bennett et al. 1995). Additionally, the TrkA receptor mediates the hyperalgesia caused by NGF because NGF can still induce thermal hyperalgesia in mice in which the low affinity p75 neutrophin-receptor is knocked out (Bergmann, Reiter et al. 1998). These findings strongly suggest that NGF is involved in regulating the sensitivity to pain in adult animals. Moreover, thermal hyperalgesia of the hind paw developed within minutes of

intraplantar NGF injection indicating that gene transcription was not involved (Lewin, Ritter et al. 1993).

In order to examine the direct effects of NGF on nociceptors from adult animals' experiments were carried out on dissociated DRG neurons in culture. A rapid enhancement of the capsaicin-induced current was observed with NGF application in these isolated DRG neurons (Shu and Mendell 1999, Shu and Mendell 2001). These findings clearly indicate that one of the targets of NGF is TRPV1. Subsequent experiments, using DRG neurons and expression systems, to try and elucidate the signaling pathways involved in sensitization by NGF have so far yielded conflicting results.

Tanezumab is a humanized monoclonal antibody, which binds and inhibits nerve growth factor that has been tested for the treatment of pain in patients with osteoarthritis. Patients experienced a reduction in joint pain and an improvement in function. However, the FDA suspended further studies of tanezumab because a small number of patients experienced worsening symptoms that eventually required surgery (Lane, Schnitzer et al. 2010). More recently the FDA concluded in 2012 that additional trials with Tanezumab are warranted but with new restrictions and protocols aimed at tracking down the cause of the joint damage.

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Chapter 5 Na^+, K^+, Ca^{++} and HCN Channels

After the noxious stimulus is transduced into a receptor potential the response must be transformed or encoded into a train of action potentials, which carry the signal to the synaptic terminal culminating in neurotransmitter release (i.e. information is conveyed to the spinal cord). It is during this process that voltage gated sodium, potassium, and calcium and hyperpolarization-activated, cation nonselective (HCN) channels make their contribution. To the extent that these channels are enriched in nociceptors in comparison to other regions of the nervous system and the body they may serve as targets for the development of analgesic or anesthetic drugs.

Na^+ CHANNELS

Voltage-gated sodium channels are essential for encoding the receptor potential into a series of action potentials and for conducting the action potentials along the axon. Voltage-gated sodium channels are composed of a pore-forming alpha subunit and at least one auxiliary β subunit. β-subunits are multifunctional: they modulate channel gating, regulate the level of channel expression, and function as cell adhesion molecules interacting with the extracellular matrix and the cytoskeleton. The family of pore-forming α-subunits has nine known members named $Na_v1.1$ through $Na_v1.9$; not all of these are present in DRG neurons.

Neurons differ in the shape of their action potentials and also in the rate and regularity at which they fire action potentials. Generally speaking nociceptors fire action potentials having a longer duration (several milliseconds) and a relatively slow rate typically in a range less than 10 per second.

One can distinguish two general classes of voltage-gated sodium channels based on their sensitivity to tetrodotoxin (TTX) a potent neurotoxin that blocks action potentials in nerves by binding to the pore of voltage-gated sodium channel α-subunits. Not all α-subunits are sensitive to TTX; therefore we can distinguish between TTX sensitive (TTX-S) and TTX resistant (TTX-R) sodium channels. Nociceptive sensory neurons have been shown to express both TTX-R and TTX-S sodium channels. Of the voltage-gated sodium channels, the TTX-S channels $Na_v1.1$, $Na_v1.3$, $Na_v1.6$ and $Na_v1.7$, and the TTX-R channels $Na_v1.8$ and $Na_v1.9$, have been implicated in the functioning of nociceptors in both normal and pathological states.

Characterization of the contribution of specific voltage-gated sodium channels to the functioning of DRG neurons is limited by the lack of selective channel blockers. Generation of knockout mice for specific voltage-gated sodium channels provides an alternative solution to this problem and these animals can be characterized behaviorally. Furthermore electrophysiological recordings can be made from these animals to further characterize the contribution of specific channels to the detection of painful stimuli.

The TTX-R voltage gated sodium channels $Na_v1.8$ and $Na_v1.9$ are expressed predominantly in small DRG neurons, which include nociceptive cells. Furthermore, the

voltage-gated sodium channel $Na_v1.7$ is also predominantly expressed in DRG neurons suggesting that $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ are good targets for the pharmacologic treatment of pain. What's more the findings described below strongly indicate that these channels make excellent targets for the treatment of pain.

$Na_v1.7$

In 2006 a group of individuals from three families were reported upon, who exhibited a congenital inability to perceive pain and were otherwise apparently normal (Cox, Reimann et al. 2006). The loss of the ability to sense pain was shown to result from nonsense mutations in the gene SCN9A, which encodes the α-subunit of the TTX-S voltage-gated sodium channel $Na_v1.7$ that is expressed at high levels in nociceptive DRG neurons. Before these findings in humans $Na_v1.7$ knockout mice were found to die shortly after birth, therefore $Na_v1.7$ had to be deleted in a subset of neurons, therefore yielding less useful information than the findings from the global knockout of $Na_v1.7$ in humans (Nassar, Stirling et al. 2004). The absence of serious effects, other than loss of pain sensation with a total loss of $Na_V1.7$, as demonstrated in patients with congenital inability to perceive pain, have stimulated intense efforts to develop $Na_v1.7$-specific analgesics for the treatment of pain.

There is now also evidence that point mutations in SCN9A can result in an increase in pain sensations. Patients, with the painful inherited neuropathy, inherited erythromelalgia (sometimes called erythromelalgia), experience episodes of severe chronic burning pain, skin redness, and swelling of the extremities, ears and face. Patients with primary erythromelalgia have mutations in SCN9A which encodes the α-subunit of $Na_v1.7$ (Yang, Wang et al. 2004). The electrophysiological properties of mutant $Na_v1.7$ channels having the same mutations found in patients with erythromelalgia have been studied (Cummins, Dib-Hajj et al. 2004). The mutant channels exhibited hyper-excitability brought about by a hyperpolarizing shift in activation and by a slowing in deactivation and inactivation.

Other mutations that make $Na_v1.7$ more active have been linked to additional pain conditions: paroxysmal extreme pain disorder (PEPD) and idiopathic small fiber neuropathy (I-SFN) (Faber, Hoeijmakers et al. 2011, Dib-Hajj, Yang et al. 2013).

$Na_v1.8$

$Na_v1.8$ is expressed predominantly in small diameter nociceptive afferents and $Na_v1.8$ knockout mice exhibit a pronounced increase in the threshold to noxious mechanical stimuli determined by Randall-Selitto stimulation in contrast thresholds to stimulation with Von-Frey hairs were the same in wild-type or knockout mice (Akopian, Souslova et al. 1999, Matthews, Wood et al. 2006). Before considering these findings it is necessary to digress and consider two different methods that are commonly used to study mechanical pain thresholds. Von Frey hairs have small tip diameters (typically 0.5 mm) therefore it is unlikely that the mechanical stress applied to the skin surface using them is transmitted to the deeper tissues such as muscle. Therefore Von Frey hairs are thought to measure the pain threshold of the skin. In contrast,

the Randall Selitto apparatus for applying pressure can be used with a probe having a much larger tip diameter (10 mm) hence the force applied can be transmitted more effectively to deeper tissues through the skin. Consequently, a change in the pain threshold measured with the Randall-Selitto method is considered to represent a change in the pain threshold in deeper tissue, possibly muscle, when there is a concomitant absence of a change in the Von Frey hair pain threshold.

In order to ablate neurons expressing $Na_v1.8$ the human diphtheria toxin receptor was inserted into cells expressing $Na_v1.8$. Mice in which diphtheria toxin A was then used to kill neurons expressing $Na_v1.8$ (DTA mice) showed a pronounced increase in the threshold to noxious mechanical stimuli (Abrahamsen, Zhao et al. 2008). Behavioral responses to mechanical stimuli applied with Von Frey hairs were normal in the DTA mice while behavioral thresholds measured using the Randall-Selitto apparatus were dramatically elevated. Interestingly, in these studies responses to noxious heat were similar to those in normal mice and the development of inflammatory hyperalgesia was suppressed.

These findings might be explained if $Na_v1.8$ voltage-gated sodium channels were specifically localized to mechano sensitive nociceptors and determined their excitability. In this context TTX-R sodium channels have been shown to be present at sufficiently high densities in the peripheral terminals of nociceptors to be the determinant of their excitability (Brock, McLachlan et al. 1998). Alternatively, knockout of $Na_v1.8$ might disrupt the mechano sensing apparatus located in the nociceptor terminal. Further studies of the exact role of $Na_v1.8$ in mechano sensitive nociception and the development of highly specific $Na_v1.8$ channel blockers would help to clarify the situation.

In addition to the increase in the threshold to noxious mechanical stimuli, $Na_v1.8$ knockout mice also exhibited a loss of sensitivity to noxious cold stimuli (Matthews, Wood et al. 2006, Zimmermann, Leffler et al. 2007). The loss of sensitivity to noxious cold probably occurs because $Na_v1.8$ appears to be the only voltage gated sodium channel that remains functional at very cold temperatures (Zimmermann, Leffler et al. 2007). Additionally in the cold much lower currents were needed to trigger $Na_v1.8$ generated action potentials than at 30° C for TTX treated nociceptor terminals (Zimmermann, Leffler et al. 2007). The decreased threshold for triggering action potentials at reduced temperatures probably results from an increase in the input membrane resistance due to the closure of background potassium (K_{2P}) channels (Maingret, Lauritzen et al. 2000, Reid and Flonta 2001). Remember as discussed in chapter 4 (see Figure 4-1) the closure of background potassium channels will depolarize the membrane and furthermore it will also increase the input membrane resistance, thus less inward current initiated by noxious stimuli will be needed to trigger action potentials. With respect to the TRP channels thought to be cold sensors, DTA mice (Abrahamsen, Zhao et al. 2008) exhibit a reduced expression of the cold sensor TRPA1 and they exhibit an almost complete suppression of the second phase of the formalin response, which has been ascribed to activation of TRPA1 (McNamara, Mandel-Brehm et al. 2007). In contrast the expression of the cold sensor, TRPM8 appears to be normal in DTA mice. Based on these results one can make a tentative model for the transduction of noxious cold in mouse mechano-cold

nociceptors as illustrated in Figure 5-1. Exposure to a noxious cold temperature around
0° C results in the activation of TRPA1 and the inhibition of the background potassium
channels (K_{2P}), the resulting depolarization excites $Na_v1.8$ and action potentials
propagate to the spinal cord. In the model of Figure 5-1 the mechano sensing apparatus,
which is upstream from $Na_v1.8$ has been left out for the purpose of simplicity.

As mentioned above, there was an almost complete suppression of the second phase of
the formalin response in the DTA mice. Additionally, spontaneous pain, as well as
thermal- and mechanical- hyperalgesia induced by Freund's complete adjuvant (FCA)
injections were also lost as was carageenan and NGF evoked thermal hyperalgesia.
Consequently, neurons expressing $Na_v1.8$ play an essential role in both thermal and
mechanical hyperalgesia and spontaneous pain after an inflammatory insult. In contrast
neuropathic pain (see chapter 6) was unchanged in the DTA mice.

A mutation that makes $Na_v1.8$ more active which results in increased excitability of DRG
neurons has been linked to painful small fiber peripheral neuropathy (Huang, Yang et al.
2013).

$Na_v1.9$

Most DRG neurons that express $Na_v1.8$ also express $Na_v1.9$, although a very small
number of DRG cells express either only $Na_v1.8$ or $Na_v1.9$ (Amaya, Decosterd et al.
2000). For mice in which $Na_v1.9$ has been knocked out the behavioral response to
noxious mechanical, noxious heat and noxious cold stimulation were no different than in
wild type mice (Priest, Murphy et al. 2005, Amaya, Wang et al. 2006). Additionally,
experiments, using a skin nerve preparation, indicated that mechanical and thermal pain
thresholds were the same for $Na_V1.9$ knockout mice and wild type mice (Priest, Murphy
et al. 2005). In contrast hypersensitivity resulting from peripheral inflammation produced
by intra plantar injection of complete Freund's adjuvant is substantially reduced in the
knockout mice (Amaya, Wang et al. 2006), as is the hypersensitivity resulting from intra
plantar injection of formalin or carrageenan (Priest, Murphy et al. 2005).

As shown in chapter 4 multiple inflammatory mediators (such as PGE_2, bradykinin, ATP
and 5-HT) acting through multiple intracellular signaling pathways (for example PLC-β,
PKA and PKC) reduce the threshold for painful stimuli. In chapter 4 evidences were
presented that these intracellular pathways targeted the ion channels TRPV1, TRPA1,
TRPV4 and K_{2P} to produce the hypersensitivity. The evidence presented above suggests
that $Na_V1.9$ might also be a target for these inflammatory mediators and intracellular
signaling pathways. In this context thermal hypersensitivity resulting from intra plantar
injection of PGE_2 was significantly reduced in $Na_v1.9$ knockout mice compared to wild
type mice (Priest, Murphy et al. 2005, Amaya, Wang et al. 2006). There is as yet no
consensus as to the intracellular signaling pathway that targets $Na_v1.9$. Because the
inflammatory mediators PGE_2, bradykinin, ATP and 5-HT mentioned above act through
G-protein coupled receptors the hypothesis that activation of G-proteins was required to
regulate $Na_v1.9$ was tested (Baker, Chandra et al. 2003). They found that the amplitude of
a TTX-R Na^+ current, attributed to $Na_v 1.9$, recorded from small DRG neurons having
less than a 25 μm diameter, was increased more than 3-fold by 500 μm intracellular GTP-
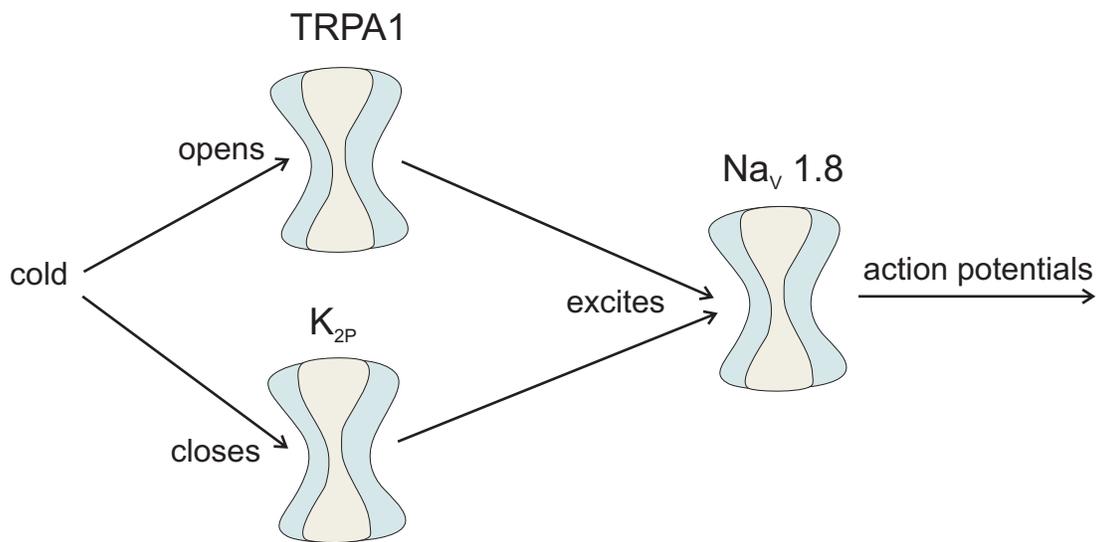
Figure 5-1. In a mechano-cold nociceptor exposure to a cold temperature of around O^oC opens TRPA1channels and closes K_{2P} channels, which in turn activate $Na_V1.8$ channels thereby exciting the nociceptor.

γ –S, a non-hydrolysable analog of GTP. Presumably, GTP- γ –S was working by binding to a G-protein and keeping the G-protein to which it is bound in the active state because the GTP- γ –S is resistant to hydrolysis to GDP (see Chapter 4). A later study showed that the TTX-R Na$^+$ current being up regulated was indeed Na$_v$1.9 (Ostman, Nassar et al. 2008). To reiterate, although these findings indicate the involvement of a G-protein they do not point to the intracellular signaling pathway that is activated by the G-protein. One possibility is that the activated G-protein, with bound GTP- γ –S, works by interacting directly with Na$_v$1.9.

As mentioned above the loss of the ability to sense pain was shown to result from **nonsense** mutations in the gene SCN9A, which encodes the α-subunit of the TTX-S voltage-gated sodium channel Na$_v$1.7. Interestingly, a **missense** mutation in the gene SCN11A which encodes the α-subunit of the TTX-R voltage-gated sodium channel Na$_v$1.9 causes loss of pain perception (Leipold, Liebmann et al. 2013). Surprisingly, the mutant Na$_v$1.9 channels were more active indicating that increased activity in Na$_v$1.9 results in pain insensitivity. It was suggested that Na$_v$1.9 is active at resting voltages resulting in a sustained depolarization of nociceptors and impaired synaptic transmission and generation of action potentials. The possibility that Na$_v$1.9 is active at resting voltages suggests a novel way to modulate pain perception.

Na$_v$ 1.3

Neuropathic pain, or "neuralgia", sometimes develops following injury or disease that damages a nerve. A variety of changes occur both in nociceptors and also in the central nervous system after nerve damage, among these changes hyper-excitability of DRG neurons is well documented after injury to DRG peripheral axons. It has been proposed that hyper excitable DRG neurons might contribute to neuropathic pain (for example see (Devor 2001)) and that upregulation of Na$_v$1.3 contributes to the abnormal hyper excitability of injured DRG neurons (Black, Cummins et al. 1999). Na$_v$1.3 is a TTX-S channel expressed in neurons throughout the embryonic nervous system that is substantially down regulated in adult animals. However, normal levels of neuropathic pain behavior develops in Na$_v$1.3 knockout mice (Nassar, Baker et al. 2006) suggesting that Na$_v$1.3 expression is not necessary for the development of neuropathic pain.

Na$_v$1.1 and Na$_v$1.6

Although Na$_v$1.1 and Na$_v$1.6 are expressed in nociceptors their specific roles if any, in nociception and pain sensation is as yet not clear.

K$^+$ CHANNELS

ATP-sensitive K$^+$ channels

For the reason that early work showed that glibenclamide, a blocker of ATP-dependent potassium channels (K$_{ATP}$), antagonizes morphine analgesia (Ocana, Del Pozo et al.

1990) this section begins with a discussion of morphine and its' receptors. The pain relieving properties of morphine, the principal active ingredient in opium has been known for centuries. Morphine's analgesic effect is primarily attributed to the activation of opioid receptors within the central nervous system. However, morphine acts both in the central nervous system and the peripheral nervous system to cause analgesia. For our purposes we are only interested in its action on nociceptors. A variety of experiments have suggested the existence of at least three types of opioid receptors, μ, δ and κ. Morphine's analgesic effects are dramatically reduced in mice lacking the μ opioid receptor suggesting that μ-receptors primarily mediate analgesia (Loh, Liu et al. 1998). Additionally, in behavioral studies in the rat the analgesic effects of peripherally administered morphine also appear to be mediated by the μ-receptor but are not readily detectable in normal tissue; the effects are only apparent during hyperalgesia when receptors are sensitized (Levine and Taiwo 1989). Utilizing a rat skin-nerve preparation the effects of morphine on the response properties of single nociceptors innervating normal and inflamed skin have been compared (Wenk, Brederson et al. 2006). Morphine had no significant effect on the response of nociceptors to mechanical or thermal stimuli in normal skin. However, nociceptors innervating inflamed skin exhibit lower thresholds for noxious mechanical stimuli and responses to noxious mechanical and thermal stimuli were elevated, and peripherally administered morphine inhibited the activity of cutaneous nociceptors under these conditions of inflammation. These findings indicate that morphine acting on opioid receptors located in the sensory transduction region of the nociceptor (see Figure 1-3) mediate analgesia during local inflammation.

It has been known for some time that high blood glucose levels antagonize morphine analgesia and it was suggested that the effect might be due to elevated intracellular ATP levels (Singh, Chatterjee et al. 1983). Subsequently, as mentioned above, it was shown that glibenclamide, a blocker of ATP-dependent potassium channels (K_{ATP}), antagonizes morphine induced analgesia in mice (Ocana, Del Pozo et al. 1990), suggesting that morphine acts by activating K_{ATP} channels. ATP-dependent potassium channels are a type of potassium channel composed of four regulatory sulfonylurea receptor (SUR) subunits and four ATP sensitive pore forming inwardly rectifying potassium channel (K_{ir}) subunits.

K_{ATP} channels act as metabolic sensors and when intracellular ATP levels are high the channels are closed. In capsaicin sensitive rat DRG neurons, diazoxide a K_{ATP} channel agonist, reversed the sensitization produced by PGE_2, indicating that activation of K_{ATP} can reverse the enhanced excitability of DRG neurons (Chi, Jiang et al. 2007). The opioid receptors are G-protein coupled receptors, which raises the question of which intracellular second messenger pathway mediates the action of morphine on K_{ATP}. It has been suggested that morphine activates K_{ATP} via the nitric oxide-cGMP-PKG pathway (Granados-Soto, Rufino et al. 1997, Sachs, Cunha et al. 2004).

GIRK channels

In mice GIRK channels (G protein coupled inwardly rectifying potassium channels) are also involved in the analgesic effects of morphine (Cruz, Berton et al. 2008, Devilliers, Busserolles et al. 2013).

K_{2P} channels

The K_{2P} channel TRESK, encoded by the gene KCNK18, has recently been implicated in the pathogenesis of migraine with aura (Lafreniere, Cader et al. 2010). Where aura refers to the early symptoms that occur before the actual headache begins in about 20% of migraine sufferers. Characterization of this mutation demonstrated that it had a dominant negative effect causing a complete loss of TRESK function. These findings point to this channel as a potential target for drug development.

In chapter 4 we considered a number of situations in which chemical mediators such as serotonin, tryptase etc. appear to excite (depolarize) nociceptors by blocking the resting background K^+ permeability (K_{2P} channels). There is yet another way in which these background K^+ channels may play a role in nociception, it turns out that the members of the TREK subfamily (TREK-1, TREK-2 and TRAAK) of the K_{2P} channels are activated by both mechanical and thermal stimuli (for example see (Kang, Choe et al. 2005)). Moreover, these channels are colocalized with the TRP channels, TRPV1, TRPV2 and TRPM8 in trigeminal ganglion neurons (Yamamoto, Hatakeyama et al. 2009). The activity of TREK-1, TREK-2 and TRAAK is very low at around 24° C and they become very active at 37° C (Kang, Choe et al. 2005). Additionally membrane stretch greatly increases the activity of TREK-1, TREK-2 and TRAAK at 37° C (Kang, Choe et al. 2005). The activity of all three channels continues to increase up to 42° C the maximum temperature for which accurate measurements could be made in the expression systems used. Remember from chapter 3 that TRPV1 is activated at temperatures above 42° C. Assuming that the activity of these K_{2P} channels continues to increase above 42° C then their activation in nociceptors would tend to counteract excitation by increasing the membrane K^+ permeability. Consequently, it was suggested that painful mechanical and thermal stimuli in nociceptors would be expected to activate members of the TREK subfamily of K_{2P} channels, thereby "tuning" or counteracting excitation (Honore 2007, Noel, Zimmermann et al. 2009). That is, activation of a nociceptor by noxious mechanical or thermal stimuli is a balance between depolarization caused by activation of a mechano or thermo sensitive excitatory ion channels and hyperpolarization caused by activation of a TREK K_{2P} channel family members.

TREK-1 is colocalized with TRPV1 and TREK-1 knockout mice are more sensitive to painful thermal and mechanical stimuli (Alloui, Zimmermann et al. 2006). Furthermore, TREK-1 knockout mice display increased inflammatory thermal and mechanical hyperalgesia. In addition to the K_{ATP} channels and GIRK channels mentioned above morphine has also been shown to induce analgesia by activating the K_{2P} channel TREK-1 (Devilliers, Busserolles et al. 2013). These findings point to TREK-1 as a likely target for drug development.

One aspect of the above findings with morphine that deserves mentioning is the effectiveness of activating a variety of K^+ channels for the induction of analgesia.

K_{Na} channels

K^+ channels activated by internal Na^+ (K_{Na} channels) were first described in DRG neurons more than 15 years ago (Bischoff, Vogel et al. 1998). Because K_{Na} channels are activated by intracellular Na^+ they are ideally suited to act as effectors of negative feedback during neuronal excitation. That is, as a result of sodium influx K^+ channels activated by internal Na^+ act to limit neuronal excitation, for example the influx of sodium that occurs during the opening of voltage gated sodium channels would increase the potassium permeability by activating K_{Na}. Consequently K_{Na} channels may play an important role in regulating DRG neuron excitability.

