Estradiol, Substance P, and the PI3K-Akt-mTOR Pathway in the Dorsal Horn of the Spinal Cord During Inflammatory Pain

Zane Ferguson
The Graduate Center, City University of New York

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Estradiol, Substance P, and the PI3K-Akt-mTOR Pathway in the Dorsal Horn of the Spinal Cord during Inflammatory Pain

Zane Ferguson

Graduate Center, City University of New York

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2017
Estradiol, Substance P, and the PI3K-Akt-mTOR Pathway in the Dorsal Horn of the Spinal Cord during Inflammatory Pain

by

Zane Ferguson

This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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THE CITY UNIVERSITY OF NEW YORK
Abstract

Estradiol, Substance P, and the PI3K-Akt-mTOR Pathway in the Dorsal Horn of the Spinal Cord during Inflammatory Pain

by

Zane Ferguson

Advisor: Vanya Quinones-Jenab

Pain is a critical survival mechanism that signals potential or actual damage, but it can become pathological when it persists beyond the injury. Chronic pain is a major health issue that affects 10-20% of the adult population and is found disproportionately in women. There are numerous, interacting mechanisms underlying this phenomenon. This study used female rats to investigate the impact of estrogens on sensory signaling by substance P, a neuropeptide that contributes to the development of chronic pain, and the PI3K-Akt-mTOR pathway, an intracellular mechanism of nervous system plasticity.
This work is dedicated to Sabrina Cox, whose love and support made it possible.

Thank you to Vanya Quinones-Jenab, an advisor of superhuman patience.

Thank you also to Judith Kubran, who saved me during the defense process.
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Estradiol, Substance P, and the PI3K-Akt-mTOR Pathway in the Dorsal Horn of the Spinal Cord during Inflammatory Pain

Pain is a critical survival mechanism that signals potential or actual damage. A properly functioning system for pain perception helps avoid unrecognized infection, injury, and self-mutilation that would reduce one’s lifespan (Dubin & Patapoutian, 2010). However, under certain conditions the system can produce pathological chronic pain that persists beyond the original injury and becomes maladaptive. Chronic pain can be conceived of as a disease in its own right (International Association for the Study of Pain (IASP) Declaration, 2001). Chronic pain is a major health issue that affects 10-20% of the adult population and costs $70 billion annually (Gatchel et al., 2007). It is a particular problem for women, who exhibit greater pain sensitivity and suffer from chronic pain conditions at higher rates than men (Amandusson & Blomqvist, 2013).

The pain system is exceedingly complex. Its features include plasticity in the primary afferents, dorsal horn, and brain; pain inhibition systems; descending modulation from the brain to the dorsal horn; changes in extracellular signaling molecules and inflammatory mediators; changes in membrane receptors and channels; interactions among various intracellular pathways; structural and functional shifts during the development of chronic pain; interactions among neuronal, glial, and immune systems; sex differences; and influence from stress, mental state, and social factors (Mendell, 2010).

The nociceptive system detects external threats, such as extreme temperature, intense pressure, or chemical irritation. Receptors in the skin react to these stimuli and send a signal to the brain via primary afferent terminals and the spinal cord. The primary afferents enter the
spinal cord and synapse onto central neurons in the dorsal horn, a major center of nociceptive processing and plasticity. The neurons involved in nociception are largely concentrated in the superficial laminae. Strong, sustained nociceptive signaling can modify the structure and function of the dorsal horn such that noxious stimuli elicit stronger responses (hyperalgesia) and non-noxious stimuli activate nociceptive pathways (allodynia), although these terms will be revisited later. When these changes persist beyond the disappearance of an injury, it constitutes chronic pain. Structural and functional changes in the central nervous system (central sensitization) are particularly important in this process (Kuner, 2010).

Experimental paradigms often use inflammatory agents to induce sustained nociceptive signaling. Carrageenan is one common agent used to induce inflammatory pain. Intraplantar injection of carrageenan leads to C-fiber, A-fiber, and central sensitization, as well as increased sensitivity to thermal and mechanical stimuli (Lisi et al., 2015).