Subsequently, it was discovered that the K_{Na} current in cultured DRG neurons is inhibited by PKA (Nuwer, Picchione et al. 2010). Additionally, PKA was known to be important for the induction of hypersensitivity during inflammation (Malmberg, Brandon et al. 1997). Taken together these findings suggest that inhibition of K_{Na} plays a role in the development of nociceptor hypersensitivity during inflammation.

Outward K^+ channels

Remember that the closing of background potassium channels will, in addition to depolarizing the membrane, will also increase the input membrane resistance; thus less exogenous current will be needed to trigger action potentials. Another way to increase the effectiveness of inward currents initiated by noxious stimuli in triggering action potentials is to inhibit depolarization activated potassium currents (K_v). In rat DRG neurons PGE_2 and the stable PGI_2 analog carbaPGI_2 suppressed the sustained type of voltage gated outward K^+ current (Nicol, Vasko et al. 1997). Also, remember from chapter 4 that bradykinin acting through its B_2 receptor, phospholipase-C and release of calcium from intracellular stores inhibits M-type K^+ channels.

Ca^{++} CHANNELS

Opioid μ-receptors have also been localized to the synaptic terminal of DRG neurons in the spinal cord (Besse, Lombard et al. 1990, Arvidsson, Riedl et al. 1995). Additionally, morphine applied to the spinal cord reduced substance P release evoked by sciatic nerve stimulation (Go and Yaksh 1987). Moreover, opioids were found to suppress excitatory but not inhibitory synaptic transmission into adult rat spinal cord neurons (Kohno, Kumamoto et al. 1999). Taken together these findings imply that opioids play a role in regulating transmitter release from nociceptor terminals. Two different mechanisms could contribute to the presynaptic inhibition of transmitter release by opioids. First, as discussed above morphine could activate K^+ channels in the terminal, by this means hyperpolarizing the presynaptic terminal and decreasing the terminal's input resistance. This would reduce the ability of invading action potentials to depolarize the terminal and activate the calcium channels, which in turn would reduce the resulting calcium influx

and consequent transmitter release. Second, the opioids could directly inhibit the voltage gated calcium channels in the synaptic terminal. Using Xenopus oocytes expressing neuronal calcium channels and opioid receptors, activation of the morphine receptor with a synthetic enkephalin (DAMGO) resulted in a rapid inhibition of calcium currents (Bourinet, Soong et al. 1996). In rat DRG neurons μ-opioid receptors are negatively coupled to three types of high-threshold calcium channels thought to play a role in synaptic transmission (Rusin and Moises 1995). It has been suggested that the opioid receptors inhibit the calcium channels via a direct action of the G-protein on the calcium channel and not via a second messenger pathway (see review by (Dolphin 1995)). In conclusion the available evidence is consistent with opiates inhibiting transmitter release by activating K_{ATP} channels and/or inhibiting calcium channels in the synaptic terminal.

HCN Channels

The membrane currents resulting from the activation of hyperpolarization-activated, cation nonselective (HCN) channels were first described in isolated DRG neurons more than 25 years ago (Mayer and Westbrook 1983). It wasn't until the last few years that the possible role of HCN channels in peripheral neuropathic pain (or peripheral neuralgia) became apparent (see review by (Jiang, Sun et al. 2008)). Neuropathic pain is discussed in the following chapter 6; here we will consider the general properties of HCN channels, their role in regulating firing frequency in some DRG neurons and also those properties that will prove useful in understanding their possible role in neuropathic pain.

Hyperpolarizing voltage steps to potentials more negative than, -50 to -60 mV activates HCN channels. This is near the resting potentials of most cells. These hyperpolarization-activated cation currents, I_h, were originally termed I_f for "funny" and I_q for "queer" because unlike the majority of voltage gated channels they were activated by hyperpolarization rather than depolarization. As shown in Figure 5-2A a hyperpolarizing voltage step activates a slowly developing inward current, I_h, the amplitude of which increases with increasing hyperpolarization (not shown). If the magnitude of I_h is plotted against membrane potential (I/V relationship) it is apparent that there is a region of "anomalous" inward rectification. By inward rectification it is meant that the channels pass current (positive charge) more easily into the cell (inward direction) and poorly in the outward direction. When first discovered inward rectification was called anomalous rectification to distinguish it from the more commonly encountered outwardly rectifying currents. HCN channels are not the only inwardly rectifying channels; remember that K_{ATP} channels have pore forming inwardly rectifying potassium channel (K_{ir}) subunits. I_h channels are permeable to both Na^+ and K^+ ions while K_{ir} channels are permeable to K^+ ions. On the one hand, activation of K_{ir} will tend to inhibit the firing of action potentials, because the membrane potential will move towards E_K ($\approx$ -85 mV). In contrast activation of HCN channels will tend to excite the nociceptor: depending on the permeability ratio of P_K/P_{Na} the membrane potential will move towards a value between E_K and E_{Na}, which will depolarize the membrane toward the threshold for firing action potentials.

As illustrated in Figure 5-2B, I_h is an inward current activated by hyperpolarization beyond the resting potential, which is manifest as the depolarizing sag of the membrane
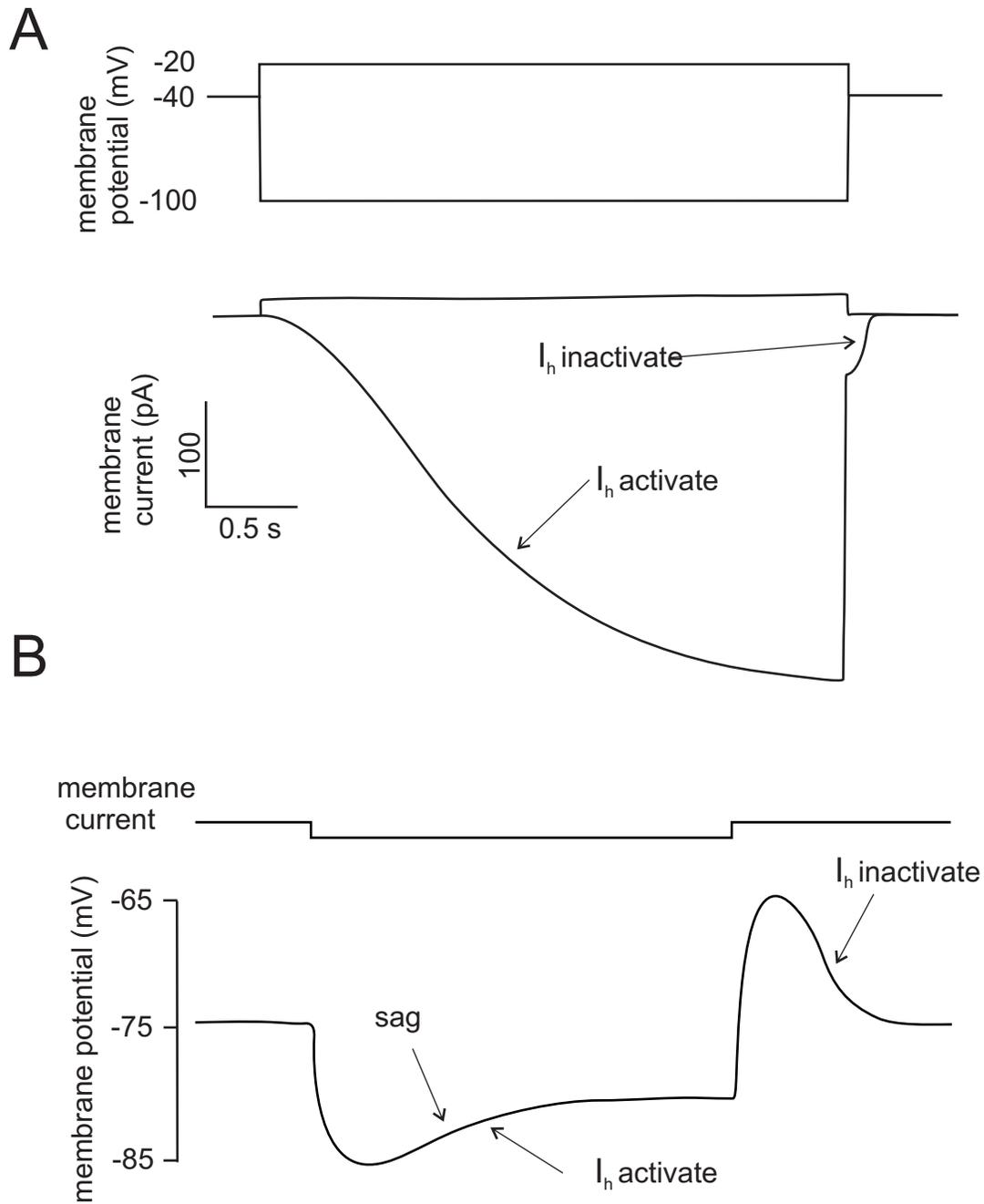
Figure 5.2. I_h activation and inactivation under voltage clamp (A) and current clamp (B) conditions. In (A) I_h is activated by a voltage step from -40 to -100 mV and inactivates when the voltage returns to -40mV. In (B) a hyperpolarizing current pulse activates I_h causing a depolarizing sag during the membrane hyperpolarization.

potential during hyperpolarizing current. Since the I_h current does not inactivate at a given voltage, a sustained inward I_h current will play a role in determining the resting potential and input resistance. Because the net membrane current at the resting potential is zero, an equal outward current must balance the I_h inward current. A resting background K^+ current carried by K_{2P} channels might contribute this outward current. Participation of I_h in determining the resting potential has the consequence that it may play a role in determining the neuron's excitability. When I_h is present in a cell together with a low threshold voltage gated sodium channel and an appropriate amount of background potassium channels there is the potential for the rhythmic firing of action potentials. At a permissive resting potential the after hyperpolarization following an action potential may be sufficient to activate I_h which may be sufficient to depolarize the membrane back to the action potential threshold (Robinson and Siegelbaum 2003). It is this potential for rhythmic firing that is thought to play a role in neuropathic pain (see chapter 6).

A family of four mammalian genes encodes the HCN1–4 channel subunits responsible for I_h currents. Each HCN subunit is composed of six transmembrane segments, with a voltage sensor and a pore forming P region. One important feature of HCN channels is their cyclic nucleotide-binding domain, which underlies their regulation by cAMP and does not require protein phosphorylation. HCN2 and HCN4 are strongly modulated by increased concentrations of cAMP, with the voltage of activation shifted up to more positive potentials by 10–20 mV, while HCN1 and HCN3 channels are relatively insensitive to cAMP. Consequently, with increased cAMP a hyperpolarizing voltage step activates HCN2 and HCN4 more completely.

Remember from chapter 4 that PGE_2 and PAR2 activate PKA via a rise in cAMP. This raises the possibility that these chemical mediators might sensitize nociceptors by shifting the voltage of activation of HCN2 or HCN4 channels via a rise in cAMP. This possibility was tested in DRG neurons using a variety of techniques including ZD7288, a potent I_h blocker (not selective among HCN1-4) (Momin, Cadiou et al. 2008). These authors found that there was a population of DRG neurons, with smaller diameter cell bodies, that had a cAMP-sensitive I_h. Moreover, they found for these small nociceptive neurons that elevating cAMP levels shifted the voltage activation curve of I_h to more depolarized potentials and caused a steady depolarization of the resting membrane potential which was blocked by ZD7288. These findings suggest that modulation of I_h via a rise in cAMP plays an important role in the nociceptor sensitization caused by PGE_2 (Momin, Cadiou et al. 2008).

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Chapter 6   NEUROPATHIC PAIN

As mentioned in the introduction the capacity to experience pain provides a rapid warning to the nervous system to initiate a motor response to minimize physical harm. That is, acute or nociceptive pain is a necessary protective mechanism; in contrast chronic pain serves no obvious survival or helpful function. Among the different types of chronic pain is peripheral neuropathic pain (or peripheral neuralgia) the essential feature of which is pain resulting from a wound or damage to a primary nociceptor. Neuropathic pain is often intense and unrelenting and resistant to relief by available therapies. Chronic pain without any evidence of a lesion or damage to the primary nociceptor as in a migraine is not considered neuropathic pain. The injury may be in any part of the nociceptor and may be the result of any of a number of possible insults to the nociceptor. Although the insult in peripheral neuropathic pain is to the primary nociceptor the changes underlying the neuropathic pain syndrome may include changes to the peripheral nervous system, the spinal cord and central nervous system. In this chapter we will focus on those changes thought to occur in the peripheral nervous system.

Symptoms of peripheral neuropathic pain can include persistent or paroxysmal pain, burning, prickling, itching or tingling that is independent of any obvious stimulus. There can also be abnormally heightened sensations such as allodynia (pain resulting from a normally innocuous stimulus) and hyperalgesia (an enhanced response to a normally painful stimulus). Intuitively, one might think that when an afferent nerve is injured it would fail to transmit information to the spinal cord. That is, one might reasonably expect a loss in sensations rather than a heightened or persistent sensation. That is what happens when a telephone line is cut; one cannot make or receive telephone calls. To the extent that there may be some loss of sensation associated with peripheral neuropathic pain the analogy to the telephone line holds true. However, the enhanced response and the presence of pain in the absence of a stimulus imply that there is something fundamentally different between a damaged neuronal axon and a cut telephone cable. The question then becomes what kinds of changes occur when a nerve is damaged that might give rise to neuropathic pain?

Functionally the spinal roots are classically divided into dorsal roots for sensory transmission and ventral roots for motor transmission. The ventral roots are thought to be composed of the axons of myelinated motor neurons. However, in humans and other mammals on the order of one third of all axons in the ventral roots are unmyelinated, have their cell bodies in the dorsal root ganglion, and are predominantly nociceptive (see Figure 6-1). This probably explains why dorsal rhizotomy, a procedure in which the spinal nerve root between the dorsal root ganglion (DRG) and the spinal cord is severed, sometimes fails to provide relief from chronic pain. Furthermore, these types of lesions where the dorsal roots are severed have not been found to cause neuropathic pain in humans. <u>Therefore one can say that not all lesions to nociceptors result in neuropathic pain.</u>
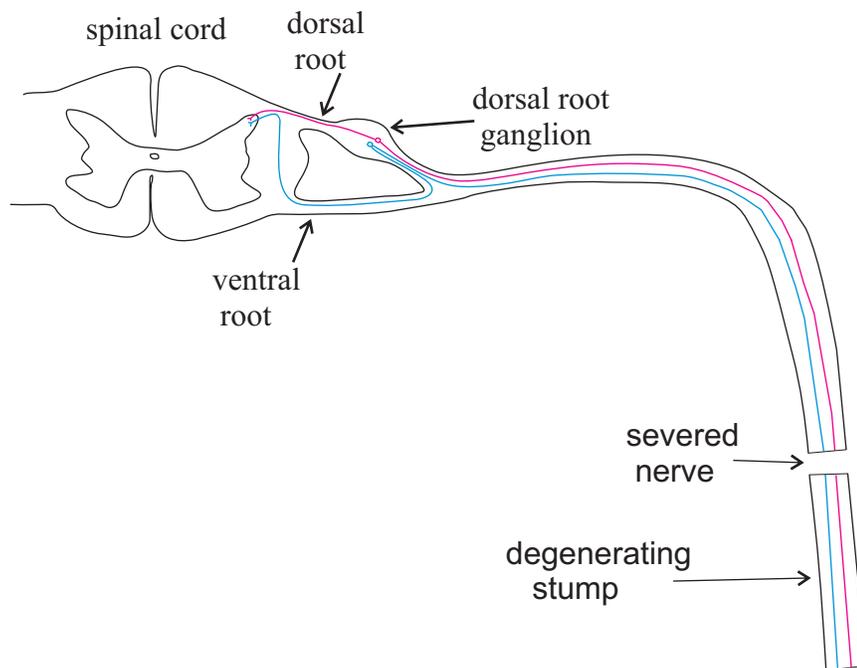
Figure 6-1. Schematic drawing showing two nociceptors in the dorsal root ganglion. For the one shown in red its axon enters the spinal cord through the dorsal root, and for the one shown in blue its axon enters the spinal cord through the ventral root. Also the nerve formed by the two roots is shown as being severed distal to where the nerve leaves the spinal canal.

Animal models of nerve injury used to study neuropathic pain

When a nerve bundle is severed distal to the DRG (as illustrated in Figure 6-1) the proximal stump that is the portion still connected to the cell bodies seals off, and under the most favorable conditions nerve fibers sprout and will regenerate and reform the appropriate peripheral connections. However if the regeneration is blocked somehow, as in an amputation, the sprouts can form a tangled mass or "neuroma". The part of the axons, of the severed nerve bundle, separated from the nerve cells nucleus degenerate in a process called "Wallerian degeneration" also known as anterograde (occurring in the normal or forward direction) degeneration. When only a fraction of the nerve fibers in a nerve bundle are damaged and undergoing Wallerian degeneration the remaining intact axons will be exposed to the products from the degenerating axons and also to agents released from the surrounding tissue that is responding to the degenerating axons. During Wallerian degeneration it might be thought that the part of the axon separated from the nerve cell nucleus degenerates simply because it no longer receives the nutrients normally supplied by the nucleus. However, in the mutant mouse strain of slow Wallerian degeneration (Wlds) axonal degeneration is delayed following nerve lesion, the distal axonal stump remains structurally intact exhibiting prolonged electrical conductance after nerve injury. The development of neuropathic pain is attenuated and slowed in Wlds mice, that is, they slowly develop an attenuated hyperalgesia.

In animals in which an experimental neuroma is made by axotomy (severing) of the sciatic nerve, abnormal spontaneous activity can be recorded from dorsal roots and the nerve fibers just above the neuroma. The spontaneous discharge is also ectopic: originating in an abnormal place, rather than the normal location the peripheral nerve ending. The spontaneous activity may be related to the development of the persistent pain or paroxysmal pain that occurs with neuropathic pain (see discussion below). After sciatic nerve axotomy a large number of genes are either up or down regulated in DRG neurons (Costigan, Befort et al. 2002; Xiao, Huang et al. 2002). These changes in gene expression most likely lead to an increase in the excitability of the DRG neurons, which is the simplest explanation for the abnormal spontaneous activity. One would predict that an up regulation of excitatory voltage gated Na^+ and Ca^{++} channels and a down regulation of K^+ channels, which oppose the excitatory channels, might be responsible for the increased excitability of DRG neurons following peripheral nerve injury.

The effects of axotomy of the sciatic nerve on Ca^{++}, K^+ and HCN channel currents has been studied in isolated rat DRG neurons from roots (L4 and L5) that give rise to the sciatic nerve (Abdulla and Smith 2001). Currents carried by all three channel types were decreased in DRG neurons following sciatic axotomy, suggesting that increases in neuronal excitability are associated with decreases in K^+ channel currents.

Models of mechanical nerve injury

One of the problems in studying neuropathic pain experimentally and treating it clinically is that it can be caused by various neuropathies; consequently the symptoms and treatments may depend upon the particular cause and on which nerve, or nerves, are involved. Mechanical nerve injury can result from acute or chronic nerve compression or

more severely by the partial or complete severing of a nerve as described above. Several mechanical nerve injury models (illustrated in Figure 6-2) have been developed and have proved useful in the experimental study of neuropathic pain in rodents. As illustrated in Figure 6-2 nerve roots can be either ligated and or severed. The sciatic nerve can be partially ligated or a chronic constriction can be placed around the sciatic. Also, in the spared nerve injury model nerves emanating from the sciatic are severed and one nerve is spared. These procedures have in common that the innervation of the foot remains partially intact allowing tests for allodynia and hyperalgesia to be done. Similar nerve injury models can be used with the trigeminal nerve.

One of the earliest events following nerve injury is the influx of calcium, which triggers the fusion of intracellular vesicles with both ends of the damaged axons thereby sealing the endings of the injured axons. The rise in intracellular calcium also activates calcium-dependent proteases such as m-calpain. However, m-calpain levels return to their normal values within few hours suggesting that m-calpain may act as a trigger for the initiation of Wallerian degeneration. Inhibition of m-calpain protects axons from the effects of injury and reduces pain (Kunz, Niederberger et al. 2004). Therefore, m-calpain appears to trigger the initial phase of Wallerian degeneration leading to neuropathic pain.

Matrix metalloproteases and Wallerian degeneration

Following ligation of the L5 spinal nerve, matrix metalloprotease -9 (MMP-9) activity was greatly increased in rat DRG neurons within one day of nerve injury (Kawasaki, Xu et al. 2008). Matrix metalloproteases (MMPs) are a family of zinc-dependent extracellular proteases that participate in the modification of the extracellular matrix as well as a number of cell surface and soluble proteins including cytokines and chemokines. The rapid increase in MMP-9 activity correlates with the initiation of Wallerian degeneration, and treatment with metalloproteinase inhibitors delays the onset of Wallerian degeneration. In MMP-9 knockout mice, the early-phase of mechanical allodynia during the initial days following L5 spinal nerve ligation was reduced, however, neuropathic pain symptoms fully developed by the tenth day. It may be that compensatory adaptations may have occurred in the knockout mice.

Decreased expression of K$^+$ channels

A possible mechanism by which nerve injury could lead to a decrease in the expression of K$^+$ channels has been identified (Zhao, Tang et al. 2013). Spinal nerve ligation, of the fifth lumbar nerve (see Figure 6-2) increased the expression of a long noncoding RNA named Kcna2, which was antisense for the RNA of a voltage dependent potassium channel Kcna2. Over expression of Kcna2 antisense RNA in DRG neurons, reduced Kcna2 and reduced the total voltage-gated potassium current and produced neuropathic pain symptoms. Blocking the increase of Kcna2 antisense RNA induced by nerve injury–reversed the decrease of Kcna2 and attenuated neuropathic pain. These findings point to Kcna2 antisense RNA as a potential target for the treatment of neuropathic pain.

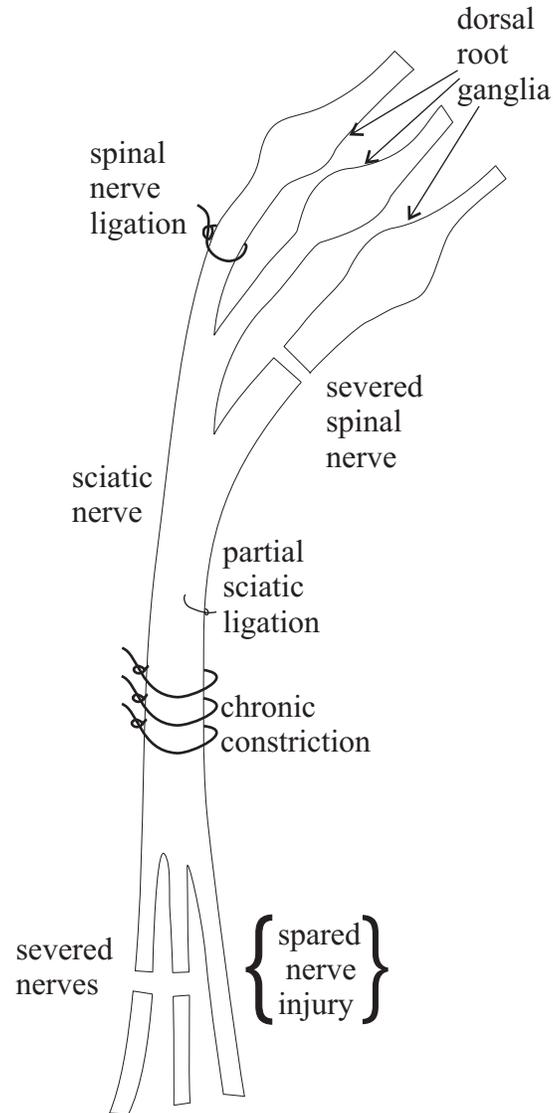### Up regulation of voltage gated Na$^+$ channels

Figure 6-2. Several different ways for mechanically injuring nerves innervating the rodent foot. These procedures have in common that some of the nerve fibers innervating the foot remain intact and allow for testing hyperalgesia and allodynia.

A potential mechanism by which nerve injury could lead to an up regulation of excitatory voltage gated Na^+ channels has been recognized (Laedermann, Cachemaille et al. 2013). Using the spared nerve injury model (see Figure 6-2) to induce neuropathic pain in rats it was shown that the E3 ubiquitin ligase NEDD4-2, which targets proteins for degradation by the proteasome, was decreased (Cachemaille, Laedermann et al. 2012). Subsequent experiments in mice (Laedermann, Cachemaille et al. 2013) showed that spared nerve injury resulted in an increased amplitude of $Na_V1.7$ and $Na_V1.8$ currents in DRG neurons and a redistribution of $Na_V1.7$ channels toward peripheral axons.

Other mechanisms of neuropathic pain

Remember from chapter 5 that it was suggested that up regulation of $Na_v1.3$ contributes to the abnormal hyper-excitability and spontaneous activity of injured DRG neurons (Black, Cummins et al. 1999). However, normal levels of neuropathic pain behavior develops in $Na_v1.3$ knockout mice (Nassar, Baker et al. 2006) suggesting that increased $Na_v1.3$ expression is not necessary for the development of neuropathic pain.

It is important to keep in mind that allodynia and hyperalgesia may occur in the nociceptor and/or more centrally, and it is therefore necessary to determine whether the hypersensitivity in a nerve injury-induced neuropathic pain state is in whole or in part the result of changes in the properties of nociceptors. In rats with unilateral ligation of L5/L6 lumbar spinal nerves, which produced allodynia and hypersensitivity in the ipsilateral leg, single fiber recordings were made distal to the ligation from the nerves innervating the leg (Shim, Kim et al. 2005). Response thresholds to mechanical stimuli were lower and the magnitude of responses to suprathreshold mechanical stimuli was greater for both the C- and Aδ-fibers in these animals than in sham operated animals. Only C-fibers were responsive to heat stimuli and their thresholds were lower in the animals with nerve ligation than in the sham-operated animals. These findings indicate that following nerve injury nociceptors innervating the skin become sensitized to both mechanical and thermal stimuli, thereby providing evidence that nociceptor sensitization can contribute to neuropathic pain.

 Mechanical trauma is not the only way a nerve can be injured. Injury can also occur as a result of a metabolic disorder (diabetes), infection (postherpetic neuralgia), autoimmune disorders, or a chemically induced nerve trauma resulting from the use of chemotherapy drugs. Complicating the situation further is that about a third of peripheral neuropathies are considered to be "idiopathic" that is resulting from an obscure or unknown cause. Even though chronic pain with allodynia may occur in most types of neuropathy it is very unlikely that a single molecular change will uniformly characterize neuropathic pain states. A couple of briefly described examples to illustrate the diversity of neuropathic pain syndromes are given below.

Two examples of neuropathic pain

COMPLEX REGIONAL PAIN SYNDROME TYPE II (CAUSALGIA)

The civil war physician Silas Weir Mitchell first used the term causalgia in 1864 to describe the intense burning pain and marked sensitivity to vibration or touch in the distribution of an injured peripheral nerve following military injuries. In the 1930-1940's causalgia was associated with the sympathetic nervous system and it is now generally accepted that pain associated with sympathetic efferent function is classified as sympathetically maintained pain. Under normal conditions activity in postganglionic sympathetic fibers does not produce pain, nor is it capable of activating nociceptors; however, after nerve injury nociceptors in the injured nerve can become excited by epinephrine and by stimulating the sympathetic trunk. However, it is not clear whether there is a direct sympathetic effect on the nociceptor or if the effect is indirect. Sympathectomy is an effective treatment especially for patients who show a positive response to sympathetic block.

TRIGEMINAL NEURALGIA

Trigeminal neuralgia, also called tic douloureux, is a chronic pain condition characterized by sudden bursts (paroxysms) of facial pain in the areas supplied by the trigeminal nerve: the cheeks, jaw, teeth, gums, lips and less frequently around the eye or forehead. These episodes of pain can be triggered by a light touch around the mouth or face or even by talking or eating. It may be caused by irritation or stimulation of the trigeminal nerve by a blood vessel pressing on it as it exits the brainstem. In some cases it is the associated with multiple sclerosis or a tumor. The first choice treatment option for trigeminal neuralgia is medication: anticonvulsants such as carbamazepine (trade name Tegretol) are generally effective. For those patients unable to tolerate the side effects of the medications or those that become resistant to medication surgery is the next option.

Some examples of the diversity of the treatments for neuropathic pain

GABAPENTIN

The anti epileptic drug gabapentin (trade name Neurontin) was initially synthesized to mimic the chemical structure of the neurotransmitter gamma-aminobutyric acid (GABA). However gabapentin is not currently thought to act on the same brain receptor as GABA. Its use as an analgesic for neuropathic pain resulted from clinical case reports of its analgesic effects in patients with well-documented histories of neuropathic pain. Pregabalin (trade name Lyrica) was designed as a more potent successor to gabapentin and like gabapentin was found to be useful for the treatment of neuropathic pain.

A high affinity binding protein for [^{3}H]gabapentin was subsequently isolated and then identified as the $\alpha_2\delta$-1 subunit of a voltage gated calcium channel (Gee, Brown et al. 1996). The expression of the $\alpha_2\delta$-1 subunit was increased 17-fold in the DRG ipsilateral to nerve injury but not in the contralateral DRG (Luo, Chaplan et al. 2001). The increased expression of the $\alpha_2\delta$-1 subunit was found to precede the onset of allodynia in the experimental animals and to diminish while animals were recovering. These results imply that up regulation of $\alpha_2\delta$-1 in nociceptors could potentially play a role in the development of neuropathic pain following nerve injury. However, mechanical hypersensitivity

develops after partial sciatic nerve ligation, albeit with a delay, in $\alpha_2\delta$-1 knocked out mice (Patel, Bauer et al. 2013).

If the analgesic effects of gabapentin and pregabalin result from their binding to the $\alpha_2\delta$-1 subunit of the voltage gated calcium channel and if the binding could somehow be blocked or eliminated the analgesic effect should be greatly diminished. It turns out that substitution of alanine for arginine at position 217 in the $\alpha_2\delta$-1 molecule prevents gabapentin and pregabalin binding (see (Field, Cox et al. 2006)). Utilizing gene targeting techniques a mutant mouse was produced having alanine at position 217 in the $\alpha_2\delta$-1 molecule (Field, Cox et al. 2006). The mutant mice exhibited normal pain responses, however the analgesic effect of pregabalin during the late phase of formalin induced pain or allodynia following chronic ligature constriction of the sciatic nerve was lost, thereby conclusively demonstrating that the analgesic actions of pregabalin are mediated via the $\alpha_2\delta$-1 subunit of voltage gated calcium channels. It should be kept in mind that the mutation in the $\alpha_2\delta$-1 subunit greatly decreased pregabalin binding throughout the nervous system and not just in the DRG ipsilateral to the nerve injury. Furthermore, the analgesic effect of pregabalin, following partial sciatic nerve ligation, was lost in $\alpha_2\delta$-1 knockout mice (Patel, Bauer et al. 2013).

In the study (Luo, Chaplan et al. 2001) showing that the increased expression of the $\alpha_2\delta$-1 subunit preceded the onset of allodynia following nerve injury; the injury was produced by ligation of the L5/L6 lumbar spinal nerves at a point distal to their DRGs and proximal to their union to form the sciatic nerve. In a subsequent study (Luo, Calcutt et al. 2002) three types of mechanical nerve injury were utilized to determine whether $\alpha_2\delta$-1 subunit up-regulation in the DRG correlated with the induced allodynia. In addition to the ligation of L5/L6 as described above, L5/L6 were transected at the same location and thirdly the sciatic nerve on one side was constricted by a series of ligatures around the nerve. In all three situations there was a significant increase in the expression of the $\alpha_2\delta$-1 subunit in the DRG and a corresponding tactile allodynia, which was inhibited by gabapentin. These authors also studied animals exhibiting tactile allodynia resulting from diabetic neuropathy and toxic neuropathy induced by vincristine (an agent used in cancer chemotherapy which works by inhibiting microtubule assembly and its main side-effect is peripheral neuropathy). In these animals there was no significant change in the expression of the $\alpha_2\delta$-1 subunit in the DRG, suggesting that the level of the $\alpha_2\delta$-1 subunit in the DRG is not the determining factor for the tactile allodynia during all neuropathic pain states. Interestingly, in animals with diabetic neuropathy there was a tactile allodynia that was inhibited by gabapentin suggesting the involvement of $\alpha_2\delta$-1 subunits in locations other than the DRG. Although gabapentin and pregabalin act via the $\alpha_2\delta$-1 subunit of a voltage gated calcium channel, and the expression of this subunit is up regulated in the DRG, there is as yet no compelling evidence that their site of action as an analgesic for neuropathic pain is on the nociceptor and not in the spinal cord or more centrally.

ARTEMIN

Current therapies for the treatment of neuropathic pain have been described as inadequate since in many cases they are only of limited benefit and they have a high incidence of undesirable side effects. In the continuing search for new therapies, artemin, one of the members of the glial cell derived neurotrophic factor (GDNF) family was considered. It signals through the GDNF family receptor GFRα3, which complexes with the tyrosine kinase receptor RET. GFRα3 expression in adults is largely restricted to small diameter DRG cells which have unmyelinated axons many of which are nociceptors. The effects of subcutaneous administered artemin on thermal and tactile hypersensitivity as a result of spinal nerve ligation in rats were examined. It was found that systemic, intermittent artemin administration produced a dose dependent reversal of nerve ligation induced thermal and tactile hypersensitivity which was reestablished after cessation of artemin administration (Gardell, Wang et al. 2003). In a subsequent study the effects of artemin following nerve injury were specifically studied on C fibers because the expression of the GFRα3 receptor is predominantly found in small diameter DRG neurons having unmyelinated axons (Bennett, Boucher et al. 2006). They showed that artemin protected against injury-induced changes in the histochemistry and electrophysiological properties of C fibers.

CANNABINOIDS

Marijuana was widely used for medicinal purposes (including analgesia) in the United States before it was classified as a Schedule I drug, which classified it as a drug with high abuse risk and no accepted medical use. However, the passage of referenda in several states has allowed the use of marijuana for medicinal purposes. Animal studies have indicated that cannabinoids produce analgesic effects at peripheral sites as well as spinal and supra spinal sites. Nevertheless, the use of cannabinoids as analgesics in humans is hindered by their potential for adverse effects, such as hallucinations, euphoria, or dysphoria, in the patients who use them. What is needed is a cannabinoid agonist that produces analgesia but having minimal adverse effects.

The effects of cannabinoids are mediated via binding to two G protein coupled receptors (CB1 and CB2) that inhibit adenylate cyclase leading to decreased cAMP levels in most tissues and cells. In order to determine the extent to which CB1 receptors located in nociceptors contributed to analgesia in inflammatory and neuropathic pain CB1 was deleted in nociceptors in the peripheral nervous system of mice, while preserving its expression in the central nervous system (Agarwal, Pacher et al. 2007). These authors used a model of neuropathic pain in which they lesioned two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves) leaving the remaining sural nerve intact. They examined the analgesic effects of systemic administration of the cannabinoid receptor agonist WIN 55,212-2 on the response latency to thermal stimuli and the mechanical threshold in animals following nerve lesion. WIN 55,212-2 significantly increased response latency to thermal stimuli and raised the mechanical threshold, however the effects were significantly weaker in the knockout mice. They concluded that CB1 receptors expressed on nociceptors mediate a significant proportion of the cannabinoid-induced analgesia produced in neuropathic pain. They reached the

same conclusion for the analgesia produced by WIN 55,212-2 during inflammatory pain. These findings strongly argue for the development of peripherally acting analgesics based on synthetic cannabinoids that do not cross the blood-brain barrier.

Finally, it is worth considering how CB1 receptor agonists that inhibit adenylate cyclase leading to decreased cAMP levels might cause analgesia for inflammatory pain and neuropathic pain. First consider the inflammatory mediators discussed in chapter 4 that activate the enzyme adenylate cyclase (AC) via the stimulatory G-protein (G_S). As shown in Figure 6-3 the AC activators are PGE_2 and PAR2. Activated AC catalyzes the conversion adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP), which in turn modulates the activity of four ion channels (HCN, TRPV1, TRPV4, TRPA1). The activity of TRPV1, TRPV4 and TRPA1 is modulated by phosphorylation via the cAMP activated protein kinase (PKA). On the other hand HCN channels have a cAMP-binding domain, which regulates the channel and does not require protein phosphorylation. Both PAR1 and cannabinoids (CB) inhibit AC via the inhibitory G-protein G_i, and thereby they will tend to inhibit nociceptor activation by the inflammatory mediators PGE_2 and PAR2 and in that way they may inhibit inflammatory pain. How then might CB1 receptor agonists cause analgesia for neuropathic pain? One possible mechanism shown in Figure 6-3 is via the HCN channels, which have been implicated in neuropathic pain (see below).

An alternate strategy for activating cannabinoid receptors is by elevating the concentration of endogenous cannabinoids (endocannabinoids) by prolonging their lifetime. This might be accomplished through the inhibition of the endocannabinoid catabolic enzymes, such as fatty acid amide hydrolase (FAAH). The effect of PF-04457845, a potent and selective FAAH inhibitor, on pain due to osteoarthritis of the knee has been investigated. PF-04457845 was well tolerated by the patients, and there was **no evidence of an analgesic effect** although four 4 endogenous substrates of FAAH were substantially increased (Huggins, Smart et al. 2012). The reasons for this failure are presently unclear

HCN CHANNELS

The procedure of placing a ligature around a spinal nerve to produce a neuropathy was originally developed to have an animal model for neuropathic pain in which there was chronic pain with allodynia and hyperalgesia (Bennett and Xie 1988). Using this model it was shown that A-fibers appear to mediate mechanical allodynia and hypersensitivity while C-fibers the thermal hyperalgesia (Shir and Seltzer 1990). One mechanism that could give rise to the chronic pain of the neuropathic pain state is the presence of spontaneous activity which could activate and/or sensitize spinal neurons and thereby contribute to pain, as well as allodynia and hyperalgesia. As mentioned above, in animals in which an experimental neuroma is made by severing the sciatic nerve high levels of spontaneous ectopic neuronal activity can be recorded. In order to determine the type of fibers contributing to spontaneous activity during neuropathic pain in which there was chronic pain with allodynia and hyperalgesia a simple experiment was performed (Kajander and Bennett 1992). Ligatures were placed around the sciatic nerve and at
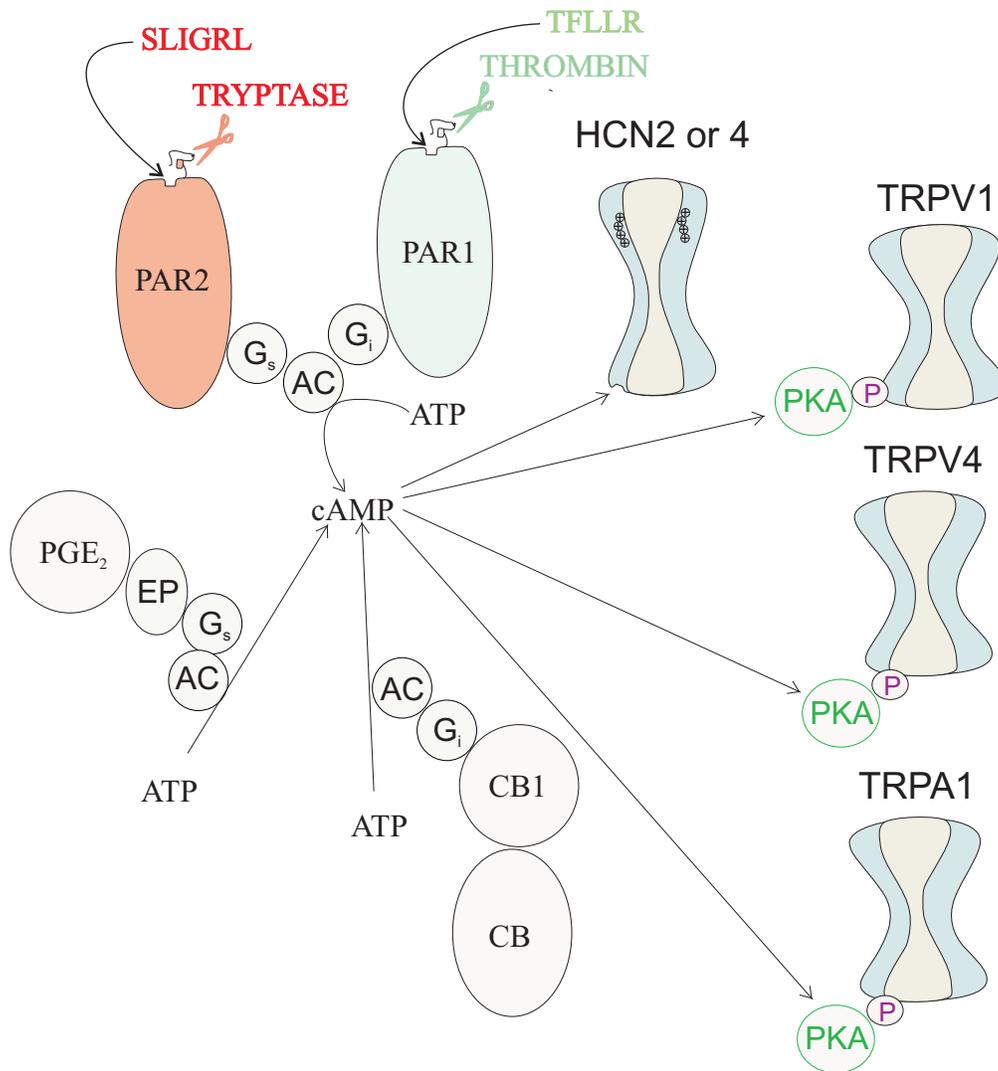
Figure 6-3. Regulation of nociceptor function by cAMP and PKA. PAR2 and PGE_2 activate AC via G_s thereby leading to an increase in cAMP concentration and thereby to activation of PKA. In contrast PAR1 and CB1 inhibit AC via G_i thereby leading to a decrease in cAMP concentration and a decrease in the activity of PKA.

different times post operatively nerve fiber recordings were made from the lumbar roots, which contribute to the sciatic nerve (see Figure 6-4). Stimulating electrodes were placed distal and proximal to the site of ligation. The conduction velocity of spontaneously active and silent axons could be determined using the proximal stimulating electrodes. The distal stimulating electrodes were used to determine which fibers conducted through the ligation site. Spontaneous discharges were observed in 35% of Aβ fibers (55 fibers 89% of which did not conduct through the ligation site), 15% of Aδ fibers (20 fibers 65 % of which did not conduct through the ligation site) and only 3% of C fibers (2 fibers which did conduct through the ligation site). A subsequent experiment determined that the spontaneous activity could some times originate at or near the site of ligation (Tal and Eliav 1996) in addition to the DRG. These findings indicate that after mechanical trauma many of the injured nerve's Aβ and Aδ fibers and some C fibers become spontaneously active, and the spontaneous activity can originate at the DRG or at or near the site of injury. Furthermore, some of the fibers having spontaneous activity still innervated the region affected by the damaged nerve.

Many of the spontaneous active Aβ and Aδ fibers described above exhibited a regular rhythmic firing pattern strongly suggesting the possibility that the firing pattern results from an underlying pacemaker current. As discussed in chapter 5, pacemaker currents (I_h) carried by HCN channels are found in DRG neurons. In rats in which spinal nerves L5 and L6 were ligated the resulting tactile allodynia was dose dependently suppressed by ZD7288 a drug originally thought to be a specific I_h blocker (not selective among HCN1-4) (Chaplan, Guo et al. 2003). Furthermore, ZD7288 decreased spontaneous discharges from Aβ and Aδ fibers. Finally nerve injury increased the pacemaker currents in large DRG neurons and the resting membrane potential of these neurons was significantly more positive than controls. These results suggest that increased I_h plays a role in the tactile allodynia of neuropathic pain. Moreover these findings support the idea that spontaneous discharges in DRG neurons play a causal role in neuropathic pain. In disagreement with the behavioral and electrophysiological findings described above; both HCN mRNA and protein was decreased in DRG neurons on the same side as the ligation. In a subsequent study abundant axonal accumulation of HCN channel protein was found at the sites of injury along with a slight decrease in DRG neuronal cell bodies (Jiang, Xing et al. 2008). These findings suggest that accumulation of HCN channels at the axonal site of injury gives rise to the spontaneous ectopic firing of action potentials, which contributes to the mechanical allodynia of neuropathic pain. The conclusions linking I_h to the induction of the tactile allodynia of neuropathic pain depended on the specificity of ZD7288 for I_h, which has been called into question with the discovery of the nonspecific effects of ZD7288. Hence, characterization of the contribution of specific HCN channels to neuropathic pain is therefore limited by the lack of highly specific blockers of these channels. Generation of knockout mice for specific HCN channels provides an alternative solution to this problem and these animals can be characterized behaviorally.

The role for HCN1 in neuropathic pain was investigated by ligation of the sciatic nerve in HCN1 knockout mice (Momin, Cadiou et al. 2008). Following nerve ligation mechanical hyperalgesia and cold allodynia was present in control animals and in the HCN1
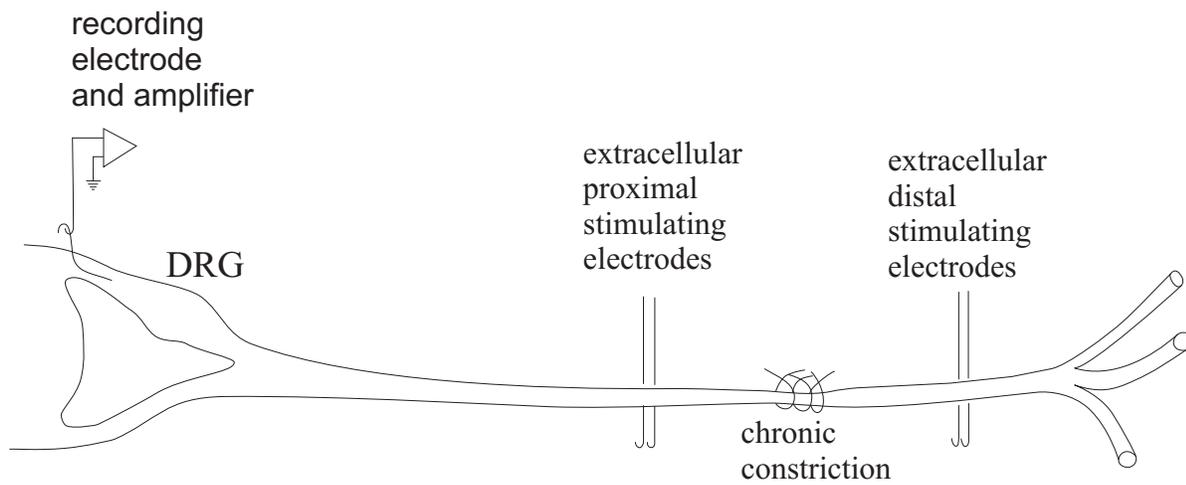
Figure 6-4. Experimental system used for determining the types of nerve fibers firing spontaneously during neuropathic pain.See text for further details.

knockout mice the mechanical hyperalgesia was similar however there was a significant decrease in the cold allodynia of more than 50% in the knockout mice. These findings suggest a causal role for HCN1 in the induction of cold allodynia in neuropathic pain.

Unfortunately, mice in which HCN2 is knocked out were found not to live beyond about 6 weeks. To get around this problem HCN2 was knocked out from the subpopulation of sensory neurons expressing $Na_V1.8$ (Emery, Young et al. 2011). These knockout mice had normal pain thresholds, and inflammatory mechanical hyperalgesia was normal but inflammation did not result in heat hyperalgesia. Remember that in mice in which neurons expressing $Na_V1.8$ were killed also abolished thermal hyperalgesia. Neuropathic pain, in the HCN2 knockout mice, following chronic constriction injury to the sciatic nerve was abolished. This finding would seem to imply that neuropathic pain is dependent upon HCN2 channels functioning in nociceptors expressing $Na_V1.8$ sodium channels. However, remember that neuropathic pain was unchanged in mice in which neurons expressing $Na_V1.8$ were killed (Abrahamsen, Zhao et al. 2008). The reason for this apparent discrepancy is unclear. Because HCN channels are involved cardiac rhythm and vision an analgesic compound targeting HCN for the treatment of neuropathic pain would have to be highly specific in order to prevent effects on cardiac rhythmicity and vision.

What is the role of spontaneous activity?

It was mentioned earlier that following nerve injury nociceptors innervating the skin become sensitized to both mechanical and thermal stimuli. Thereby providing evidence that nociceptor sensitization can contribute to the neuropathic pain state. Since allodynia and hyperalgesia can result from changes that occur centrally, it might be that spontaneous activity leads to centrally mediated hyperalgesia. It has been shown that electrical stimulation of C fibers in humans can lead to hyperalgesia, indicating that electrical activity in C fibers is sufficient to produce centrally mediated hyperalgesia (Klede, Handwerker et al. 2003). Ongoing spontaneous activity in the injured neuron is not necessary to produce neuropathic pain. An L5 ganglionectomy in which all the L5 afferents are removed resulted in mechanical hyperalgesia comparable to that for spinal nerve ligation (Sheth, Dorsi et al. 2002). The authors proposed, that "interaction between degenerating neurons of the injured nerve and intact afferent fibers of neighboring nerves play a critical role for both initiation and maintenance of the mechanical hyperalgesia in neuropathic pain".