Glutamate is the primary signaling molecule in this system, but there are many others involved. Substance P is a tachykinin neuropeptide that acts as both an inflammatory mediator in the periphery and a signaling molecule at the dorsal horn. It contributes to the development of hyperalgesia and central sensitization. When noxious stimuli activate primary afferents, they release substance P at their central terminal. Substance P acts on NK-1 receptors in the superficial laminae and activates several intracellular mechanisms, the most important being increases in intracellular calcium levels. Calcium is critical to central sensitization. It contributes to synaptic plasticity through multiple intracellular signaling pathways to modify receptors and ion channels (Youn, Gerber, & Sather, 2013).

The phosphatidylinositol 3-kinase (PI3K)–Akt–mammalian target of rapamycin (mTOR) signaling pathway is important in chronic pain. It regulates protein synthesis and synaptic
plasticity, which contribute to central sensitization during inflammation-induced hyperalgesia (Lisi et al., 2015).

The PI3K pathway is active in superficial dorsal horn projection neurons that express the neurokinin 1 (NK-1) receptor. These neurons are activated in overlapping areas on the same timecourse. Substance P-induced hyperalgesia is also blocked by PI3K pathway inhibition. These results suggest that inflammation-induced hyperalgesia is mediated by substance P and dependent on the PI3K pathway to some degree (Xu et al., 2011).

Important sex differences have been observed in nociception, and these are attributed in part to gonadal steroid hormones. Estradiol is one of the most investigated of these hormones and is thought to modulate both the perception of pain and the development of chronic pain states. However, the literature on its relationship to pain is complex and contains many contradictory results (Amandusson & Blomqvist, 2013).

Estradiol interacts with the PI3K pathway, suggesting that it influences the synaptic plasticity that underlies sensitization. Estradiol has been found to activate the PI3K pathway in the context of pain and inflammation (Ghisletti et al., 2005; Peng et al., 2010). Estradiol also interacts with substance P. It reduces substance P protein levels and mRNA expression in DRG neurons in the context of an anti-nociceptive estradiol effect, which may reduce the release of substance P at the dorsal horn (Sarajari & Oblinger, 2010). Together, these results suggest that estradiol’s impact on nociception and sensitization may be mediated by substance P and the PI3K pathway to some degree. This study investigated that possibility.

**History.** Pain is a fundamental part of the human experience. Prehistoric notions of pain are lost, but examination of primitive societies gives some clues about those beliefs. Pain was conceptualized in magical or religious terms and often imagined as an intrusion of an object or
spirit into the body. It was first described over 2,000 years ago in both Western and Eastern civilization. In traditional Chinese medicine, pain was explained as an imbalance of yin and yang. The medical treatise *Huang di Nei Jing (The Medical Classic of the Yellow Emperor)* was supposedly written around 2600 BCE, but is dated to around 300 BCE. However, as a collection of preexisting writings and oral history, it is the earliest known description of nociception and inflammatory pain. In classical Greece, pain was first mentioned in Homer’s epics (8th century BCE), followed by the medical and philosophical studies of Hippocrates (5th century BCE), Aristotle (4th century BCE), and Galen (2nd century CE). In the 11th century, the Persian philosopher Avicenna wrote his *Canon of Medicine*, which served as a foundational medical text for several centuries. In it, he proposed that pain may be independent of touch or temperature sense, which would be supported by research eight centuries later. Several important differences existed among these perspectives, including the nature of pain as an emotional or sensory phenomenon, the location of pain perception in the heart or the brain, the independence of pain from touch and temperature sense, and the causes of pain as divine retribution, infiltration of external agents, or mechanistic operation of the body. Some of these questions are still being investigated (Dallenbach, 1939; Perl, 2011).