The possibility that the spontaneous ongoing pain of neuropathic pain is caused by spontaneous firing of nociceptive neurons has been studied in rats (Djouhri, Koutsikou et al. 2006). Spontaneous foot lifting behavior as a result of nerve damage was used as an indicator of spontaneous pain (Choi, Yoon et al. 1994). A correlation was found between spontaneous foot lifting and the firing rate of C nociceptors following nerve injury and complete Freund's adjuvant treatment, a finding consistent with the possibility of a causal relationship between the two (Djouhri, Koutsikou et al. 2006). Although there may be situations in which chronic neuropathic pain is the result of spontaneous firing of

nociceptive afferents, this should not be taken to imply the converse that all cases of ongoing neuropathic pain result from spontaneous firing of nociceptors.

But what about the spontaneous activity of Aβ afferents described above, could the spontaneous firing of these neurons be the cause of chronic pain as well as secondary allodynia and hyperalgesia? Remember from chapter 1 that a substantial fraction of the A-fiber nociceptors appear to conduct in the Aβ conduction velocity range (Lawson 2002; Djouhri and Lawson 2004). Thus the spontaneous firing of Aβ fibers may very well be the cause of ongoing pain, as well as secondary allodynia and hyperalgesia. At the present time this question remains to be unanswered.

In summary, multiple sites are altered following nerve injury. Abnormalities can occur in both injured and uninjured nociceptors innervating the affected region. These effects include spontaneous activity, as well as allodynia and hyperalgesia. Central effects specifically sensitization following nerve injury can also occur, though their mechanisms are not considered here.

BOTULINUM TOXIN TYPE A

Botulinum toxin type A (trade name BOTOX) binds to the pre-synaptic nerve terminal where it is internalized into the cell and then interferes with vesicle docking thereby inhibiting acetylcholine release and muscle contraction. This has made BOTOX useful for the treatment of medical disorders arising from excessive muscle contraction, which are sometimes painful. However, a dissociation between muscle relaxation and analgesia was sometimes observed clinically; suggesting that BOTOX might have analgesic effects independent of its muscle relaxing effects.

The use of BOTOX as an analgesic agent was tested in rats in which ligation of the sciatic nerve was performed to induce neuropathic pain. A single ipsilateral intra plantar non toxic dose of BOTOX injected either 5 or 12 days after ligation of the sciatic nerve was able to significantly reduce mechanical allodynia for at least 3 weeks (Luvisetto, Marinelli et al. 2007). These findings strongly support the suggestion that BOTOX has analgesic effects independent of its muscle relaxing effects.

Double blind placebo controlled studies for the use of BOTOX to treat neuropathic pain have been carried out in patients with post traumatic/post operative pain or post herpetic neuralgia (Ranoux, Attal et al. 2008) and another group with painful diabetic neuropathy (Yuan, Sheu et al. 2009). In both studies BOTOX significantly reduced neuropathic pain for periods lasting up to 3 months.

No improvement in phantom limb pain (PLP) was observed in a double-blinded study to examine the effect of Botox injection on PLP and residual limb pain (RLP) in a group of 14 amputees with intractable RLP and/or PLP who failed in conventional treatments. However a significant reduction in RLP was found in the amputees. The relief of RLP was significant and seemed to be more effective in the first 3 months after Botox injection, and was still effective for up to 6-months (Wu, Sultana et al. 2012).

DEMYELINATION, LYSOLECITHIN AND LYSOPHOSPHATIDIC ACID

Neuropathic pain (i.e. spontaneous pain, hyperalgesia, and allodynia) is also associated with human peripheral demyelinating neuropathies such as some types of Charcot-Marie-Tooth disease and Guillain-Barre´ syndrome. Traumatic nerve injury, such as that resulting from placing a ligature around a nerve will also lead to demyelination of the injured nerve. Consequently, it is reasonable to consider whether or not demyelination might contribute to the development of the neuropathic pain state. The possible contribution of demyelination to the development of neuropathic pain was studied using the demyelinating agent lysolecithin (lysophosphatidyl choline) applied to peripheral nerves (Wallace, Cottrell et al. 2003). These authors found that topical application of lysolecithin caused focal demyelination, without any morphological or immunological indications of axonal loss. Functionally they found the occurrence of low frequency spontaneous action potentials, with no significant peripheral allodynia or hyperalgesia but with central mechanical allodynia and thermal hyperalgesia. These findings suggest that demyelination, and not axonal damage, of afferent A-fibers induces central neuropathic pain.

At about the same time that the work with lysolecithin described above was being done another independent group was investigating the role of lysophosphatidic acid (LPA) in neuropathic pain (Inoue, Rashid et al. 2004). These authors found that intrathecal injection of LPA induced behavioral allodynia and hyperalgesia with demyelination in the dorsal root similar to that found for animals after nerve ligation. Keep in mind that the intrathecal space surrounds the spinal cord and the dorsal root ganglion. Moreover they found that mice lacking one of the LPA receptors (LPA1) did not develop behavioral allodynia, hyperalgesia and demyelination after nerve injury. They concluded that receptor-mediated LPA signaling is crucial in the initiation of neuropathic pain. In order to clarify the situation with regard to lysolecithin and LPA the same group examined the effects of lysolecithin in LPA1 knockout mice (Inoue, Xie et al. 2008). They found that in contrast to normal mice those lacking the LPA1 receptor did not develop behavioral allodynia and hyperalgesia after intrathecal injection of lysolecithin. They concluded that lysolecithin is converted to LPA, which then activates the LPA1 receptor to initiate neuropathic pain. Remember from chapter 4 that autotaxin, also known as ectonucleotide pyrophosphatase/phosphodiesterase 2 (NPP2 or ENPP2), is a secreted enzyme important for generating LPA. It was discovered that autotaxin also has lysophospholipase D activity that converts lysophosphatidylcholine into LPA. This enzyme probably provides most of the extracellular lysophosphatidic acid from lysophosphatidylcholine.

More recently it was shown that the injection of LPA into the trigeminal ganglia of rats (Ahn, Lee et al. 2009) induced mechanical allodynia both ipsilateral and contralateral to the injection site and mechanical hyperalgesia was only observed ipsilateral to the injection site.

In addition to LPA1 the LPA receptor 5 (LPA5) is expressed at high levels in the DRG suggesting its possible involvement in pain signaling. Moreover, it was found that partial

sciatic nerve ligation failed to produce mechanical allodynia in LPA5 knockout mice (Lin, Rivera et al. 2012). Additionally, nerve injury induced demyelination was undiminished in LPA5 knockout mice indicating that LPA5 loss unlike LPA1 loss does not prevent demyelination, despite protecting against the development of neuropathic pain. These results indicate that LPA signaling is involved in the development of neuropathic pain and that LPA1, LPA5, and perhaps other LPA receptors subtypes are potential targets for the development analgesic compounds for the treatment of neuropathic pain.

The impetus for studying the role of LPA in neuropathic pain (Inoue, Rashid et al. 2004) was the earlier finding by the same group that intrathecal injection of botulinum toxin C3 (BoTXC3) before peripheral nerve injury inhibited the development of hyperalgesia in mice (Ye, Inoue et al. 2000). BoTXC3 inhibits the RhoA/Rho kinase (ROCK) pathway by ADP ribosylation of RhoA, and RhoA is activated by LPA signaling through the G-protein G12/13α (Inoue, Rashid et al. 2004). Furthermore, they showed that the induction of mechanical allodynia and thermal hyperalgesia by intrathecal LPA injection was dose dependently inhibited by BoTXC3 and also by Y-27632, a reversible inhibitor of ROCK. A possible role for the RhoA/Rho kinase pathway in injured neurons was indicated by the finding that neuronal RhoA mRNA and the proportion of L5 DRG neurons that express RhoA rises following distal axotomy (Cheng, Webber et al. 2008).

As mentioned above botulinum toxin type A has also been found to inhibit neuropathic pain, moreover it has been shown to work in humans. Botulinum toxin type A inhibits acetylcholine release at peripheral cholinergic synapses by proteolytically cleaving the SNAP-25 protein, which is essential for release of transmitter. However, it has been shown that botulinum toxin type A also targets RhoB for degradation by proteasome (Ishida, Zhang et al. 2004). RhoB like RhoA is activated by LPA signaling through its G-protein coupled receptor.

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Chapter 7 PROCESSING OF NOCICEPTOR SIGNALS IN THE SPINAL CORD

Interposed between the initial detection of a noxious stimulus by nociceptors and the conscious appreciation of pain is a complex series of mechanisms whereby the noxious stimulus is encoded and progressively transmitted to and processed by higher centers of the nervous system until it is perceived as pain. This process begins in the periphery, in the dorsal horn (DH) of the spinal cord, which is the subject of this chapter.

Considerations to keep in mind when interpreting experimental findings

Historically, basic research scientists have extensively utilized the rat and the mouse as mammalian model systems for the study of pain. Most recently, the mouse has become popular for the study of pain, largely because the mouse is advantageous over the rat when it comes to genetic studies. However, it should be kept in mind that animal models, such as the rat and the mouse, might not always be good models for pain in humans. For example, exogenous substance P, when applied to rat dorsal horn neurons has a prolonged excitatory action that resembles the pattern of excitation observed after noxious stimulation. However antagonists of the tachykinin NK1 receptor (receptor for substance P) have failed to show analgesic effects in humans. ***One must therefore be very cautious in extrapolating experimental findings in lower animals to humans.***

Functionally the spinal roots are classically divided into dorsal roots for sensory transmission and ventral roots for motor transmission. The ventral roots are thought to be composed of the axons of myelinated motor neurons. ***However, in humans and other mammals on the order of one third of all axons in the ventral roots are unmyelinated, have their cell bodies in the dorsal root ganglion, and are predominantly nociceptive.*** This probably explains why dorsal rhizotomy, a procedure in which the spinal nerve root between the dorsal root ganglion (DRG) and the spinal cord is severed refer to Figure 7-2) sometimes fails to provide relief from chronic pain.

Nociceptors differ in many ways: the growth factors they are dependent upon, their response to different noxious stimuli, the receptors and ion channels they express, the conduction velocity of their axons, their capacity for sensitization by inflammation or injury, the neurotransmitters they release from their synaptic terminals, etc. One of the problems in trying to understand the processing of nociceptor signals by the spinal cord is deciding which characteristics of the nociceptor input are relevant. First consider the noxious stimuli: nociceptors respond to noxious cold, noxious heat and high threshold mechanical stimuli as well as a variety of chemical mediators. However, not every nociceptor responds to each and every one of these stimuli. The question of whether a given nociceptor responds to a particular noxious stimulus can be problematic because the apparent lack of a response may result because the stimulus intensity is insufficient. Moreover the application of a high intensity stimulus of one modality may alter the response properties of the nociceptor to other modalities. Consequently it is not possible to generate a comprehensive list of all the different types of nociceptors and the noxious stimuli and chemicals each one responds to. ***Therefore when studying the second order nociceptive cells of the spinal cord one does not know the specific properties of all the***

nociceptors that synapse onto it. Moreover, second order nociceptive cells are usually classified in terms of the noxious stimuli applied to their nociceptive inputs and not to the chemicals that activate their nociceptive inputs.

Spinal cord processing of nociceptive signals

One can think of the spinal cord as a black box with inputs and outputs: where input information from nociceptors is processed in the spinal cord and output nociceptive information is sent to higher centers of the brain involved in the sense of pain. In order to understand how the spinal cord processes signals emanating from nociceptors it is important to identify all the spinal cord inputs and outputs involved in nociceptive signaling. As illustrated in Figure 7-1 the nociceptor input to the spinal cord comes from dorsal root ganglion (DRG) neurons, which enter the spinal cord via Lissauer's tract. Pain and temperature afferents entering through the dorsal roots enter the spinal column and travel one or two segments up or down the cord before penetrating the gray matter of the dorsal horn where they synapse on second order neurons. The trigeminal ganglion (not shown) is analogous to the dorsal root ganglia of the spinal cord and is responsible for painful sensation in the face.

The anterolateral system, which is composed of a bundle of fibers located in the ventrolateral aspect of the spinal cord, is typically described as transmitting nociceptive and thermal information to higher brain centers (see Figure 7-1). The nerve fibers of the anterolateral system originate from cell bodies of projection neurons in the contralateral dorsal horn, which give off axons that decussate via the anterior white commissure. The anterolateral system consists of the spinothalamic tract, spinoreticular tract, spinomesencephalic tract and spinohypothalamic tract. Anterolateral cordotomy, surgical division of the pain-conducting tracts in the anterolateral quadrant of the spinal cord, provides the selective loss of pain and temperature perception several segments below and contralateral to the segment at which the lesion is placed. This procedure is performed on patients experiencing severe pain due to cancer or other diseases for which there are no cure. Although cordotomy is effective in the relief of pain, the effect is usually temporary and pain tends to recur after cordotomies in the form of **central pain,** that is, pain resulting from a lesion or dysfunction of the central nervous system. The use of cordotomy may be appropriate for the treatment of dying patients with intractable pain.

The anterolateral system is not the only nociceptive output of the spinal cord. Historically, the dorsal column pathway was not thought to be involved in pain perception. However, data from clinical studies have shown that the dorsal column pathway is involved in relaying visceral nociceptive information. These studies have shown that small lesions, that disrupt fibers of the dorsal columns (see Figure 7-1) that ascend close to the midline of the spinal cord, significantly relieve pain originating in visceral organs. The nerve fibers of these dorsal column neurons originate from the cell bodies of projection neurons many of which are located in the vicinity of the central canal.
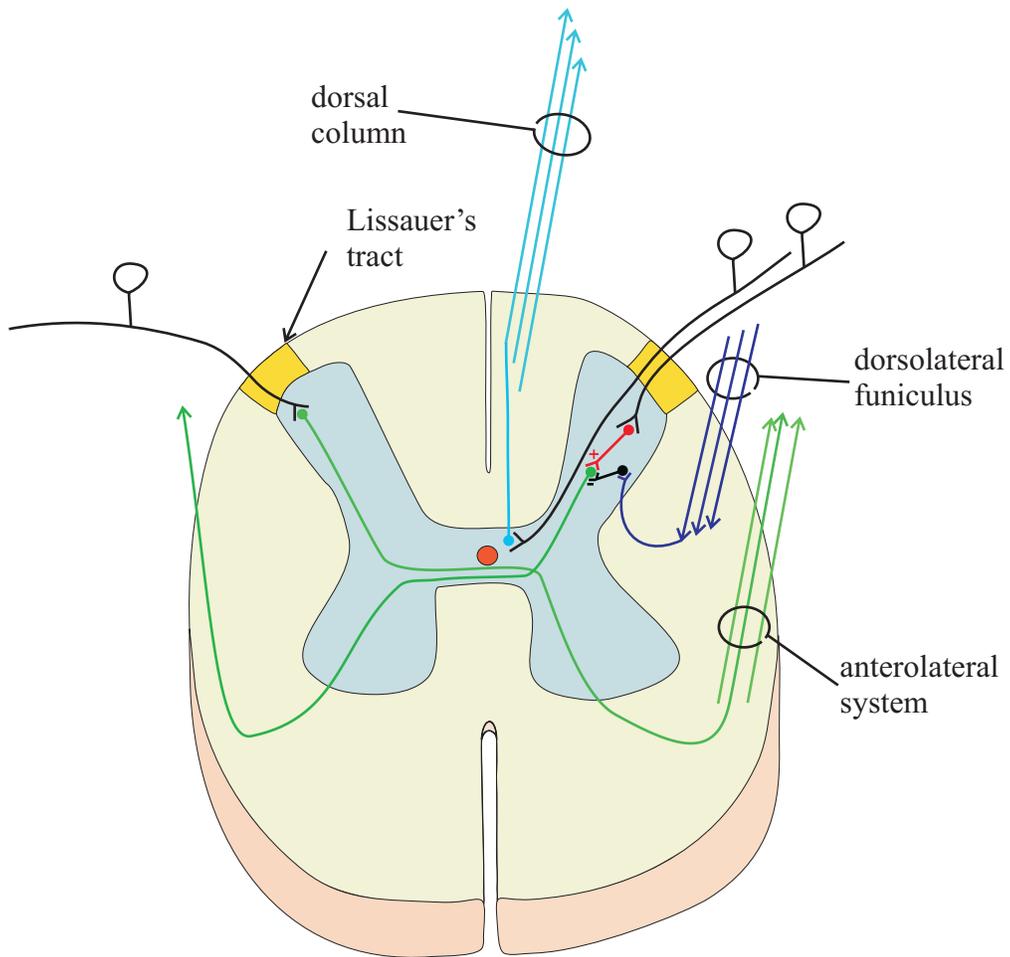
Figure 7-1. Spinal cord input and output pathways involved in nociceptive signaling. See text for further details.

It has become clear that as well as these two ascending pathways that transmit nociceptive information to higher brain centers there are also descending connections from supra spinal centers to the spinal cord. These descending pathways may either block or facilitate transmission of pain information at the level of the dorsal horn. The nerve fibers of the descending pathway travel in the dorsolateral funiculus (see Figure 7-1) and originate in the rostroventral medulla, the nucleus tractus solitarius, the parabrachial nucleus, the dorsal reticular nucleus, the hypothalamus and the cortex.

In addition to these ascending and descending pathways there are also interneurons in the dorsal horn whose axons do not leave the spinal cord. Both excitatory and inhibitory interneurons (see Figure 7-1) are involved in the processing of nociceptive signals before they leave the spinal cord. No description of spinal cord processing of nociceptive signals would be complete without a consideration of these interneurons.

Spinal projection of nociceptors and lamina organization of dorsal horn

Nociceptors enter the spinal cord laterally, and typically travel in Lissauer's tract one or two segments up or down the spinal cord before synapsing ipsilaterally on second-order neurons. Lissauer's tract contains centrally projecting axons carrying pain and also temperature information (see Figure 7-1). The superficial dorsal horn of the spinal cord, including the marginal layer and the substantia gelatinosa (or laminae I and II of Rexed, respectively), are the two of the areas, where nociceptors terminate, that have been most heavily studied (see Figure 7-2).

Neurotrophic factors and the development of nociceptive circuitry

Sensory neurons are dependent for their survival on the production of neurotrophic factors by targets of innervation such as skin and muscle. Most nociceptors require the neurotrophin nerve growth factor (NGF) for survival and differentiation during early embryonic development. NGF binds to and activates its high affinity receptor TrkA (stands for tropomyosin-receptor-kinase, pronounced "Track A") on the surface of nociceptors. Mutations in the human TRKA gene result in a severe autosomal recessive genetic disorder called congenital insensitivity to pain with anhidrosis (CIPA). This disorder affects more neurons than just nociceptors and is characterized by anhidrosis (inability to sweat), the absence of reaction to noxious stimuli, and mental retardation. The lack of the ability to experience pain can cause very serious health problems such as self-mutilation, auto-amputation, and corneal scarring. Both A(δ-β)-fibers and C-fibers are NGF sensitive nociceptors and they express the neurotransmitters calcitonin gene-related peptide (CGRP) and substance P (SP) in addition to their major neurotransmitter glutamate (GLU). First consider the NGF sensitive C-fibers: their central afferents terminate primarily in the most superficial region of the dorsal horn of the spinal cord chiefly in the marginal zone or lamina I of Rexed and also the outer region of the substantia gelatinosa or lamina IIo of Rexed. In Figure 7-2, a schematized view of a C-fiber nociceptor sensitive to NGF (containing the TrkA receptor) and terminating in laminas IIo and I is shown in red. The NGF sensitive A(δ-β)-fibers terminate primarily in

spinal cord

dorsal
root

I
II
III
IV
V

dorsal root
ganglion

trkA,GLU,
CGRP, SP,
TRPV1

NGF

A(-)   C

C

Ret,GLU, CGRP
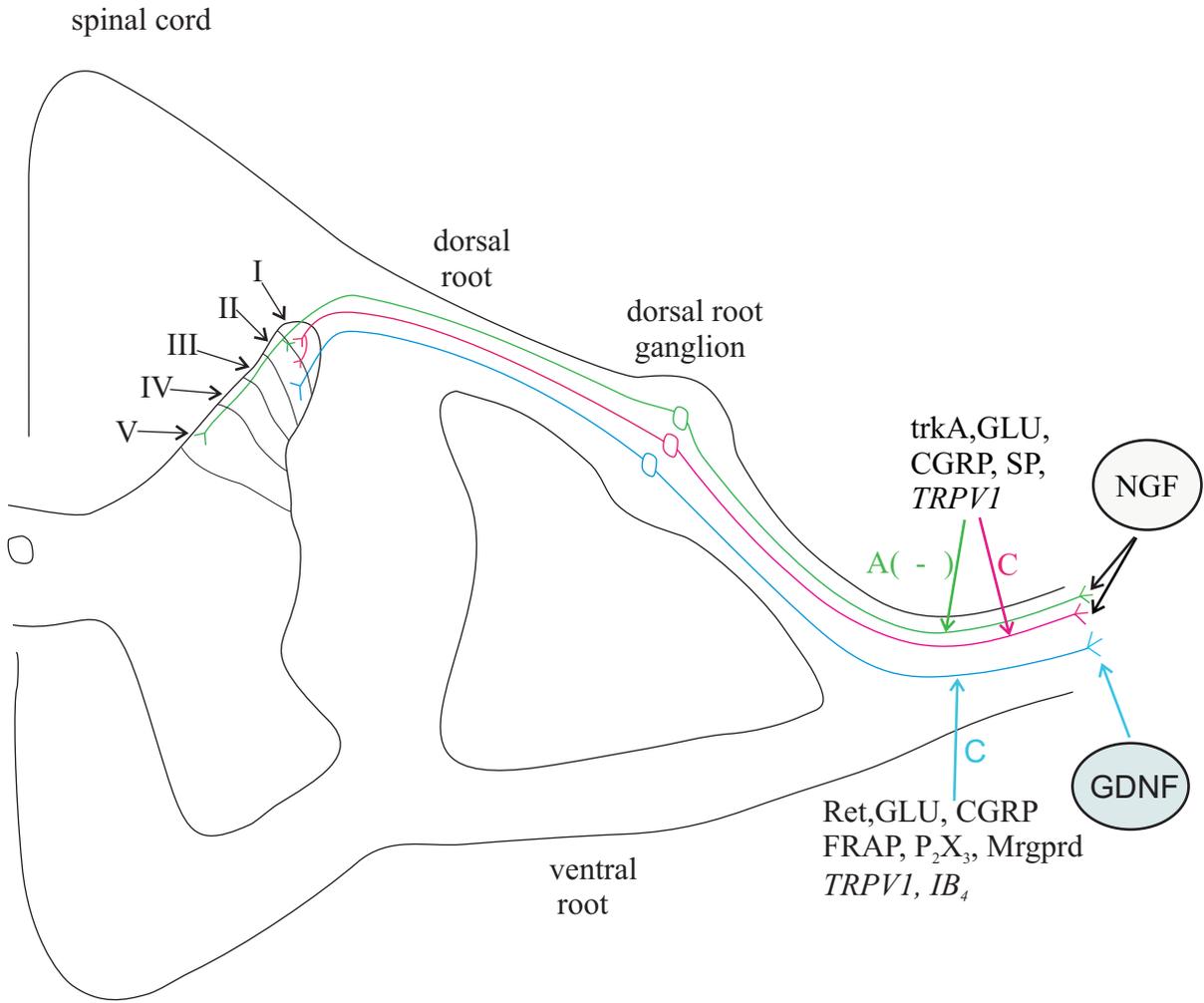FRAP, P_2X_3, Mrgprd
TRPV1, IB_4

GDNF

ventral
root

Figure 7-2.The termination of two types of nociceptive C-fibers and one type of nociceptive A fiber within the dorsal horn of the spinal cord. See text for further details.

lamina I sometimes overlapping into lamina II and also extending collaterals into lamina V, as illustrated in Figure 7-2, such a fiber is shown in green.

A large subpopulation of NGF dependent C-fiber nociceptors lose their NGF dependency as they develop and become responsive to glial cell line-derived neuro-trophic factor (GDNF) a member of the transforming growth factor β (TGF-β) family. These C-fiber nociceptors terminate almost exclusively in the deeper part of the substantia gelatinosa or lamina IIi of Rexed. In Figure 7-2 shown in blue, is a schematized view of a C-fiber nociceptor containing the GDNF receptor Ret, a member of the receptor tyrosine kinase superfamily. As shown these nociceptors express a fluoride-resistant acid phosphatase (FRAP) and subsets express the peptide neurotransmitter CGRP and the $P2X_3$ subtype of the purinergic receptor (see Chapter 4). These nociceptors also express a binding site for the IB_4 lectin, however some TrkA expressing neurons also express a binding site for the IB_4 lectin, therefore it is not a good idea to use IB_4 binding as a marker of non-NGF dependent nociceptors, as is sometimes done (Kashiba, Uchida et al. 2001). Subsets of both types of C-fibers shown in Figure 7-2 express the TRPV1 capsaicin receptor, most likely making them responsive to similar types of noxious stimuli.

Nociceptors expressing the Mrgprd receptor

A group of mouse nociceptors express the Mrgprd receptor (mas-related G-protein receptor d) in a subset of the IB_4 labeled nociceptors. The Mrgprd receptor is a member of a family of G-protein coupled receptors, the mas-related G-protein coupled receptors, a subset of which are found in small diameter mouse DRG neurons that innervate the stratum granulosum of the epidermis, as shown in Figure 7-3 (Dong, Han et al. 2001, Zylka, Rice et al. 2005). In order to ablate neurons expressing the Mrgprd receptor the human diphtheria toxin receptor was inserted into cells expressing the Mrgprd receptor. **Adult** mice in which diphtheria toxin was used to kill neurons expressing Mrgprd exhibit a reduced behavioral sensitivity to noxious mechanical stimuli determined with von Frey hairs but not to heat or cold stimuli. Interestingly, no deficits in mechanical pain sensitivity were found in mice in which diphtheria toxin was used **at birth** to kill neurons expressing Mrgprd. Apparently the young mice can compensate for the loss of Mrgprd expressing nociceptors (Cavanaugh, Lee et al. 2009). Almost all Mrgprd expressing nociceptors are polymodal; responding to noxious mechanical and thermal stimuli and to 50 μM ATP and are relatively insensitive to other nociceptor agonists. These properties led to the suggestion that Mrgprd expressing nociceptors are specialized to detect extracellular ATP and they **may** detect noxious mechanical stimuli by detecting ATP released from keratinocytes into the skin. However, this interpretation does not explain why they also respond to thermal stimuli. Interestingly the region of the epidermis innervated by the Mrgprd expressing nociceptors is distinct from the region innervated by the peptidergic CGRP (calcitonin gene-related peptide) containing nociceptors (see Figure 7-3) (Zylka, Rice et al. 2005). Moreover, the Mrgprd expressing and the CGRP expressing cells terminate in adjacent laminae of the dorsal horn (see Figure 7-3).

As mentioned above ablation of Mrgprd expressing neurons results in a reduced behavioral sensitivity to noxious mechanical stimuli determined with von Frey hairs and
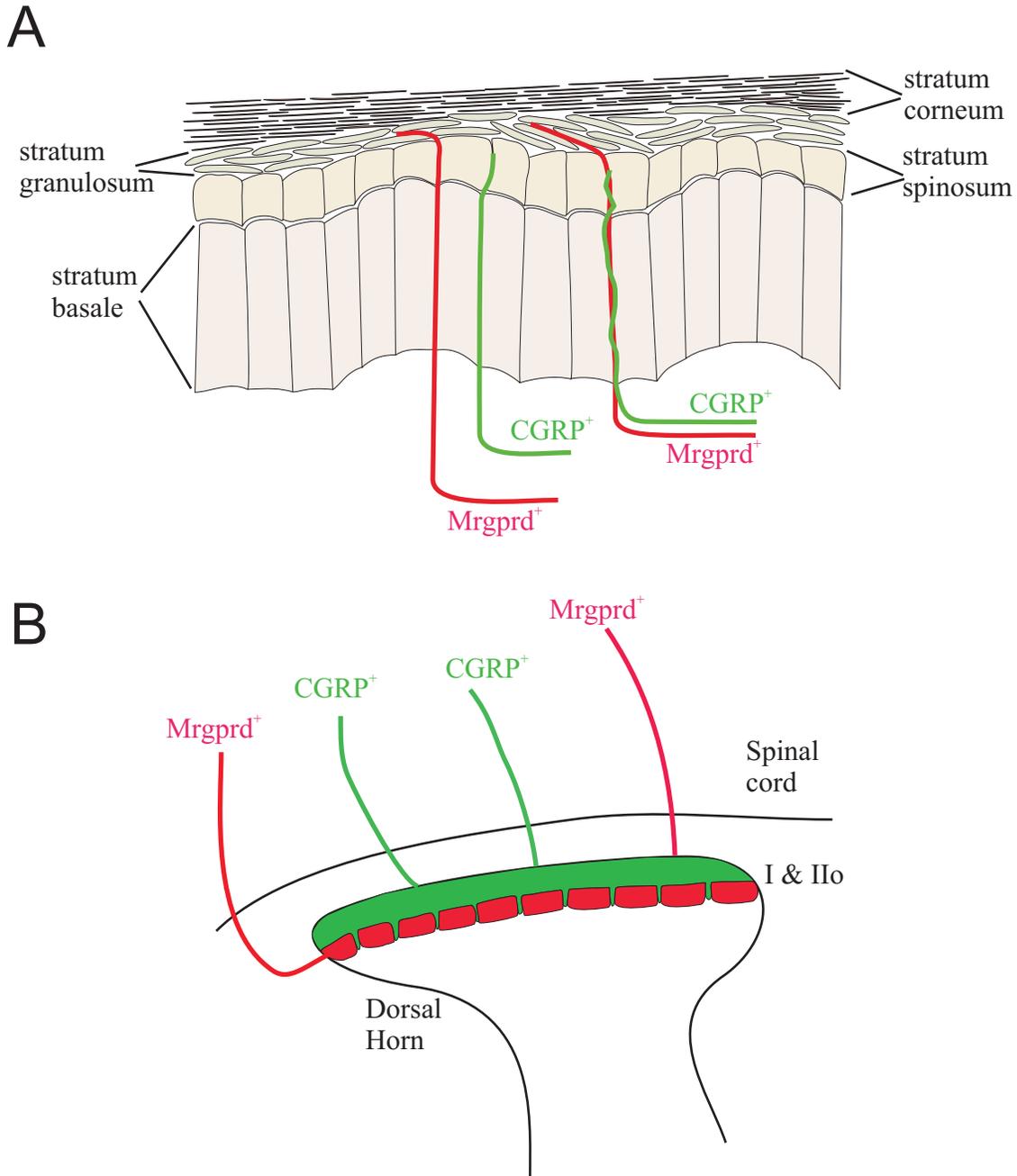
Figure 7-3. Mrgprd$^+$ and CGRP$^+$ nociceptors innervate distinct regions of the epidermis (A) and project to distinct laminae of the dorsal horn of the spinal cord (B). Mrgprd$^+$-fibers terminate in the stratum granulosum and CGRP$^+$-fibers terminate in the stratum spinosum. As shown in (A) sometimes the Mrgprd$^+$ and CGRP$^+$ fibers were found to be closely intertwined with each other. CGRP$^+$-fibers and Mrgprd$^+$-fibers terminate in adjacent laminae (I & IIo) of the dorsal horn, with CGRP$^+$-fibers sometimes intermingling with Mrgprd$^+$-fibers in lamina IIo.

desensitization lasted for at least 31 days. In contrast ablation of IB$_4$ labeled neurons (see Figure 1-1), of which Mrgprd expressing neurons are a subset, using an IB$_4$-saporin (saporin is a protein that inactivates ribosomes, shutting down protein synthesis and resulting in cell death) conjugate transiently reduced both mechanical and heat pain sensitivity for 21 days (Vulchanova, Olson et al. 2001). It may be that ablation of neurons with IB$_4$-saporin is not as effective as using diphtheria toxin.

Nociceptors expressing TRPV1

Remember from chapter 3 that mice in which TRPV1 is knocked out exhibit deficits in their response to hot temperatures above 50°C whereas TRPV1 is activated at temperatures at or above 42°C. These animals in which TRPV1 is knocked out also exhibit little thermal hypersensitivity in the setting of tissue inflammation whereas wild type mice exhibit normal hypersensitivity. On the other hand previous studies indicated that ablation of TRPV1 expressing afferents by intrathecal (into the space surrounding the spinal cord) resiniferatoxin or capsaicin injection causes a partial loss of heat pain sensitivity (Karai, Brown et al. 2004, Cavanaugh, Lee et al. 2009). This treatment blocks inflammatory hyperalgesia and neurogenic inflammation as well.

Fewer than 10% of Mrgprd positive neurons express TRPV1 in vivo and respond to capsaicin in vitro. *Therefore TRPV1 positive and Mrgprd positive neurons form largely non overlapping populations*, furthermore, many TRPV1 positive neurons also express CGRP. Therefore many TRPV1 expressing DRG neurons terminate in the same laminae of the dorsal horn as CGRP positive DRG neurons, and as shown in Figure 7-4 this region is largely non overlapping with the region where Mrgprd positive nociceptors terminate.

As mentioned above, intrathecal administration of resiniferatoxin or capsaicin blocks inflammatory hyperalgesia and neurogenic inflammation. This finding led to the novel suggestion that deletion of TRPV1 expressing nociceptors, by intrathecal resiniferatoxin administration, be used for pain control (Karai, Brown et al. 2004). It should be kept in mind that intrathecal resiniferatoxin will also lead to the deletion of dorsal horn neurons expressing TRPV1 in addition to TRPV1 expressing nociceptors.

The possibility that TRPV1 receptors in the central nervous system play an important role in pain was tested by comparing in vivo the analgesic effect for various types of pain of two TRPV1 antagonists with similar *in vitro* potency but different CNS penetration (Cui, Honore et al. 2006). Both compounds blocked acute pain resulting from intraplantar capsaicin injection with the same potency. However, when administered orally, the TRPV1 antagonist with good CNS penetration had a much greater analgesic effect on mechanical allodynia resulting from intraplantar complete Freund's adjuvant injection. The results of this study demonstrated that TRPV1 receptors in the CNS play an important role in pain and that penetration of the CNS is necessary for a TRPV1 antagonist to produce broad-spectrum analgesia.
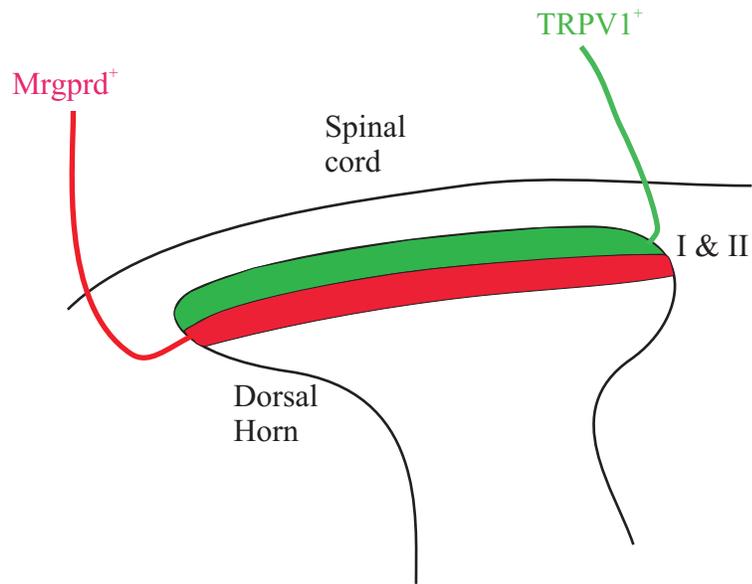
Figure 7-4. Mrgprd$^+$ and TRPV1$^+$ nociceptors project
to largely non over lapping laminae of the dorsal horn
of the spinal cord with fewer than 10% of Mrgprd positive
neurons expressing TRPVI.

Remember that mice, in which diphtheria toxin A was used to kill neurons expressing TRPV1, were unresponsive to painful and non painful hot and cold thermal stimuli and exhibited defective body temperature control but retained normal touch and mechanical pain sensation. Furthermore, nociceptive responses to ATP injection are also lost in TRPV1-DTA mice. These findings do not explain why, in adult mice, neurons expressing Mrgprd normally respond to thermal stimuli and to ATP.

Nociceptors expressing the voltage gated sodium channel $Na_v1.8$

For mice in which diphtheria toxin was used to kill neurons expressing the voltage gated sodium channel $Na_v1.8$ (DTA mice) there was almost no detectable IB_4 staining in lamina II, whereas approximately 12% of CGRP expressing neurons were still present (Abrahamsen, Zhao et al. 2008). Behavioral responses to low-threshold mechanical stimuli applied with Von Frey hairs were normal in the DTA mice while behavioral thresholds measured using the Randall-Selitto apparatus were dramatically elevated. These findings suggest that nociceptors expressing $Na_v1.8$ are necessary for sensing mechanical pain from deeper tissue. No difference was observed in behavioral thresholds to noxious thermal stimuli although there was a significant loss in TRPV1 expressing nociceptors. Both mechanical and thermal hyperalgesia following injection of Freund's complete adjuvant were greatly reduced. However, remember that neuropathic pain was unchanged in mice in which neurons expressing Nav1.8 were killed. These findings point to $Na_v1.8$ as a potential target for the treatment of inflammatory hyperalgesia. As mentioned above almost no detectable IB_4 staining remained in lamina II in the DTA mice while some of the CGRP expressing neurons remained. In Figure 7-5 I have tried to summarize the findings with respect to the laminae of termination of nociceptors expressing TRPV1, Mrgprd and $Na_v1.8$ respectively.

Dorsal horn neurons, which contain PKCγ

Protein kinase C (PKC) isoenzymes, including PKCγ are found in neurons of the spinal cord and PKCγ -immunoreactivity is found primarily in a limited population of small neurons in laminae II and III of the dorsal horn, but does not appear to be present in afferent neurons (Polgar, Fowler et al. 1999). Figure 7-6 summarizes the findings with respect to the laminae of termination of nociceptors expressing CGRP and Mrgprd relative to the location of spinal cord neurons expressing PKCγ. Mice which lack PKCγ display normal responses to acute pain stimuli, but they show reduced signs of neuropathic pain after partial ligation of the sciatic nerve (Malmberg, Chen et al. 1997). These findings point to PKCγ as a potential target for the treatment of neuropathic pain.

Neurotransmitters at the nociceptor synapse in the spinal cord

Glutamate

The amino acid L-glutamate (GLU) is recognized as the major excitatory neuro-transmitter for both A(δ-β)-fiber and C-fiber nociceptors, which also express the minor neurotransmitters calcitonin gene-related peptide (CGRP) and substance P (SP).
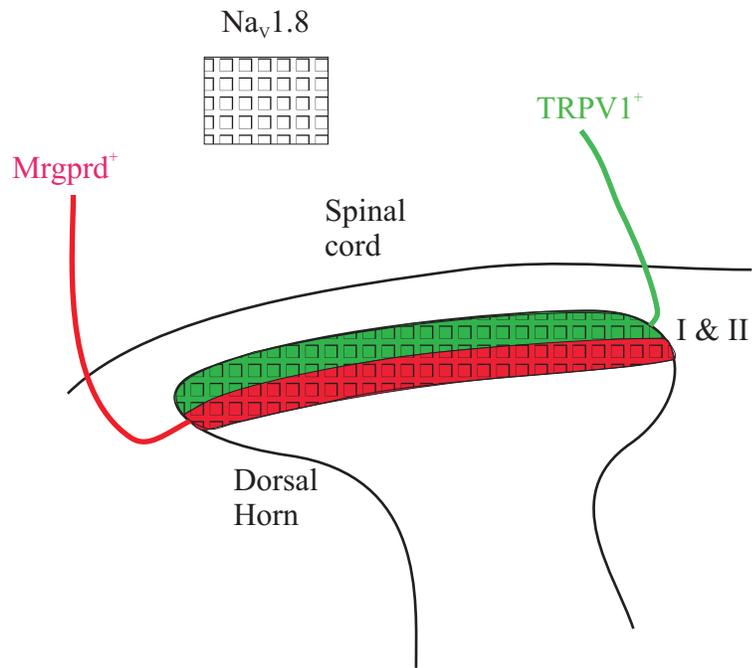
Figure 7-5. $Na_v1.8$ expressing nociceptors overlap with both Mrgprd[+] and TRPV1^{+} expressing nociceptors.
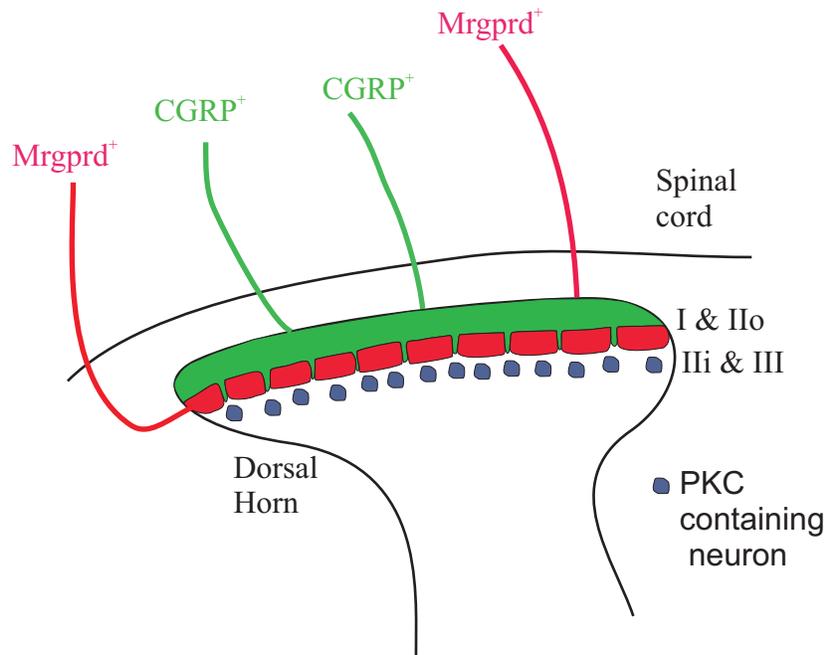
Figure 7-6. CGRP⁺-fibers and Mrgprd⁺-fibers terminate in adjacent laminae (I & IIo) of the dorsal horn, with CGRP⁺-fibers sometimes intermingling with Mrgprd⁺-fibers in lamina IIo. PKC neurons are found primarily in laminae IIi and III.

Receptors to glutamate include both ionotropic and metabotropic receptors (see Chapter 2). Ionotropic glutamate receptors can be distinguished pharmacologically by the use of specific agonists: N-methyl-D-aspartate (NMDA), kainic acid (KA), and a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA).

Blockade of non-NMDA ionotropic receptors using specific antagonists results in a nearly complete elimination of the responses of anterolateral system neurons to noxious and non-noxious stimuli. On the other hand blockade of NMDA ionotropic receptors, using specific antagonists, do not modify baseline responses to acutely painful stimuli and prevents the development of central hypersensitivity from the injection of formalin. These findings support the role of glutamate as the primary transmitter from nociceptors to spinal second order neurons and for a role of NMDA receptors in the generation of hypersensitivity.

Vesicular glutamate transporters (VGLUTs 1–3) use the proton gradient to transport the neurotransmitter glutamate into synaptic vesicles that can then be released into the synapse. VGLUT3 is expressed in unmyelinated neurons in the DRG that project to lamina I and lamina IIi of the dorsal horn suggesting that these neurons might convey information about painful stimuli. Mice in which VGLUT3 is knocked out show a decreased behavioral response to intense noxious **mechanical** stimuli and a selective decrease in the **mechanical** hypersensitivity produced by inflammation, nerve injury and trauma (Seal, Wang et al. 2009). Surprisingly, intracellular recordings fromVGLUT3 positive neurons in the DRG identify them as a population of unmyelinated, low threshold mechanoreceptors. One could imagine how sensitization of the input, to spinal nociceptive neurons, from unmyelinated low threshold mechanoreceptors could give rise to mechanical allodynia (see Peripheral sensitization versus central spinal sensitization). However, it is not clear how their absence would lead to a decreased response to intense noxious mechanical stimuli. On the other hand, it is possible that a small population of high threshold VGLUT3 positive neurons exists and was not sampled in their study. Lastly, remember that VGLUT3 is knocked out in all cells and not just in the unmyelinated low threshold mechanoreceptors in the DRG. Therefore, VGLUT3 activity in other parts of the nervous system may control mechanical hypersensitivity and the response to intense noxious mechanical stimuli (Lou, Duan et al. 2013).

Substance P

The situation with respect to the role of substance P (SP) in transmitting nociceptive information to spinal second order neurons is less clear than with GLU. Please remember that as mentioned above antagonists of the tachykinin NK1 receptor (receptor for substance P) which block the action of substance P have failed to show analgesic effects in humans.

Recall that intense noxious stimuli resulting in tissue damage often lead to hypersensitivity, that comprises both primary hypersensitivity an increased sensitivity within the injured area predominantly due to peripheral nociceptor sensitization, and secondary hypersensitivity, an increased sensitivity in the

surrounding uninjured area thought to be mediated centrally. In chapters 4 & 5 a number of intracellular biochemical pathways that could be involved allodynia and hyperalgesia in peripheral nociceptors were considered. What about hypersensitivity that is mediated centrally, possibly in the spinal cord? The method used for determining the involvement of TRPV1, Mrgprd containing neurons and IB_4 positive neurons in nociception was to selectively destroy the cells chemically (see above). As mentioned above antagonists of the tachykinin NK1 receptor have failed to show analgesic effects in humans. Nevertheless a conjugate of substance P and saporin (where saporin is a protein that inactivates ribosomes, shutting down protein synthesis and resulting in cell death) can be used to selectively kill cells containing the substance P receptor. Following intrathecal injection of such a conjugate responses, to highly noxious stimuli, were markedly attenuated as were mechanical and thermal hyperalgesia (Mantyh, Rogers et al. 1997). In subsequent experiments loss of substance P receptor neurons was shown to lead to an apparently permanent reduction of thermal hyperalgesia and mechanical allodynia associated with both neuropathic and inflammatory pain (Nichols, Allen et al. 1999). In contrast in mice in which the tachykinin NK1 receptor was knocked out acute nociceptive thresholds were unaffected as was hyperalgesia (De Felipe, Herrero et al. 1998). Consequently, neurons expressing the substance P receptor play an essential role in the maintenance of allodynia and hyperalgesia and the communication of highly painful stimuli. However, binding of substance P to its receptor does not appear to be essential for these phenomena.

Calcitonin gene related peptide and migrane headaches

It was originally proposed that neurogenic inflammation (see chapter 4) in the dura mater mediated by substance P released from trigeminal nerve endings was responsible for migraine headaches. However, antagonists of the tachykinin NK1 receptor were not effective in the treatment of patients suffering migraine attacks. On the other hand there has been support for the involvement of another component of neurogenic inflammation, calcitonin gene related peptide (CGRP), making a contribution to the development of migraine. Recently a clinical trial studying telcagepant (MK-0974), a CGRP receptor antagonist, at doses of 300 mg to 600 mg was effective in treating acute migraine pain. At these doses the plasma concentration is greater than 1 μmol/L, more than two orders of magnitude higher than the concentration needed to inhibit CGRP-induced vasodilation in human arteries (see review (Edvinsson and Ho 2010)). As a surrogate for evaluating the penetration of telcagepant through the blood brain barrier the levels of telcagepant were measured in the CSF and plasma of monkeys after oral dosing. A CSF/plasma ratio of about 1% was determined which suggests that telcagepant may possibly reach sufficient levels to inhibit CGRP receptors in the spinal cord or more centrally. These findings support the role of CGRP in somehow mediating migraine pain. However, some patients taking telcagepant developed high levels of liver enzymes (transaminases) and the clinical trials were ended. We can expect a continued search for additional CGRP-receptor antagonists for the treatment of both migraine pain and other forms of pain.

The findings discussed above are summarized below in Table 7-1.

Nociceptive neurons that contribute to the anterolateral system and dorsal columns

The nociceptive neurons that contribute to the anterolateral system are found primarily in lamina I, the outer layer of lamina II and in laminae IV, V and VI of the dorsal horn. Nociceptors sensitive to noxious visceral stimuli and which contribute to the dorsal columns are located primarily in laminae IV and V and, as illustrated in Figure 7-1, in the vicinity of the central canal (lamina X). Numerous studies have identified several categories of cells having partial or complete nociceptive properties in these laminae. Nociceptive specific (NS) cells are cells that are only activated by noxious stimuli such as noxious heat or noxious mechanical stimuli or both. An example of an NS cell activated by noxious heat is given in Figure 7-7A. In contrast to NS cells wide dynamic range (WDR) cells respond to both noxious and non-noxious stimuli. An example of a thermal WDR cell is given in Figure 7-7B where it is compared to an NS cell. A third category of cells that are sensitive to noxious heat, pinch and noxious cold are referred to as HPC cells. An example of the thermal properties of an HPC cell is given in Figure 7-7C. In many studies it was not determined whether these nociceptive cells were spinal interneurons or projection neurons that contribute to anterolateral system or the dorsal columns.

The axons of lamina I projection neurons ascend in the anterolateral system (see Figure 7-1), therefore presumptive recordings from lamina I projection neurons can be confirmed by antidromic activation of their axons by stimulating electrodes placed in anterolateral system projection areas. Studies of antidromically identified lamina I projection neurons have revealed these cells to have the properties of WDR, NS and HPC cells. In addition to the lamina I neurons with nociceptive properties, that we are interested in, there are also a group of lamina I neurons sensitive to warm and cool innocuous thermal stimuli and a group of lamina I neurons sub serving the sense of itch. There is as yet no general consensus as to the relative contributions to pain sensation of NS, WDR and HPC cells.