Questions about the nature of pain and sensory phenomena were often handled differently by philosophers as opposed to physicians and scientists. Aristotle described pain as an emotional phenomenon and spoke of it in terms of pleasantness-unpleasantness. This view was dominant for nearly 2,000 years, with minor modifications (Figure 1). The Aristotelian model was overturned during the scientific revolution, when new conceptions of the natural world were emerging. During the 17th century, René Descartes formalized the notion of pain as an internal, mechanistic process in which a channel conducted the pain signal from the periphery to the
ventricles of the brain (Figure 2). Thomas Willis studied the anatomy of brain and argued that it is the seat of pain perception. The philosophers Isaac Newton and later David Hartley proposed that nerves carry information, though they hypothesized that they did so through vibration. These developments laid rudimentary foundations of the modern theory of pain (Dallenbach, 1939; Perl, 2011).
During the 19th century, the theory of pain underwent fundamental changes in response to discoveries of the structure and function of the nervous system. Charles Bell (1811) and Francois Magendie (1822) separated the spinal cord into the motor ventral horn and the sensory dorsal horn (the so-called Bell-Magendie law). Bell (1811) and Johannes Müller (1840) discovered modality-specific nerves. Emil du Bois-Reymond (1848-1849) founded the field of electrophysiology, showing that sensory signaling is an electrochemical phenomenon. Modality-specificity in a system that operates with a common impulse mechanism suggested that the peripheral and central nerve endings must somehow encode the stimulus characteristics in order to produce specific sensations in the brain. Charles-Édouard Brown-Séquard (1860) proposed a
model of neural inhibition and, with later contributions by William Gowers, discovered contralateral signaling through partial denervation and lesion studies in the spine (Dallenbach, 1939; Grant, 2006; Perl, 2011).

The separation or unification of pain and other touch sensation was debated as early as Aristotle in classical Greece, continued through the Latin and Islamic medieval eras, and was more clearly understood beginning in the late 18th century. Two major theories about the organization of the nervous system and its relationship to pain sensation have traded dominance since the 1800s. The sensory or specificity theory posited that pain is separate from touch, and they are activated independently. The intensive theory posited that pain and touch were unified, such that strong stimulation causes pain, regardless of sensory modality. The specificity and intensive theorists were more empirically and experimentally inclined, whereas the pleasure-pain theorists were more philosophical (Dallenbach, 1939).

The specificity theory describes pain as a distinct sensation, separate from other touch sensations. Erasmus Darwin (1794) made an important observation of modality-specificity. A man suffering from fever and violent cramps of his legs became insensitive to mechanical stimulation of his feet, including pinpricks and pinching. However, his sensitivity to intense heat remained intact. Hermann Lotze (1852) and Moritz Schiff (1858) argued for specificity theory based on studies of the central nervous system. They demonstrated that pain and tactile sensations are separate through lesion studies of the spine. Schiff found that destruction of gray matter eliminated pain, but touch was left intact. Destruction of white matter eliminated touch, but left pain intact. Magnus Blix (1882) and Adolf Goldscheider (1884) argued for specificity theory based on studies of the peripheral nervous system. They found discontinuous sensation areas in the skin where separate spots in the skin produced sensations of different modalities,
regardless of how they were stimulated. Max von Frey (1894, 1895, 1896) extended this work by relating these different sensory areas to neural structures, such that their response characteristics could be paired with structurally-distinct nerve endings. These discoveries confirmed Avicenna’s 11th century proposal of a separate pain sensation (Dallenbach, 1939).

The intensive theory describes pain as a central interpretation of signals of varying strength, independent from modality. William Erb (1874) argued for the intensity theory of pain based on the observation that stimuli of various modalities can elicit pain if applied at a sufficient intensity. After their previous support for specificity theory, Blix and Goldscheider changed positions and concluded that the pain does not comprise a separate sense. Instead, it is part of the touch system, which involved only warm, cold, and pressure sensors. Goldscheider actually started in the intensive camp, switched to specificity for several years, then switched back to intensive theory (Blix, 1884; Goldscheider, 1891, 1894). He was influenced by the finding that rapid, repeated innocuous stimulation could bring about pain by summation (Naunyn, 1889). However, later electrophysiological research by Adrian et al. (1931) contradicted this finding. They selectively activated mechanical fibers by repeated puffs of air. This stimulation did not cause pain, despite eliciting maximal action potential frequency in the mechanical fibers (Dallenbach, 1939; Perl, 2011).