Primary hypersensitivity and secondary hypersensitivity

Remember that intense noxious stimuli resulting in tissue damage often lead to an increase in the response to subsequent painful stimuli, called hypersensitivity. Two areas of hypersensitivity are recognized according to their location relative to the site of injury, primary hypersensitivity, an increased sensitivity within the injured area partially due to peripheral nociceptor sensitization, and secondary hypersensitivity, an increased sensitivity in the surrounding uninjured area (see diagram below). A number of studies have shown that primary hypersensitivity involves sensitization to both mechanical and heat stimuli. On the other hand, the area of secondary hypersensitivity is characterized by sensitization to mechanical stimuli only.
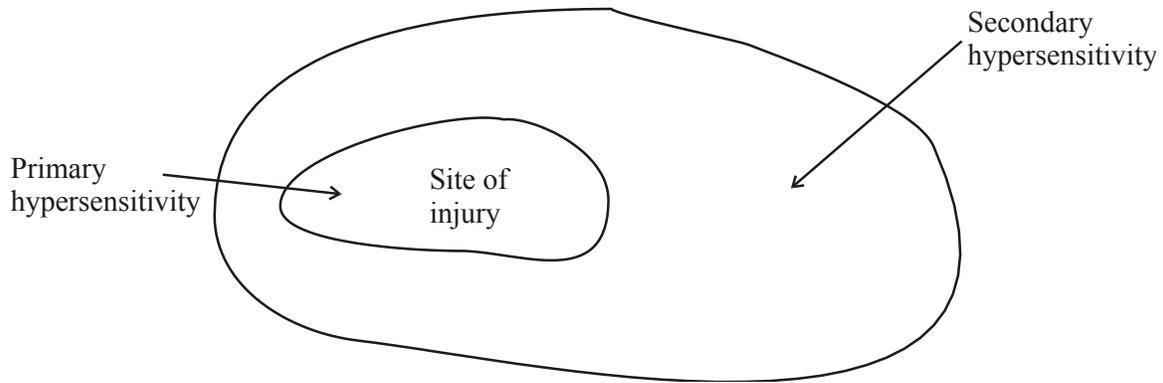
It is well documented (see Chapters 3,4 & 5) that the sensitivity of nociceptors to heat stimuli can be increased by a wide variety of stimuli such as direct tissue injury or the application of chemicals such as bradykinin, serotonin, capsaicin and mustard oil. This has led to the widely held view that the sensitization of nociceptors to heat stimuli may be sufficient to account for the heat hypersensitivity component of primary hypersensitivity.

One might think that within the region of primary hypersensitivity, the mechanisms for mechanical hypersensitivity and heat hypersensitivity are identical. However, this is not the case, for example, intradermal bradykinin injection in humans evokes hypersensitivity to heat stimuli with no measurable hypersensitivity to mechanical stimuli measured with von Frey hairs (Manning, Raja et al. 1991). Although sensitization of nociceptors to mechanical stimuli has been documented it is currently thought that it cannot quantitatively account for mechanical hypersensitivity in the zone of primary hypersensitivity, and that central sensitization may contribute to the mechanical hypersensitivity.
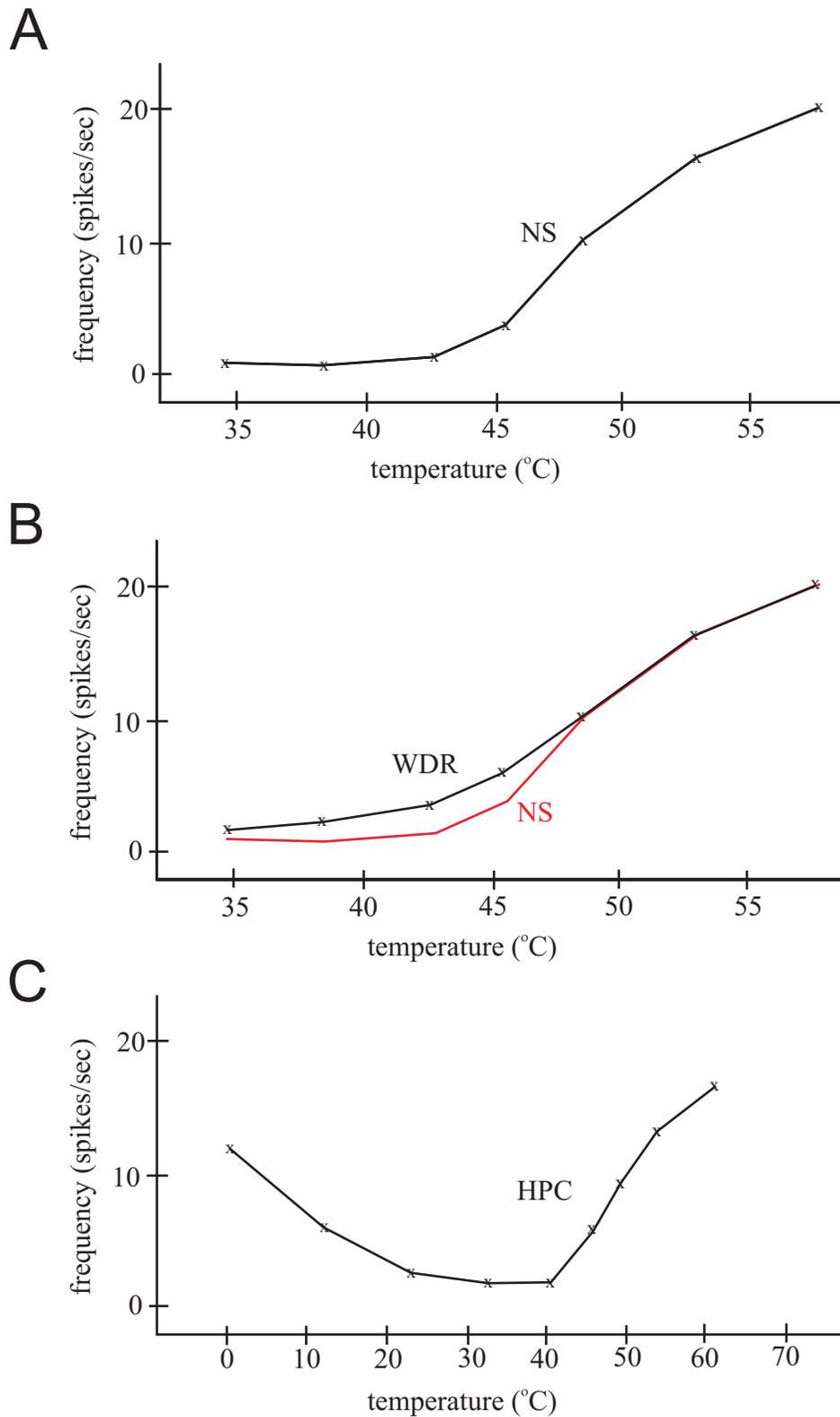
Figure 7-7. Three types of lamina I neurons sensitive to noxious thermal stimuli. (A) Nociceptive specific (NS) cells, (B) wide dynamic range (WDR) cells, (C) noxious heat, pinch and noxious cold (HPC) cells. See text for further details.

Table 7-1

Molecule studied	Molecule knocked out in mice	Neurons expressing the molecule are killed	Acute pain thresholds	Allodynia and hyperalgesia
Mrgprd, G-protein coupled receptor		XXX	↓ sensitivity to noxious mechanical stimuli determined with von Frey hairs	
TRPV1	XXX		↓ response above 50°C	↓ thermal hyperalgesia induced by inflammation
TRPV1		XXX	Unresponsive to painful and non painful hot and cold thermal stimuli	Blocks inflammatory thermal hyperalgesia & neurogenic inflammation
Na$_v$1.8		XXX	↓ sensitivity to noxious mechanical stimuli determined with the Randall-Selitto apparatus	↓ mechanical and thermal hyperalgesia following injection of Freund's complete adjuvant
PKCγ	XXX		normal	↓ neuropathic pain
VGLUT3 Vesicular glutamate transporter	XXX		↓response to intense noxious **mechanical** stimuli	↓ **mechanical** hypersensitivity produced by inflammation and nerve injury
tachykinin NK1 receptor		XXX	↓ responses to highly noxious stimuli	↓ thermal hyperalgesia and mechanical allodynia associated with both neuropathic and inflammatory pain
tachykinin NK1 receptor	XXX		Normal	Normal

Peripheral sensitization versus central spinal sensitization

Peripheral sensitization, (allodynia and hyperalgesia) of nociceptors occurs when the peripheral terminals of the nociceptors are exposed to damaged and/or inflamed tissue and consequently, is limited to the site of injury and/or inflammation. Peripheral sensitization of nociceptors contributes to pain hypersensitivity at inflamed sites (primary hyperalgesia); it appears to play a major role in altered heat but not mechanical sensitivity, which is a major feature of central spinal sensitization.

In contrast to peripheral sensitization, central spinal sensitization, allows low-threshold mechanoreceptor afferents to mediate pain although these afferents do not normally cause pain. In this respect, central spinal sensitization represents a dramatic functional shift in the way we perceive somatosensory inputs: low threshold stimuli, which may have been pleasant previously, are now painful. In this situation we are experiencing the pain as coming from outside stimuli, although the actual stimuli are not themselves noxious. It is important to keep this in mind because the target for treatment in this circumstance is not the periphery but is actually the central nervous system.

That the sensitization to mechanical stimuli of secondary hypersensitivity is mediated centrally rather than peripherally is not at all obvious. It could be that molecules, which enhance pain, are released from the injured tissue and spread beyond the site of injury to render remote nociceptors hyper excitable. To prove that the hypersensitivity is mediated centrally micro stimulation of nerves was performed in human subjects (see Figure 7-8). Electrical nerve stimulation was used to evoke a **non-**painful tactile sensation from a small area of the skin (see Figure 7-8A). A zone of secondary hyperalgesia was induced, by capsaicin injection, which included the small area of skin from which the electrical stimulation now evoked the tactile sensation and also a painful sensation (see Figure 7-8B). After the secondary hyperalgesia wore off the electrical nerve stimulation again evoked a purely **non-**painful tactile sensation. These findings indicate that secondary hyperalgesia to touch is mediated by low-threshold mechanoreceptors that normally signal non painful touch sensation (Torebjork, Lundberg et al. 1992).

Two forms of mechanical allodynia have been distinguished; one form is called stroking allodynia or dynamic allodynia and is apparent when the skin is gently stroked with a cotton swab. The second form is referred to as punctuate allodynia and occurs when punctuate stimuli such as von Frey probes are applied to the skin. Experimental evidence from patients with complex regional pain syndrome (also called causalgia and reflex sympathetic dystrophy) suggests that their mechanical stroking and punctuate allodynia is mediated in part by input from large-diameter, rapidly conducting Aβ low-threshold mechanoreceptor afferents (for example see (Gracely, Lynch et al. 1992)). This finding does not indicate where in the central nervous system Aβ low-threshold mechanoreceptor input gains access to the pain pathway or where in the pathway central sensitization occurs.
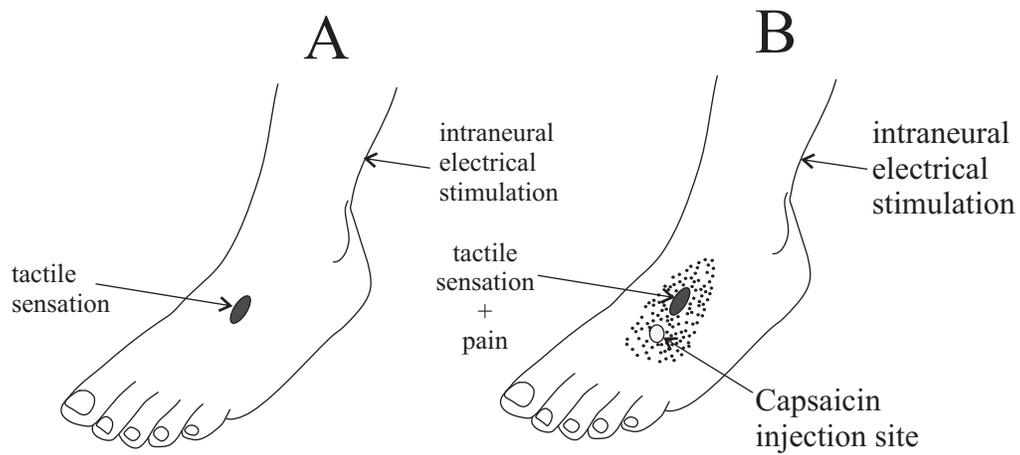
Figure 7-8. Change in the perception of the response to intraneural electrical stimulation after development of secondary hyperalgesia. A. Intraneural electrical stimulation evokes a non painful tactile sensation projected to a small area of the foot as shown. B. Following the injection of capsaicin (as shown) secondary hyperalgesia developed in the dotted area and intraneural electrical stimulation elicited a tactile sensation accompanied by pain.

Animal experiments indicate that non-nociceptive mechanoreceptor inputs gain access to the central pain pathway in lamina I of the dorsal horn. Recent experiments have shown that in rat WDR lamina I projection neurons, the low threshold mechanoreceptor input comes from C-fiber low-threshold mechanoreceptors that are best stimulated by gentle brushing of the skin (Andrew 2010). Remember that in mice in which the vesicular glutamate transporter VGLUT3 is knocked out the mechanical hypersensitivity that accompanies inflammation, nerve injury and trauma is impaired, and that VGLUT3 positive neurons are unmyelinated, low threshold mechanoreceptors (Seal, Wang et al. 2009) that project to lamina I and lamina IIi of the dorsal horn. Interestingly, stimulation of unmyelinated low-threshold mechanoreceptors by soft brush stroking is perceived as pleasant touch in humans (Loken, Wessberg et al. 2009). Taken together these findings would suggest that when we experience stroking allodynia pleasant touch is converted to painful touch. Leading to the question of where in the central nervous system does this change in perception occur?

The dorsal horn of the spinal cord is a likely candidate for one of the sites where central sensitization may occur. Moreover, dorsal horn nociceptive specific (NS) and wide dynamic-range (WDR) neurons are the most likely candidates for the neurons in which central sensitization might occur. In order to determine whether central sensitization occurs in either or both of these cell types, responses of WDR and NS dorsal horn neurons projecting into the spinothalamic tract of the anterolateral system were studied in monkeys (Simone, Sorkin et al. 1991). Capsaicin was injected into a location adjacent to the site of mechanical test stimuli to see if the test site exhibited secondary hyperalgesia as a result of the capsaicin injection. The responses at the test site of WDR and NS neurons to the mechanical stimuli were significantly increased, suggesting that increased responsiveness of both WDR and NS neurons contributes to mechanical hyperalgesia produced by capsaicin injection. NS neurons were functionally converted into WDR neurons, that is, they became responsive to low intensity mechanical stimuli. Whereas, WDR neurons simply sensitized, that is, they became more sensitive to the mechanical stimuli they were already sensitive to. Responses to both innocuous stroking and to light punctate stimuli were either weak or absent before capsaicin and increased dramatically following capsaicin injection. Indicating that both stroking and punctuate allodynia contribute to the allodynia in these neurons. Responses of these WDR and NS dorsal horn neurons to electrical stimulation of the proximal end of severed dorsal rootlets increased significantly after capsaicin injection, suggesting that the hypersensitivity of these neurons is in part due to their increased excitability. ***These findings support the idea that central sensitization is in part the result of increased excitability of WDR and NS dorsal horn neurons that project into the spinothalamic tract of the anterolateral system.***

What is the role of central sensitization in patients with peripheral neuralgia? In a study of patients with unilateral carpal tunnel syndrome, bilateral widespread pressure hypersensitivity was found, suggesting that extensive central sensitization occurred. Presumably as the result of peripheral drive from the injured nerve (Fernandez-de-las-Penas, de la Llave-Rincon et al. 2009).

Potential spinal mechanisms for allodynia and hyperalgesia

As described in Figures 7-2 through 7-5 the central terminals of functionally different types of nociceptors project to specific locations in the dorsal horn. Their targets, the dorsal horn neurons, can be divided into two general classes: projection neurons and propriospinal neurons. Projection neurons convey information from the spinal cord to the brain and are the major output emanating from the spinal cord. The axons of propriospinal neurons stay within the spinal cord, their axons can either stay within the same spinal cord segment or project from one segment to another. Pain scientists distinguish two aspects of sensitization: allodynia (pain resulting from a normally innocuous stimulus) and hyperalgesia (an enhanced response to a normally painful stimulus). In what follows spinal mechanisms that have been studied because they are thought to be involved in primary and/or secondary hypersensitivity will be considered. Simply put, we will consider mechanisms that can lead to an increase in the excitability of dorsal horn projection or propriospinal neurons, such that normal spinal inputs are able to evoke an enhanced response resulting in central hypersensitivity.

Wind up

Pain wind up is a progressive buildup of the response of dorsal horn neurons caused by repetitive low frequency (1-3 Hz) firing of C fibers, as illustrated in Figure 7-9. An important feature of wind up is that the enhancement of the response of the dorsal horn neuron decays rapidly when the stimuli are discontinued. Additionally wind up is not a property of the nociceptive C fibers but of the synapse of the C fibers with their post-synaptic dorsal horn neuron.

Wind up; Presynaptic mechanism

It has been suggested that wind up is the result of a presynaptic mechanism, where a buildup of intracellular free calcium within the presynaptic terminal, with each incoming action potential, results in an increase in the number of transmitter vesicles (quanta) released by subsequent action potentials. The C fibers release both glutamate (stored in small clear vesicles) and peptide neurotransmitters such as substance P (SP) (stored in large, dense core vesicles). Therefore, if wind up is due to an increase in transmitter release, the increase could be due to either glutamate or a peptide such as SP, since the two types of transmitter are stored and released separately.

Wind up; Postsynaptic mechanism

Numerous studies have shown partial blockade of wind up by NMDA receptor antagonists both in vitro and in vivo. Because of the partial block it has been suggested that molecules other than glutamate, such as peptides might also contribute to wind up. The available evidence from NK1 receptor knockout mice and using NK1 receptor antagonists shows that activation of NK1 receptors is necessary for wind up to occur. However, in these knockout mice nociceptive thresholds were unaffected as was

presynaptic
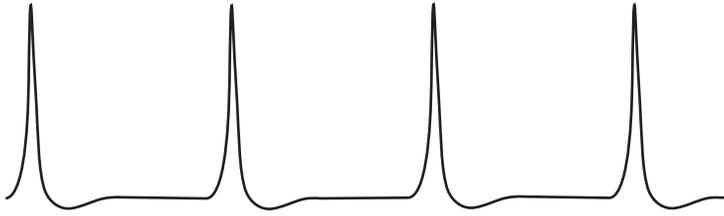
postsynaptic
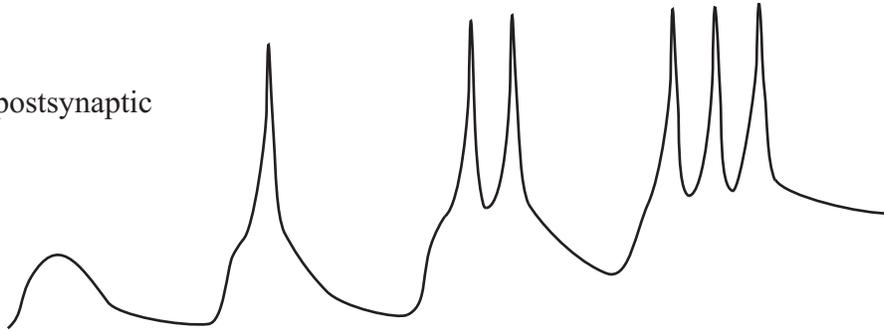
0.2s

Figure 7-9. Repeated action potentials in presynaptic nociceptor
leads to a buildup of the response in the postsynaptic dorsal horn neuron.

hyperalgesia. It has been suggested that other ion channels, such as Ca^{2+} channels and K^+ channels may be involved in wind up but there is no consensus as to their identity.

Phenomena similar to wind up in humans

As mentioned in Chapter 1, when a brief noxious thermal stimulus is applied to the volar surface of the arm the pain sensation has two components separated in time. First there is a sharp pricking sensation followed after a lull by a second burning feeling. First and second pain correspond to the arrival in the spinal cord of impulses in A(δ-β) and C fibers nociceptors respectively. If the stimulus is repeated at intervals less than 3 seconds the intensity of the second pain sensation increases. Similar results were obtained using noxious mechanical stimuli. These findings appear to be the perceptual correlate of wind up in human subjects.

Windup can be described as a potentiation of post-synaptic potentials in dorsal horn neurons due to repeated low frequency stimulation of nociceptive C-fibers. The consensus of thinking from numerous studies is that wind up is not equivalent to the spinal component of primary or secondary hypersensitivity following tissue injury. However, wind up may share some common mechanisms with central spinal sensitization.

Homosynaptic and heterosynaptic synaptic facilitation

Central sensitization is a form of functional synaptic plasticity resulting in pain hypersensitivity that is induced by a variety of intense noxious stimuli. The plasticity is evoked by conditioning stimuli that include high-frequency electrical stimulation of C fibers, damaging heat stimuli, and chemical activation of nociceptors by compounds such as mustard oil and formalin, both of which act via TRPA1 (see Chapters 3 and 4) in addition to capsaicin, which acts via TRPV1 (see Chapter 3). These stimuli have in common that they are intense and sustained stimuli which activate many fibers. A single painful stimulus, such as a pinch or a needle stick, which does not meet these requirements, will not induce central sensitization.

Central sensitization resembles long-term potentiation (LTP) in that it is a long-lasting enhancement in synaptic transmission between neurons resulting from brief bursts of high frequency stimulation. However, LTP is a form of homosynaptic facilitation where it is only the sensitivity of the activated synapses that is changed. On the other hand, central sensitization is a form of heterosynaptic sensitization, where the activity in one set of synapses enhances the activity in nonactivated synapses. This apparently occurs by sensitizing the entire neuron, with the result that NS neurons are functionally converted into WDR neurons, that is, they became responsive to low intensity mechanical stimuli. Furthermore, during central sensitization WDR neurons are sensitized so that they became more sensitive to the mechanical stimuli they were already sensitive to. This change in the responsiveness of NS and WDR dorsal horn neurons is accompanied by an expansion of the spatial extent of their inputs. That is the size of their receptive field increases so that synaptic inputs from silent or ineffective synapses are now effective.

Homosynaptic facilitation is a type of use dependent facilitation of a synapse evoked by activation of that same synapse. Windup as previously described is a form of homosynaptic facilitation in which the response of a dorsal horn neuron to a low frequency discharge of action potentials in C fibers get larger with successive stimuli (see Figure 7-9). As mentioned above both NMDA receptors and NK1 receptors appear to be involved in windup. Moreover, the available evidence from NK1 receptor knockout mice and using NK1 receptor antagonists shows that activation of NK1 receptors is necessary for wind up to occur. However, in these knockout mice nociceptive thresholds were unaffected as was hyperalgesia (see Table 7-1).

NMDA receptors and central hypersensitivity

Since central hypersensitivity has features that resemble LTP it is not surprising that a similar mechanism might underlie both processes. In the hippocampus NMDA receptors have been shown to be crucial for the development of LTP. Likewise, as mentioned above, NMDA receptor antagonists reduce the initiation and maintenance of central sensitization. Moreover, conditional deletion of the NR1 subunit of the NMDA receptor in the lumbar spinal cord reduces NMDA synaptic currents by more than 85% and reduces pain resulting from intraplantar formalin injection by 70% (South, Kohno et al. 2003). These findings indicate that NR1 subunit of the NMDA receptor is crucial for the development of central spinal sensitization. The next obvious question is how activation of NMDA receptor ion channels leads to central spinal sensitization.

At resting membrane potentials NMDA receptor channels located in postsynaptic membranes are blocked by a magnesium ion, sitting in the receptor pore that prevents the entry of calcium into the postsynaptic cell. Sufficient membrane depolarization of the postsynaptic cell is required to drive magnesium out of the pore thereby relieving the blockade of the NMDA receptor by magnesium and allowing calcium influx. This is referred to as a voltage dependent block of the NMDA receptor mediated by magnesium. At the nociceptor synapse brief bursts of high frequency stimulation release sufficient amounts of glutamate, substance P and CGRP that leads to sufficient membrane depolarization to force magnesium to leave the NMDAR pore thereby relieving the block. When the block is removed synaptic efficacy is increased allowing for the entry of calcium, which then activates numerous intracellular pathways that contribute to the induction of central spinal sensitization.

Of particular importance for the **induction** of central sensitization are several protein kinase enzymes, including calcium calmodulin dependent protein kinase II (CaMKII), protein kinase C (PKC) and the mitogen-activated protein kinase (MAPK) pathway that involves the extracellular signal-regulated kinases (ERK1 and ERK2, $ERK_{1/2}$). More over the induction of central sensitization involves protein kinase A (PKA) and the nitric oxide (NO) pathway, which involves soluble guanylate cyclase and the production cGMP which -in turn activates protein kinase G (PKG). The reason that these protein kinases are important is that the NMDA and AMPA receptors have several key residues that can be phosphorylated thereby changing their activity as well as their trafficking to or from the

plasma membrane. The net effect of these changes is to increase the efficiency of synaptic transmission via an increase in the total current carried by AMPA and NMDA receptors.

Additionally the $ERK_{1/2}$ pathway brings about a decrease in potassium currents via phosphorylation of the potassium channel Kv4.2 thereby increasing in membrane excitability. Mice in which Kv4.2 is knocked out exhibit baseline hypersensitivity to noxious mechanical and thermal stimuli (Hu, Carrasquillo et al. 2006). Furthermore these knockout mice exhibit reduced hyperalgesia in the second phase of the formalin test and after intraplantar carrageenan injection.

The changes discussed above strengthen nociceptive transmission and recruit non-nociceptive inputs to the pain pathway. Central sensitization can be thought of as being protective, because it creates a situation in which innocuous stimuli become painful thereby limiting use of the injured region-allowing repair to occur.

Ketamine, which is thought to act by blocking the NMDA receptor, although other mechanisms are possibly involved, has sometimes been found to be useful clinically as an analgesic in the treatment of neuropathic pain. However, its usefulness is limited due to its side effects such as hallucinations and other cognitive disturbances, such as impaired judgment and illusions.

Disinhibition and central sensitization

A decrease in tonic inhibition, i.e. disinhibition, of dorsal horn output neurons could account for central sensitization just as well as an increase in the total current carried by AMPA and NMDA receptors in these neurons. In the dorsal horn the amino acids γ-aminobutyric acid (GABA) and glycine are the major inhibitory transmitters. Both transmitters are present in the synaptic terminals of dorsal horn interneurons and they also coexist in a proportion of those terminals. Experiments in monkeys have shown that inhibition of spinothalamic tract neurons of the anterolateral system by spinal glycine and GABA is reduced during central sensitization (Lin, Peng et al. 1996). This work also suggested that PKC in the spinal cord was involved in the desensitization of glycine and GABA receptors, thereby contributing to the development of allodynia and secondary hyperalgesia that underlie central sensitization.

Experiments in mice have shown that the α3 subunit of strychnine sensitive glycine receptors is predominantly expressed in lamina II of the dorsal horn (Harvey, Depner et al. 2004). Moreover, mice in which the glycine receptor α3 subunit was knocked out showed a reduction in pain sensitization induced by intrathecal PGE_2 injection or peripheral inflammation. Therefore, the glycine receptor α3 subunit is a potential target for the treatment of pain.

It is very possible that neuropathic pain and central sensitization may share common mechanisms in the spinal cord. However it should be kept in mind that whereas central sensitization can develop in minutes after an intense noxious stimulus (Torebjork,

Lundberg et al. 1992) neuropathic pain typically takes days to develop. Nevertheless it has been proposed that in some cases, neuropathic pain is dynamically maintained by ongoing peripheral nociceptive afferent input which accounts for allodynia, spontaneous pain, and other abnormalities (Gracely, Lynch et al. 1992). Given these reservations remember, that as previously described, the induction of neuropathic pain by peripheral nerve injury results in tactile allodynia. Moreover, in rats peripheral nerve injury also results in a depolarizing shift in the reversal potential of GABA and glycine currents of lamina I neurons in the dorsal horn, causing **disinhibition** and, in some cases, converting the $GABA_A$ receptor and glycine receptor mediated inhibition into excitation (Coull, Boudreau et al. 2003). This might explain the allodynia of neuropathic pain and if something similar happened during central sensitization it might explain the allodynia of central sensitization as well.

The inhibitory neurotransmitters GABA and glycine act on ionotropic, chloride permeable $GABA_A$ or glycine receptors or metabotropic (G protein-coupled) $GABA_B$ receptors. Under normal conditions the intracellular concentration of chloride ions in neurons is kept low by the potassium chloride cotransporter 2 (KCC2), which extrudes chloride. Consequently the opening of $GABA_A$ or glycine activated chloride channels results in membrane hyperpolarization. Peripheral nerve injury reduces the level of KCC2 to cause a depolarizing shift in the reversal potential of GABA-mediated currents in dorsal horn neurons (Coull, Boudreau et al. 2003). The question then becomes what is the mechanism by which nerve injury reduces the level of KCC2 in dorsal horn neurons. The available evidence suggests that nerve injury increases NMDAR activity leading to Ca^{2+} influx which causes cleavage of KCC2 by means of the calcium activated protease μ-calpain (Zhou, Chen et al. 2012). These findings linking glutamate mediated activation of NMDA receptors to the disinhibition of dorsal horn neurons provides a new framework for understanding the mechanisms underlying synaptic modifications that occur in neuropathic pain.

The evidence, just described, indicating the importance of KCC2 activity in neuropathic pain suggests that drugs aimed at enhancing KCC2 activity may be useful as analgesics. The discovery of a compound, CLP257, which lowers intracellular chloride by enhancing chloride transport and also alleviated hypersensitivity in a rat model of neuropathic pain, confirms the concept of targeting KCC2 for the treatment of neuropathic pain (Gagnon, Bergeron et al. 2013).

Transplantation and integration of GABAergic interneurons, from immature mice, into the adult mouse spinal cord reversed the mechanical hypersensitivity produced by peripheral nerve injury (Braz, Sharif-Naeini et al. 2012). This finding supports the thinking that a lack of inhibition (i.e. disinhibition) of second order pain fibers is a mechanism of central hypersensitivity.

Descending inhibitor and facilitator effects

Most considerations of the descending spinal pathways focus on their role in the inhibition of pain. However it has been shown that these descending pathways may either block or facilitate transmission of pain information at the level of the dorsal horn. As mentioned earlier the nerve fibers of the descending pathway travel in the dorsolateral funiculus (see Figure 7-1) and originate in the rostroventral medulla, the nucleus tractus solitarius, the parabrachial nucleus, the dorsal reticular nucleus, the hypothalamus and the cortex. The effects of these descending pathways are largely mediated by monoaminergic neurons that utilize serotonin, norepinephrine, or dopamine.

The serotonergic inputs to the dorsal horn originate in neurons of the rostral ventromedial medulla (RVM), which receives a projection from the midbrain periaqueductal gray (PAG). The PAG is thought to be the primary site of action of opiates in producing analgesia and the RVM serotonergic pathway is thought to mediate these opiate analgesic effects. The descending serotonergic output fibers of the RVM synapse directly on presynaptic terminals of primary afferent fibers in the dorsal horn and on second order projection neurons and also on intrinsic interneurons within the spinal cord.

Seven families of 5-HT receptors (5HT1–7) have been identified along with several subtypes within these families. In mice secondary hypersensitivity, following intraplantar capsaicin injection was prevented by 5-HT_7 receptor agonists Furthermore systemic administration of 5-HT_7 receptor antagonists promoted mechanical hypersensitivity (Brenchat, Romero et al. 2009). Finally, spinal administration of a 5-HT_7 receptor antagonist blocked the analgesic effect of morphine injected into the RVM.

Remember from chapter 4 that the second phase of the formalin test was significantly reduced in 5-HT3A-knock out mice or in normal mice following intrathecal (inside the spinal canal) administration of a 5-HT3-receptor antagonist. Additionally, lesions to the serotonergic nucleus raphe magnus (NRM) located in the RVM block the second phase of the formalin test. These findings are consistent with a descending serotonergic facilitator pathway from the RVM to the dorsal horn mediated by 5-HT3-receptors, contributing to central sensitization.

These studies suggest that descending pain inhibitory or facilitator pathways from the RVM act ultimately in the spinal cord through activation of 5-HT_7 and 5-HT_3 receptors, respectively. Therefore the 5-HT_7 and 5-HT_3 receptors make excellent targets for the treatment of pain.

Spinal Glial Cells

There are a number of studies describing the effectiveness of glial inhibitors in preventing and/or reversing pain in a variety of models for neuropathic pain. It should be kept in mind that there are a number of problems with these studies. The major problem being the lack of specificity of the inhibitors, they may also affect neurons. Another problem with these studies is that there is no general consensus as to exactly what constitutes resting and activated glial cells, is there only one activated state for glial cells or do they exhibit different degrees of activation? Is glial activation determined by morphological changes or by differential gene expression or both?

In a variety of models of neuropathic pain there is general agreement that microglial and astroglial cells in the spinal cord exhibit an "activated" state. They assume a different morphology and change their expression of certain markers, over a time course of days to weeks, which is concomitant with the presence of hyperalgesia and allodynia. On the other hand the inflammatory mediators that cause the central sensitization of secondary hypersensitivity are weaker inducers of glial activation, and there are far fewer studies of glial activation by these mediators. In fact some studies have reported the absence of an obvious glial reaction associated with inflammation resulting from complete Freund's adjuvant injection.

It is important to remember that neuropathic pain arises following damage to peripheral nerves, thereby raising the question of what causes the glial response when nociceptors are damaged. A likely candidate is the activity of the injured nerve and the associated release of neurotransmitters and/or neuro-modulators. These include glutamate, substance P, calcitonin gene related peptide (CGRP), brain-derived neurotrophic factor (BDNF), ATP, neuronal chemokines, and other yet unidentified activators of glial cells.

P2X$_4$ receptors

Among the potential glial activators the most extensive evidence indicates that ATP plays a major role in glial activation. After nerve injury, the expression of the P2X$_4$ ionotropic ATP receptor increases dramatically in hyperactive microglia in the ipsilateral spinal cord, but not in neurons or astrocytes (Tsuda, Shigemoto-Mogami et al. 2003). Furthermore, intrathecal injection of antisense oligodeoxynucleotide targeting the P2X$_4$ receptor decreased the induction of P2X$_4$ receptors and suppressed tactile allodynia after nerve injury. Moreover, intrathecal injection of microglia in which P2X$_4$ receptors had been induced and stimulated, produced tactile allodynia in naive rats. However, in mice in which the P2X$_4$ receptor is knocked out the morphological changes of microglial activation as well as the up regulation of P2X$_7$ receptor expression (see below) occurs following nerve injury (Ulmann, Hatcher et al. 2008). These findings indicate that the P2X$_4$ receptor does not initiate glial activation. Nevertheless P2X$_4$ receptor knockout mice do not exhibit hyperalgesia following peripheral nerve injury indicating that the P2X$_4$ receptor contributes to central sensitization.

P2X$_7$ receptors

A number of neurodegenerative conditions exhibit enhanced P2X$_7$ receptor expression with coexisting activated microglia. In mice in which the P2X$_7$ gene is deleted, inflammatory hypersensitivity following complete Freund's adjuvant injection and the hypersensitivity of neuropathic pain following partial nerve ligation are completely absent (Chessell, Hatcher et al. 2005). That is the hypersensitivity to noxious thermal and mechanical stimuli in models of both chronic neuropathic and inflammatory pain is absent. However, there was no difference in microglial activation between wild type mice and mice in which the P2X$_7$ receptor is knocked out (Sharp, Polak et al. 2008). As with the P2X$_4$ receptor these findings indicate that the P2X$_7$ receptor does not initiate glial activation but it does contribute to central sensitization.

A-740003 is a highly potent competitive antagonist of $P2X_7$ receptors (IC_{50}=18 nM for rat) that dose dependently reduces tactile allodynia in three models of neuropathic pain, spinal nerve ligation, chronic constriction injury of the sciatic nerve and vincristine induced neuropathy (Honore, Donnelly-Roberts et al. 2006). Additionally, A-740003 reduced thermal hyperalgesia observed following intraplantar injection of carrageenan or complete Freund's adjuvant, demonstrating that selective $P2X_7$ receptor antagonists significantly reduce allodynia and hyperalgesia for in-vivo models of both neuropathic and inflammatory pain.

$P2Y_{12}$ receptors

In addition to the two ionotropic ATP receptors ($P2X_4$ and $P2X_7$) discussed above, spinal microglia constitutively express $P2Y_{12}$ a metabotropic ATP receptor. Together, the $P2X_4$ and the $P2Y_{12}$ receptors play a role in ATP induced microglial chemotaxis (Ohsawa, Irino et al. 2007). It was also found that intrathecal injection of AR-C69931MX a $P2Y_{12}$ receptor antagonist prevented the development of tactile allodynia following L5 spinal nerve ligation (Tozaki-Saitoh, Tsuda et al. 2008). Furthermore $P2Y_{12}$ knockout mice exhibited greatly impaired tactile allodynia after L5 spinal nerve ligation. Therefore, it would appear that $P2Y_{12}$ is necessary for glial activation which is an essential step leading to central sensitization.

A variety of other molecules found in nociceptors for which there are receptors that are expressed on microglia have also been implicated in the development of neuropathic pain. Some of these are considered below.

CCR2 receptors

Monocyte chemoattractant protein-1 (CCL2) is expressed in the cell bodies of DRG neurons and their axon terminals in the dorsal horn and CCR2, the receptor for CCL2, is expressed in microglia. Furthermore, microglial activation and mechanical allodynia following partial sciatic nerve ligation in CCR2 knockout mice fails to develop (Abbadie, Lindia et al. 2003, Zhang, Shi et al. 2007). Thus signaling from CCL2 to CCR2 is necessary for both microglial activation, and the development of tactile allodynia following peripheral nerve injury.

Fractalkine receptors

Fractalkine (CX3CL1) is a chemokine (**chemo**tactic cyto**kine)** which is expressed in the dorsal root ganglia and its receptor (CX3CR1) is expressed by microglia. At present there are conflicting reports regarding the role of signaling from CX3CL1 to CX3CR1 in neuropathic pain. Intrathecal injection of fractalkine in rats leads to mechanical allodynia and thermal hyperalgesia (Milligan, Zapata et al. 2004). However, injection of fractalkine into the sciatic nerve was found to attenuate allodynia in a spared nerve injury model of neuropathic pain (Holmes, Arnott et al. 2008). The spared nerve injury model involves lesioning two of the three terminal branches of the sciatic nerve leaving the remaining

nerve intact (see Figure 6-2). Furthermore, CX3CR1 knockout mice display an increase in allodynia in the spared nerve injury model compared to control mice (Holmes, Arnott et al. 2008). While CX3CR1 knockout mice in a partial nerve ligation model of neuropathic pain were reported to exhibit reduced allodynia (Staniland, Clark et al. 2010). Until the discrepancies between these studies are resolved it is not possible to draw any conclusions regarding the role of signaling from CX3CL1 to CX3CR1 in neuropathic pain.

BRAIN DERIVED NEUROTHROPHIC FACTOR (BDNF)

The situation with BDNF is interesting because BDNF is released from both stimulated nociceptive fiber terminals and also from spinal microglia cells following peripheral nerve injury. It has been claimed that BDNF released from microglia modulate pain processing in the dorsal horn by causing a depolarizing shift in the anion reversal potential (E_{anion}) in lamina I neurons, which results in disinhibition (Coull, Beggs et al. 2005). In this context it was shown that knockout of BDNF, in nociceptors expressing $Na_v1.8$, attenuated inflammatory secondary hyperalgesia but had no effect on mechanical allodynia following nerve ligation (Zhao, Seereeram et al. 2006). If BDNF in the spinal cord does play a role in neuropathic pain it does not come from nociceptors expressing $Na_v1.8$.

CYTOKINES

Cytokines are small intracellular polypeptides, which are typically classified on the basis of their biological activity as either proinflammatory or anti-inflammatory cytokines. They are effective at very small (pico molar) concentrations and are involved in various functions in immunology and inflammation. Findings in both humans and animals have implicated the pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) in neuropathic pain. For example: the intrathecal injection of either IL-1β or TNF-α results in both thermal and mechanical hypersensitivity. While the intrathecal administration of the IL-1 receptor antagonist (IL-1ra) is sufficient to transiently reverse nerve injury induced mechanical hypersensitivity. Furthermore, the intrathecal injection of TNF soluble receptors (sTNFR) before peripheral nerve injury is sufficient to prevent the development of neuropathic pain.

Several TNF-α inhibitors have been developed, including Enbrel (etanercept), Remicade (Infliximab), Humira (adalimumab) and Simponi (golimumab) that have been approved for the treatment of immune disorders including rheumatoid arthritis and psoriatic arthritis. There is as yet no convincing evidence that these agents, when administered systemically, are effective for the treatment of neuropathic pain.

The FDA has approved the IL-1β inhibitor canakinumab (Ilaris) for the treatment of systemic juvenile idiopathic arthritis and anakinra (Kineret) a recombinant form of the naturally occurring interleukin-1 receptor antagonist for the treatment of rheumatoid arthritis. As with the TNF-α inhibitors there is as yet no convincing evidence that these agents, when administered systemically, are effective for the treatment of neuropathic pain.

These IL-1β and TNF-α inhibitors would not be expected to pass the blood brain barrier.

The above findings support the *hypothesis* that increases in proinflammatory cytokine production by activated glia might be responsible for the hypersensitivity of neuropathic pain. However, the findings with the TNF-α and IL-1β inhibitors indicate that what need to be developed are IL-1β and TNF-α inhibitors that pass the blood brain barrier.

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Chapter 8 PAIN IN THE BRAIN

Ascending pain pathways

Before considering the higher mechanisms involved in the perception of pain we will briefly examine the ascending pathways that carry nociceptive information from the spinal cord to higher brain centers. As described in chapter 7 and illustrated in Figure 7-1 the anterolateral system, which is composed of a bundle of fibers, located in the ventrolateral aspect of the spinal cord, has been recognized as conveying nociceptive, thermal and innocuous touch information to higher brain centers. The cell bodies of the second order neurons that give rise to the anterolateral system are found primarily in lamina I, outer layers of lamina II and in laminas IV and V of the dorsal horn. These ascending second order projection neurons send axons and axon collaterals that decussate and terminate in the brainstem and thalamus. As described in chapter 7, studies of antidromically-identified lamina I projection neurons have revealed these cells to have the properties of WDR (wide dynamic range), NS (nociceptive specific) and HPC (noxious heat, pinch and noxious cold) cells. In addition to the lamina I projection neurons with nociceptive properties, there are also a group of lamina I neurons sensitive to innocuous thermal stimuli and a group of lamina I neurons sub serving the sense of itch. There is as yet no general consensus as to the relative contributions to pain sensation of NS, WDR and HPC cells.

Anterolateral cordotomy, surgical division of the pain-conducting tracts in the anterolateral system, is sometimes performed on patients experiencing severe pain due to incurable diseases for which all other pain treatments have proved ineffective. The procedure provides the selective loss of pain and temperature perception several segments below and contralateral to the segment at which the lesion is placed. However, in most cases, these surgical ablations have only relieved pain for a few months or a few years. Pain tends to recur in the form of *central pain* that is pain that is due to a lesion or dysfunction of the CNS. This limitation led to the development of electrical and chemical procedures at every level of the nociceptive pathway for the treatment of pain. These procedures include peripheral nerve stimulation, spinal cord stimulation, deep brain stimulation and motor cortex stimulation.

The spinothalamic tract of the anterior lateral system synapses in the contralateral thalamus. The axons and axon collaterals that terminate in the brainstem synapse in the medullary reticular formation (via the spinoreticular tract), the mesencephalic periaqueductal gray (via the spinomesencephalic tract) the parabrachial nucleus (via the spinoparabrachial tract) and the hypothalamus (via the spinohypothalamic tract). Third order nociceptive neurons have been reported within the reticular formation, periaqueductal gray, parabrachial nucleus and the hypothalamus. Portions of the ascending anterolateral system are illustrated in Figure 8-1. From the brainstem the nociceptive information eventually reaches the thalamus via multiple additional brainstem synapses.

thalamus

A11

periaqueductal gray

medial lemniscus

midbrain

dopamine

gracile nucleus

noradrenaline

dorsolateral pontine tegmentum

medulla

rostro-ventral medulla

anterolateral system

serotonin

dorsal column

dorsolateral funiculus
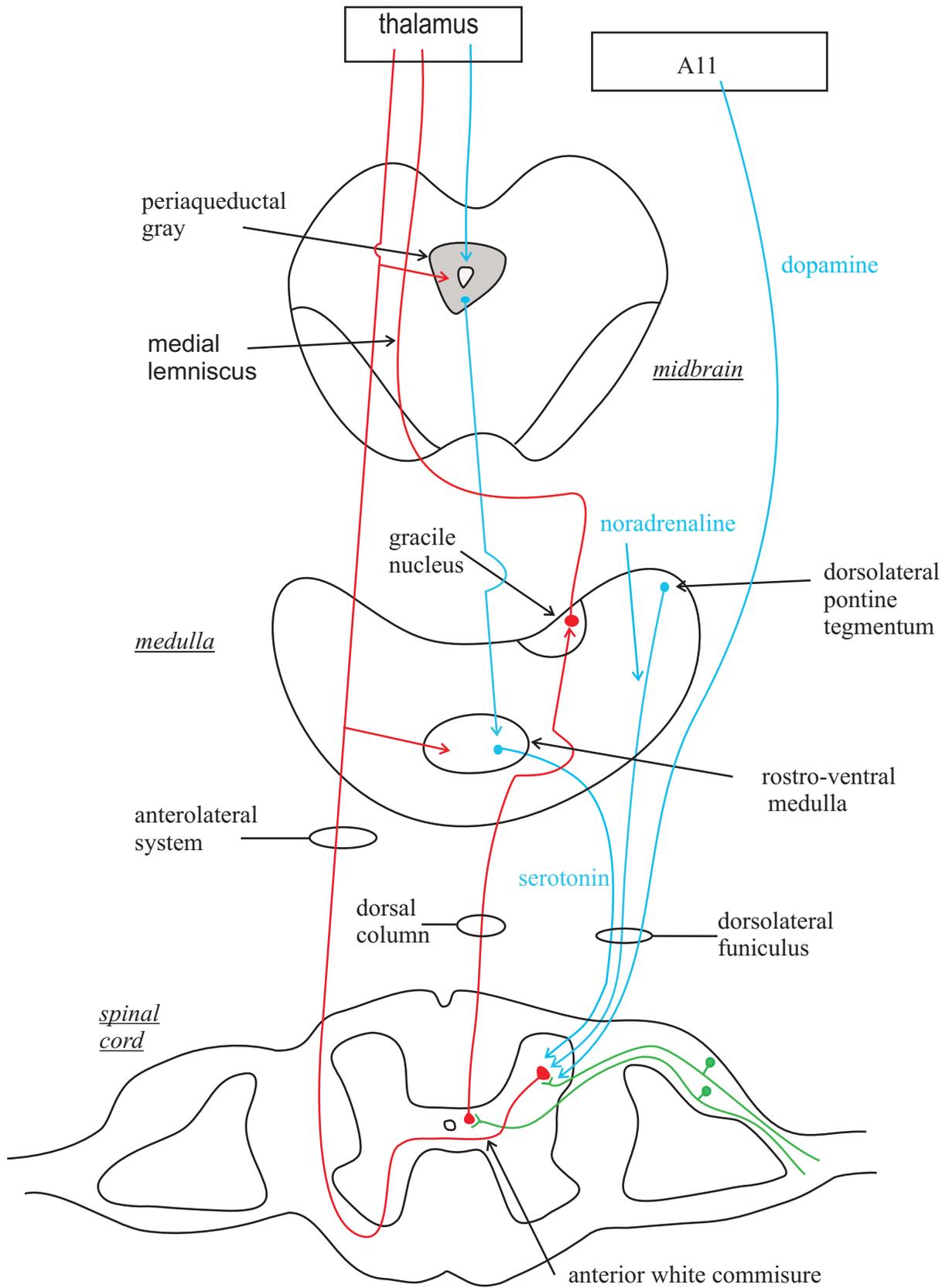
spinal cord

anterior white commisure

Figure 8-1. Ascending and descending pain parhways

Clinical and animal studies have shown that the dorsal column pathway is involved in relaying visceral nociceptive information (Palecek 2004). These studies have shown that lesions, that disrupt fibers of the dorsal columns (see Figure 8-1) that ascend close to the midline of the spinal cord, significantly relieve pain originating in visceral organs. The nerve fibers of these dorsal column neurons originate from the cell bodies of projection neurons many of which are located in the vicinity of the central canal and project to the gracile and cuneate dorsal column nuclei (as illustrated in Figure 8-1). Axons from these nuclei then decussate and travel up the brainstem as the medial lemniscus on the contralateral side.

Not shown in Figure 8-1 is the contribution of the trigeminal nerve – trigeminal ganglion to the ascending pain pathway. The trigeminal nerve projects laterally from the pons (not illustrated in Figure 8-1). The pons is bounded superiorly by the midbrain and inferiorly by the medulla. The trigeminal nerve, also called the fifth nerve, is responsible for sensation in the face, and projects to the trigeminal ganglion, which is analogous to the dorsal root ganglia of the spinal cord. The trigeminal nucleus is divided into three regions along its length; from rostral to caudal they are called the subnucleus oralis, the subnucleus interpolaris, and the most caudal region is called the subnucleus caudalis which is the site where the pain fibers synapse. The fibers from the subnucleus caudalis cross to the opposite side, and join the spinothalamic tract and medial lemniscus on their way to the thalamus.