In the 20th century, anatomical and physiological studies of the nervous system led to the modern model of nociception. During the 1890-1900s, Camillo Golgi and Ramón y Cajal debated whether the nervous system was composed of discrete cells (Cajal) or a continuous syncytium (Golgi). Ultimately, a system of discrete cells and theorized synapses connecting these cells was established. Michael Foster and Charles Sherrington (1897) made a major contribution with their model of neural inhibition and reciprocal innervation. They showed that
neural inhibition is an active mechanism rather than simply an absence of activity. These insights anticipated complex processing of sensory stimuli as a core structural and functional feature of the nervous system. Sherrington (1906) also introduced the concept of the nociceptor, a specialized sensory receptor dedicated to the detection of potentially harmful stimuli. The fact that pain is evoked by multiple stimulus modalities (thermal, mechanical, and chemical) had previously been seen as evidence against pain as a distinct sensation. Sherrington unified pain with the concept of noxious (tissue-damaging) stimuli and the signaling of those stimuli (nociception) (Grant, 2006; Perl, 2011).

In the 1920s, newly developed techniques and equipment allowed for a more detailed understanding of the nervous system than ever before. The action potential was established as the basic unit of nerve activation, and summation of action potentials was observed. Sensory fibers were separated into different classes (A and C) based on their conduction velocity. A number of researchers established the relationships between specialized subsets of nerve fibers and the reactions they trigger, including the slow fiber system. These fibers are activated by noxious stimuli, and their activation lasts beyond the original injury, which are reliable indicators of nociceptive processing. These early 20th century findings were integrated into a model of sensory signaling as a system of neurons with plastic functional connections (Adrian, 1931; Erlanger & Gasser, 1930).

Melzack and Wall (1965) argued for a convergence theory of pain, where nociceptive signals are processed in a gate control system in the spinal cord. Their theory was a major milestone in the field (Figure 3). It incorporated previous work on pattern theory, wherein interacting signals are interpreted (Noordenbos, 1959). It describes pain as an integration of diverse somatosensory signals to produce a single signal of varying intensity. This signal is then
sent to and interpreted by the brain. Separate, non-nociception-specific signals with different tonic activity and response characteristics converge on wide dynamic range (WDR) neurons in a processing circuit (the gate control system) in the superficial laminae of the dorsal horn. This system processes these signals to produce some degree of excitation in an action system that sends a signal to the brain, where it is interpreted as painful or innocuous. This theory was powerful in that it accommodated observations of spinal and descending modulation of pain sensations, hyperalgesia and allodynia, as well as interactions between pain and competing sensations, physiological and psychological states, and other phenomena (Craig, 2003; Perl, 2011). By the 1970s Perl, Iggo, and others had successfully recorded from a population of small-diameter fibers with the characteristics of nociceptive neurons. There are nociceptors sensitive to heat, cold, mechanosensation, and chemical stimuli, including polymodal nociceptors that detect multiple types (Bessou & Perl, 1969; Burgess & Perl, 1967; Iggo, 1959).
Figure 3. The gate control theory, in which separate, signals from non-nociception-specific fibers (L and S) interact and converge on WDR neurons (T) to produce an ascending signal. This signal is then interpreted by the brain as painful or innocuous. The model also accommodates descending modulation. Adapted from “Constructing and Deconstructing the Gate Theory of Pain,” by L. M. Mendell, 2014, Pain, 155, p. 213.

The modern consensus describes a widely-distributed network of brain structures that forms a neuromatrix. Pain is conceived of as a product of the activity in this system, with specific brain areas associated with particular aspects of pain (as well as other, non-nociceptive functions). In this system, the discriminatory, affective, cognitive, and motor aspects of nociception undergo extensive processing, integration, and modulation. Plasticity is a key feature of this system. It accounts for many observations, including chronic pain (Melzack, 1990). Technological advances allow real-time observation of both temporal and spatial aspects of nociception, allowing the subjective experience of pain to be correlated with nervous system activity. Today’s methodologies allow investigation at every level of the phenomenon, from genetic and molecular mechanisms to psychological and social factors (Kucyi & Davis, 2016; Prescott & Ratté, 2012).