Fast and Slow Components of the ascending pain pathway

Recall that with some painful stimuli (see Chapter 1) one can distinguish first a fast pain felt within about 0.1 second after the pain stimulus and having a sharp pricking sensation. This fast pain is then followed after a lull of about one second or more by a second slow pain having a dull burning feeling. First fast pain is mediated by A (δ-β) fibers sensitive to mechanical and thermal stimuli. The second slow dull pain is transmitted centrally by C-fibers. On entering the spinal cord the fast pain pathway and the slow pain pathway take two routes to the brain via the neospinothalamic tract and the paleospinothalamic tract respectively.

Fast pain travels via type A(δ-β) fibers which synapse primarily on dorsal horn neurons in lamina I of the spinal cord the axons of which form the neospinothalamic tract. The axons of the neospinothalamic tract after crossing the midline through the anterior white commissure, travel to the brain in the contralateral anterolateral system. These fibers of the neospinothalamic tract ascend through the medulla, pons and the midbrain and terminate primarily on the ventrobasal complex of the thalamus. The ventrobasal complex serves as a relay station that sends axons to the somatosensory cortex.

In contrast to the neospinothalamic tract, the paleospinothalamic tract runs to the medulla and other areas of the brain stem before reaching the thalamus. Slow pain is transmitted via C fibers, which synapse primarily in the substantia gelatinosa, that is, laminae II and III of the dorsal horn. The second order neurons of the substantia gelatinosa make direct and indirect synaptic connections in laminae V -VIII of the dorsal horn. The axons of

these third order neurons mostly join fibers from the fast pathway, crossing to the opposite side via the anterior white commissure, and traveling upwards along the anterolateral pathway, although some ascend ipsilaterally. These third order neurons terminate throughout the brain stem, with as few as one tenth of the fibers ending in the thalamus and the rest terminating in the medulla, pons and periaqueductal grey of the midbrain. Slow pain is poorly localized and is described as an aching, throbbing or burning pain.

Descending pain pathways

Under some circumstances we are able to block the perception of pain. For example, during battle soldiers have reported a lack of pain despite severe injuries. This suppression of the perception of pain is attributed to the descending pathway projecting to the dorsal horn (as illustrated in Figure 8-1).

Evidence for a descending *inhibitory* pain pathway comes from animal experiments in which electrical stimulation of the periaqueductal gray (PAG) reliably inhibits the tail flick reflex escape behavior produced by noxious thermal stimuli. As illustrated in Figure 8-1 the PAG provides a major input to the rostro ventral medulla (RVM), which contains cells that project to the dorsal horn via the dorsolateral funiculus (DLF). Moreover lesions to the DLF block the inhibitory effects of PAG stimulation on the escape behavior from painful stimuli. Additionally in a group of patients suffering intractable pain from cancer, stimulation of the PAG led to cessation of their pain. The PAG may therefore be a critical center for control of the perception of pain by descending activity from higher cortical regions

Descending monoaminergic pathway

Serotonin

As mentioned in Chapter 7, serotonergic inputs to the dorsal horn originate in neurons of the RVM (also see Figure 8-1) and in mice secondary hypersensitivity following intraplantar capsaicin injection was prevented by 5-HT$_7$ metabotropic receptor agonists. Consequently, and as mentioned in chapter 7, 5-HT$_7$ receptors make an excellent target for the treatment of pain.

As discussed in chapter 7 it became apparent that the descending pathway mediates both inhibition and facilitation of nociceptive information. For example, as described in chapter 4, the second phase of the formalin test was significantly reduced in 5-HT$_{3A}$ ionotropic channel knockout mice or in normal mice following intrathecal (inside the spinal canal) administration of a 5-HT$_3$-receptor antagonist. Additionally, lesions to the RVM block the second phase of the formalin test. These findings are consistent with a descending serotonergic pathway from the RVM to the dorsal horn contributing to central sensitization. In summary, the descending serotonergic pathway from the RVM to the spinal cord can mediate both inhibition and facilitation of nociceptive information via the

activation of 5-HT$_7$ and 5-HT$_3$ receptors, respectively. Therefore these receptors make excellent targets for the treatment of pain.

One can think of the synaptic circuitry of the dorsal horn as functioning as a gate for the transmission of nociceptive signals centrally. The gate can be closed thereby *inhibiting* the transmission of nociceptive signals and thereby the perception of pain. Conversely, the gate can be opened wide *enhancing* the transmission of nociceptive signals and the perception of pain. Additionally, the same transmitter acting on different receptors can either *inhibit* or *enhance* nociceptive signals.

Serotonin is not the only transmitter modulating the flow of nociceptive information in the dorsal horn. The modulation of nociceptive information by *the descending pathway is for the most part mediated by monoamines* as illustrated in Figure 8-1. The descending monoaminergic pathway includes serotonin, norepinephrine, and dopamine; acting via different receptor subtypes to either inhibit or facilitate transmission of nociceptive information at the level of the dorsal horn. The monoamines and their receptors represent a target for the pharmacologic management of pain.

Norepinephrine

The action of norepinephrine in modulating pain has been studied most extensively in the spinal cord and it is thought that the dorsal horn is the major site for its analgesic action. The source of the norepinephrine input to the dorsal horn of the spinal cord is descending axons originating in the noradrenergic nuclei of the brainstem. These noradrenergic nuclei are connected to multiple regions of the brain, including the PAG, thereby providing a pathway for descending pain modulation. Antinociception by activation of descending noradrenergic fibers has been attributed to the direct inhibition of nociceptive second order spinal neurons, presynaptic inhibition of primary afferent nociceptors (Kawasaki, Kumamoto et al. 2003) and the direct excitation of inhibitory spinal interneurons (Gassner, Ruscheweyh et al. 2009) .

Epinephrine produces analgesia when administered intrathecally, in addition, clonidine, a partial α_2 agonist has analgesic activity and what's more epidural clonidine has been shown to be effective and safe in the management of acute postoperative pain. The available evidence suggests that norepinephrine mediates antinociception by means of presynaptic inhibition of nociceptors via presynaptic metabotropic α_2-adrenoreceptors, and direct excitation of inhibitory spinal interneurons via metabotropic α_1 receptors.

Dopamine

Compared with the literature for serotonin and norepinephrine the spinal action of dopamine in modulating pain has received less attention. Dopamine acts through 5 distinct G protein-coupled receptors that positively and negatively regulate adenylate cyclase. The D2 metabotropic receptor is the major dopamine receptor subtype in the dorsal horn where it mediates the antinociceptive action of dopamine. Intrathecal

application of either dopamine or D2 receptor agonists increases thermal and mechanical nociceptive thresholds.

Antidepressants for analgesia

Before considering the use of antidepressants for analgesia it is worthwhile to consider the monoamine theory of depression that led to the development of a number of antidepressants that enhance the availability of monoamines.

Monoamine Theory of Depression

Monoamine oxidase inhibitors and tricyclic antidepressants, both of which increase brain levels of norepinephrine and serotonin, were reported to be beneficial in treating depression. Additionally it was reported that reserpine, a drug that depletes monoamine neurotransmitters, caused depression in about 15% of individuals, although this report has been considered controversial. These findings led to the monoamine theory of depression, which simply states that depression is due to a deficiency of brain monoaminergic activity and that depression is treated by drugs that increase the activity of monoamines. A major difficulty with the monoamine theory was that the increase in monoamine activity occurred almost immediately while the therapeutic effect of the antidepressant took weeks to develop.

According to the monoamine theory deficiency of norepinephrine, serotonin and dopamine are thought to be involved in mental depression. Different mechanisms may increase the availability of brain monoamines, including blocking the reuptake of the monoamine into the nerve terminal or inhibiting the metabolism of the monoamine inside the nerve terminal. Among the antidepressants there are the selective serotonin reuptake inhibitors (SSRIs), norepinephrine reuptake inhibitors (NRIs), serotonin norepinephrine reuptake inhibitors (SNRIs), noradrenaline dopamine reuptake inhibitors (NDRIs), and the tricyclic antidepressants (TCAs), which have also been shown to block the reuptake of serotonin and norepinephrine. Inhibitors of the metabolism of the monoamines in the nerve terminal by monoamine oxidase (MAO) inhibitors increase the amount of these amines available for release from the nerve terminal. Therefore these antidepressants are hypothesized to act by increasing the activity of monoamines at the synapse.

The fact that the descending pain pathway utilizes monoamine neurotransmitters suggests that antidepressants that increase the availability of monoamines might serve as analgesics. Some positive and negative findings for the use of antidepressants for the treatment of pain are given below.

Tricyclic antidepressants

TCAs have been used for the treatment of chronic pain for decades; their use and the use of other antidepressants depend on a determination of whether the benefits outweigh the

potential for adverse effects. The TCA amitriptyline is commonly used off label for the treatment of shingles pain.

Serotonin selective reuptake inhibitors (SSRIs)

It has been assumed that serotonin, norepinephrine and/or dopamine reuptake inhibitors may attenuate pain by preventing their presynaptic reuptake, leading to increased postsynaptic monoamine levels and thereby sustained activation of the descending pain inhibitory pathway. Several serotonin selective reuptake inhibitors (SSRIs) have been shown to have antinociceptive effects for acute pain in both rats and mice. The site of action of systemically administered SSRIs is equivocal, in that they may involve both supraspinal and spinal structures. On the other hand the antinociceptive effects of SSRIs in chronic pain models have been inconsistent. They have not been found to be clinically efficacious for the treatment of chronic pain syndromes.

Norepinephrine Reuptake Inhibitors (NRIs)

Neither of the NRIs reboxetine or atomoxetine has been approved for the treatment of chronic pain.

Serotonin Norepinephrine Reuptake Inhibitors (SNRIs)

The SNRIs Duloxetine, tradename Cymbalta and Desvenlafaxine tradename Pristiq, are approved by the FDA for the treatment of fibromyalgia pain.

These few positive results make one wonder whether these antidepressants are in fact working by enhancing the analgesic activity of the descending pain pathway.

Morphine

Before considering the possible role of morphine in activating the descending inhibitory pain pathway its' effects on nociceptors will be briefly reviewed. As mentioned in Chapter 5 peripherally administered morphine inhibited the activity of cutaneous nociceptors under conditions of inflammation, indicating that morphine is acting on opioid receptors located in the sensory transduction region of nociceptors. Furthermore the evidence suggests that morphine acts by activating K_{ATP}, GIRK and TREK-1 channels. In addition, opioid μ-receptors have also been localized to the synaptic terminal of DRG neurons in the spinal cord where they inhibit transmitter release from nociceptor terminals.

Turning to the role of morphine in the brain, it has been shown that direct injection of opioids into several brain regions in unanaesthetized animals initiates analgesia. These brain regions include the amygdala, substantia nigra, the periaqueductal gray (PAG) and the rostroventral medulla (RVM). The best-characterized region mediating opioid analgesia is the PAG. Injection of morphine into the PAG blocks behaviors elicited by painful stimuli and the effect of morphine is reversed by the opioid antagonist naloxone.

The exact mechanism, by which opioids act within the PAG to bring about analgesia, is unknown. However, it is possible that the mechanism of action of morphine in the PAG is similar to the mechanisms that were described for morphine in Chapter 5.

Cholecystokinin

Cholecystokinin (CCK) is an octapeptide that functions as a gastrointestinal hormone responsible for gallbladder contraction and pancreatic enzyme secretion. It is also found in the central nervous system (CNS) where it functions as a neurotransmitter and among its' CNS actions it functions as an antagonist to opiates. For example, in the rat administration of CCK to the PAG dose dependently antagonized the analgesic effect of systemically administered morphine (Li and Han 1989). Furthermore this study showed that microinjection of a CCK antagonist into the PAG enhanced morphine induced analgesia. This study and many others point to the potential for CCK antagonists to be combined with opioids for pain management.

Thalamus

The thalamus is located between the cerebral cortex and the subcortical areas of the central nervous system and has been described as the brain's sensory relay station. For our purposes its function in terms of location and connections is to relay ascending pain information to the cerebral cortex and descending pain information to the spinal cord.

The pain fibers of the neospinothalamic tract terminate primarily in the ventroposterolateral (VPL) nucleus and ventroposteroinferior (VPI) nucleus of the thalamus. While the fibers from the trigeminal nucleus, mediating fast pain, terminate primarily in the ventroposteromedial (VPM) nucleus of the thalamus. Collectively the fibers of the neospinothalamic tract bypass the brainstem and project directly from the spinal cord or the trigeminal nucleus to the thalamus. The thalamic nuclei in turn send their axons to the somatosensory cortex as illustrated in the oversimplified diagram of Figure 8-2. The primary somatosensory (SI) cortex receives nociceptive input from the both the VPL and VPM nuclei. The secondary somatosensory (SII) cortex receives input from VPL, VPM and VPI nuclei. Finally the VPM nucleus sends axons to the prefrontal cortex. Consequently, signals from the fast conducting pain system are relayed rapidly thereby producing the well-localized immediate sharp pricking sensation of first pain. It has been argued that the neospinothalamic tract is a phylogenetically new pathway found in primates and other mammals. **The human pain system consisting of the thalamic nuclei and the somatosensory cortices of Figure 8-2 is referred to as the lateral pain system**; we will consider this system in more detail below.

In contrast to the neospinothalamic tract many of the nerve fibers of the paleospinothalamic tract terminate in the brainstem. The nerve fibers that terminate in the brainstem synapse in the medullary reticular formation (via the spinoreticular tract), the mesencephalic periaqueductal gray (via the spinomesencephalic tract) the parabrachial nucleus (via the spinoparabrachial tract) and the hypothalamus (via the
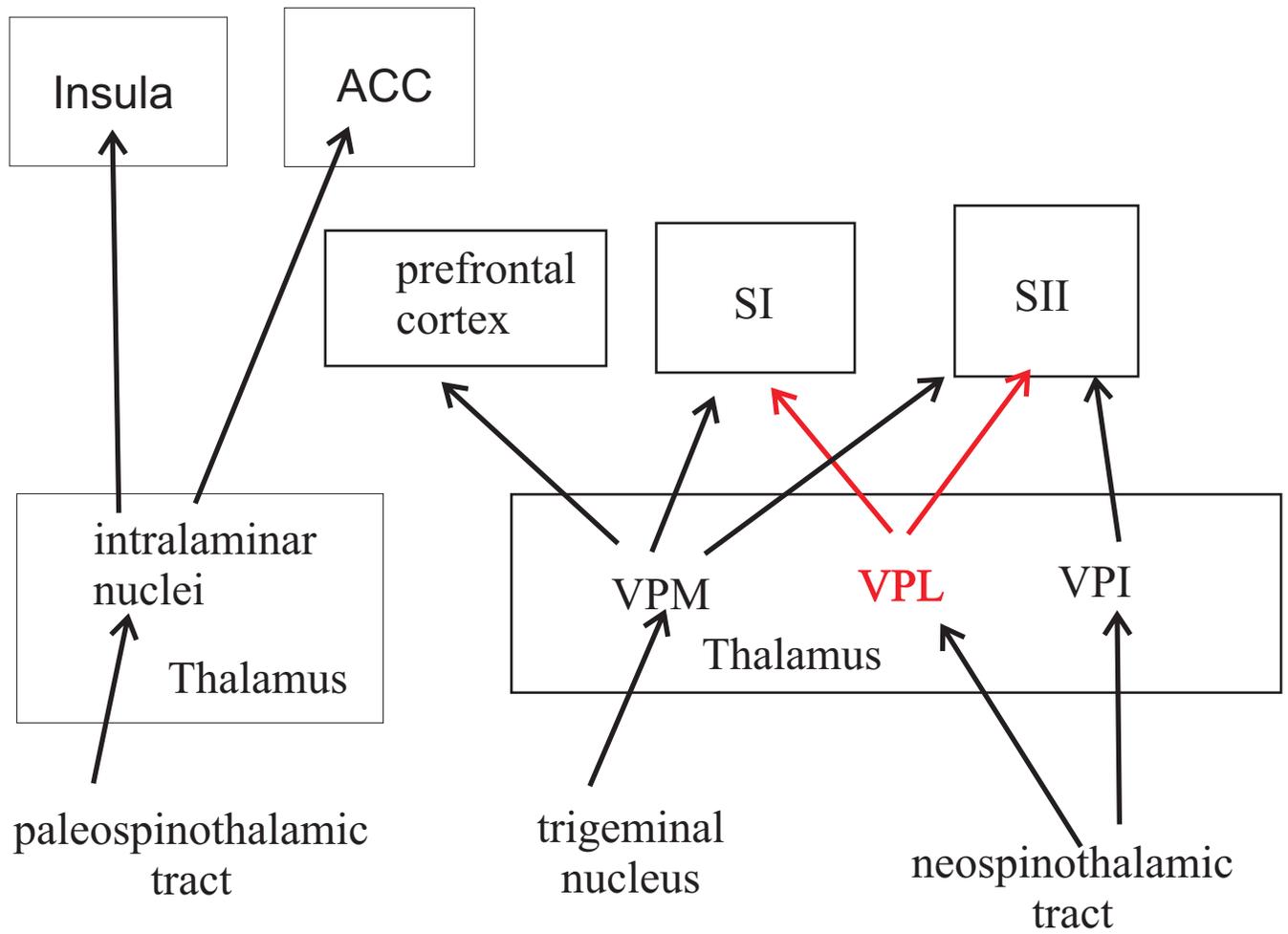
Figure 8-2. Medial and Lateral Pain Systems

spinohypothalamic tract). These sites of termination form complicated multineuronal systems that modify the signals emanating from the paleospinothalamic tracts. The brain stem nuclei, activated by the paleospinothalamic pathway, are also the origin of the descending pain pathway. This allows for the possible involvement of the paleospinothalamic tract in the regulation of the descending pain pathway. The intralaminar nuclei of the thalamus receive ascending projections from the paleospinothalamic system. The cortical efferents of the intralaminar nuclei subserving pain project to the anterior cingulate cortex, the insular cortex and other brain regions. **The human pain system that projects through medial thalamic nuclei to the anterior cingulate cortex the insular cortex and other brain regions is referred to as the medial pain system**; we will consider this system in more detail below.

Thalamic Pain Syndrome

Thalamic Pain Syndrome (Dejerine-Roussy Syndrome) is a rare neurological disorder that occurs as a later complication of a small stroke in the thalamus. The primary symptoms are chronic pain and loss of sensation on the side of the body opposite to the side of the brain that the stroke occurred in. In many cases the patient experiences allodynia, that is, the perception of innocuous sensations become painful on the involved side. The pain may decrease over time but is often permanent.

Central Pain Syndrome

Central pain is pain that is due to a lesion or dysfunction of the central nervous system (CNS). As mentioned earlier in this chapter anterolateral cordotomy, surgical division of the pain-conducting tracts in the anterolateral system was sometimes performed on patients experiencing severe pain due to incurable diseases for which all other pain treatments have proved ineffective. Although cordotomy is effective in the relief of pain, the effect is usually temporary and pain tends to recur after cordotomies in the form of *central pain.* Thalamic pain syndrome mentioned above is a form of *central pain* resulting from a lesion caused by stroke in the thalamus.

Neuropathic pain, that is pain caused by a lesion or dysfunction of the peripheral nervous system was discussed in chapter 6 and has much in common with *central pain*. Since we do not understand the pathogenesis of either type of pain they will be considered separately, although treatments for central pain are similar to those for neuropathic pain.

Cerebral Cortex and the Medial And Lateral Pain Systems

Attempts to locate a "center for pain" in the cerebral cortex have largely failed. For example, direct electrical stimulation of the cerebral cortex in areas activated by painful stimuli were found to rarely evoke a report of pain in patients that were awake. Furthermore analgesia is not a prominent symptom of cortical lesions in areas that are typically activated by painful stimuli. Rather than revealing a center for pain these

findings were originally interpreted to argue against the involvement of the cerebral cortex in the perception of pain.

The difficulty that scientists had in establishing the involvement of the cerebral cortex in the perception of pain derives from the nature of the painful experience. According to the 2011 definition of pain by the International Association for the Study of Pain (IASP); Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. Pain is therefore a sensory experience that is unpleasant. Current thinking is that the processing of nociceptive signals in the brain takes place simultaneously within two anatomically distinct systems: the medial and lateral pain systems that are associated with different aspects of the sensation of pain. ***The medial system is involved mainly in the processing of the emotional unpleasant aspects of pain and the lateral system is involved mainly in the processing of the sensory discriminative aspects of pain.*** As described above these two systems diverge at the level of the thalamus.

Medial Pain System

Asymbolia is the loss of the power to understand previously familiar symbols and signs usually as the consequence of a brain lesion. Patients with asymbolia for pain (Berthier, Starkstein et al. 1988) recognize a stimulus as being painful but lack appropriate motor and emotional responses to the stimulus. These patients feel pain but it doesn't represent something harmful or dangerous, for example painful stimulation to the arm doesn't elicit withdrawal of the arm or grimacing. In these patients there is dissociation between the sensory discriminative aspect of pain and its emotional unpleasant aspect. The extent of the area of the cortex damaged by the lesion in these patients differed, however, the insular cortex was damaged in all the patients, suggesting that damage to the insular cortex likely plays a role in the diminished emotional response to pain.

More than fifty years ago it was shown that most patients undergoing frontal cingulumotomy for intractable pain continued to have pain but it was significantly less emotionally unpleasant than before the procedure (Foltz and White 1962). This finding directly implicates the cingulum in the emotional response to pain. Taken together with the observations of patients with pain asymbolia described above these findings in humans directly implicate the insular cortex and the cingulate cortex of the medial pain system in the emotional unpleasant aspect of pain.

Hypnotic Suggestion

The concept of a medial and a lateral pain system presupposes that the sensory and emotional aspects of pain are analyzed in separate regions of the brain. This raises the possibility that it might be feasible to selectively modulate the sensory and emotional components of pain. Hypnotic suggestion has been used successfully to increase or decrease the unpleasantness of pain (Rainville, Carrier et al. 1999). Suggestions directed towards increasing or decreasing pain unpleasantness achieved a significant modulation

of the ratings for pain unpleasantness largely independent of variations in the ratings for pain intensity.

Lateral Pain System

As described above the main cortical constituents of the lateral pain system are the primary (SI) and secondary (SII) somatosensory cortices. A patient with a right sided selective ischemic lesion to the SI and SII cortices has been studied for pain perception (Ploner, Freund et al. 1999). Cutaneous laser stimulation was used to test for pain thereby avoiding the activation of tactile sensations. For the left hand no pain sensation could be elicited for stimulus intensities up to three times greater than the threshold for painful stimuli delivered to the patients' right hand. However, for stimuli delivered to the left hand, at intensities greater than twice the threshold for the right hand, the patient described an unpleasant ill localized feeling emanating from an area "somewhere between the finger tips and the shoulder", that he wished to avoid. The findings in this patient strongly support the suggestion that the lateral system is involved mainly in the processing of the sensory discriminative aspects of pain.

To reiterate, ***the medial system is involved mainly in the processing of the emotional unpleasant aspects of pain and the lateral system is involved mainly in the processing of the sensory discriminative aspects of pain.*** Under normal circumstances we experience pain as an unpleasant sensory and emotional experience associated with tissue damage. However, as the result of the specific lesions described above it is possible to ***disconnect*** the sensory and the emotionally unpleasant components of the painful experience. Moreover, through hypnotic suggestion it is possible to increase or decrease the unpleasantness of pain.

Functional Brain Imaging

Functional magnetic resonance imaging (FMRI) studies of different cortical regions have provided significant support for the existence of the medial and lateral pain systems. The blood oxygenation level-dependent (BOLD) signal, as measured by functional magnetic resonance imaging (FMRI) provides a noninvasive method for measuring brain responses in humans. The magnetic resonance (MR) signal of blood is slightly different depending on the level of oxygenation. Higher BOLD signal intensities arise from increases in the concentration of oxygenated hemoglobin. Five major cortical areas respond consistently to acute pain stimuli: anterior cingulate cortex, insular cortex, primary somatosensory cortex, secondary somatosensory cortex and prefrontal cortex. These are the areas that were identified above as belonging to the medial and lateral pain systems.

Measuring Pain

Assessment of pain is important for identifying the cause of the pain and establishing a plan to manage it. Unfortunately there is no standard objective scale in use for the measurement of a patient's pain. Currently, pain intensity is usually assessed when a patient self reports pain intensity typically on a subjective scale of one to 10. Objective

measures of pain could confirm those subjective reports and provide clues about how the brain registers different types of pain. In 2013 an advance was made in using FMRI activity to objectively measure pain intensity. A pattern of activity was found in regions of the CNS, which were known to show increased activity in association with experimentally induced pain. The regions included the ventrolateral thalamus, the secondary (SII) somatosensory cortex, the insula and the anterior cingulate cortex and other regions. The signature response measured from the included regions showed increased activity for thermally induced cutaneous pain (Wager, Atlas et al. 2013).

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