**Pain and nociception.** Pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (IASP Task Force on Taxonomy, 2008). The term “pain” is often used when describing basic research on animals, but “nociception” may be more appropriate. Nociception is the processing of noxious sensory stimulation. Arguably, the ability to report the subjective experience of pain is limited to human subjects, and therefore we should limit the discussion to nociception in animal research (Barrot, 2012).

Pain has two components: a sensory-discriminative element that communicates the nature, location, intensity, and duration of pain; and a cognitive-affective-motivational element
that underlies the aversive quality of pain. These systems can be structurally and functionally
differentiated in the brain (Basbaum et al., 2009). Pain can also be described in different
functional terms. Acute pain involves the signaling of extreme temperature, intense pressure, or
chemical irritation, and is highly adaptive. Noxious (damaging or potentially damaging) stimuli
are detected in the periphery and a signal is transmitted to the central nervous system, allowing
an animal to escape and/or avoid potential injury. Activity-dependent sensitization describes
temporary hypersensitivity of the nociceptive system in response to injury that also serves as an
adaptive mechanism. Chronic sensitization describes hypersensitivity that persists beyond an
injury and becomes pathological (Dubin & Patapoutian, 2010; Kuner, 2010).

The term "nociceptor" is used variously in the literature to refer to the pain-sensitive
receptor found in the peripheral nerve terminal, the peripheral neuron that carries the nociceptive
signal, or any neuron in the nociceptive pathway. The IASP has established that the term
"nociceptor" should refer specifically to the receptors for noxious stimuli found in the peripheral
neuron terminal (IASP Task Force on Taxonomy, 2011). However, this usage remains
uncommon in the literature. In order to be consistent with the most common usage, “nociceptor”
will refer to a nociceptive primary afferent neuron.

The nociceptive system carries information about environmental threats to the cerebral
cortex via a chain of connections. Activation of nociceptive neurons in peripheral tissue induces
somatic pain (as opposed to visceral or neuropathic pain). This signal travels through a three-
neuron chain to the cerebral cortex and is modified at various levels of the nervous system
(Figure 4). The first-order neurons have their cell bodies in the dorsal root ganglion (DRG) and
conduct the pain signal from the peripheral terminal to the dorsal horn of the spinal cord. The
second-order neurons project from the dorsal horn to the brain, primarily the thalamus and brain
stem, with collaterals connecting to other areas. The third-order neurons transmit the signal from
the thalamus and brainstem to the limbic system and cortex. Descending pain-control pathways
also exist. Modulation can occur at all levels of this system (Basbaum et al., 2009; Kuner, 2010).
It is difficult to describe the nociceptive system because understanding a given component
involves reference to others. The following description will attempt to move from peripheral to
central with minimal forward leaps and backtracking.
Figure 4. Nociceptive signals originating in the peripheral terminal of nociceptors travel to the dorsal horn of the spinal cord, ascend along tracts to multiple targets in the brain, and terminate in various areas higher in the brain. The spinothalamic tract targets the somatosensory cortex via the thalamus and is responsible for the sensory-discriminative aspect of pain. The spinoparabrachial tract targets the cingulate and insular cortices via the brainstem and amygdala and is responsible for the cognitive-affective-motivational aspect of pain. The periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) are involved in descending modulation and regulate spinal input. Adapted from “Cellular and Molecular Mechanisms of Pain,” by A. I. Basbaum, D. M. Bautista, G. Scherrer, and D. Julius, 2009, Cell, 139, p. 269.

Primary afferent nociceptors are pseudounipolar neurons that carry nociceptive signals from the periphery to the spinal cord. They have free, unmyelinated nerve endings and are generally categorized by their modality sensitivities, including polymodal (Figure 5). However, the relationship between nociceptors and nociceptive processing is not straightforward. Selective deletion of certain populations of nociceptors does not simply alter pain according to the nociceptors’ understood function (Mendell, 2010). When they detect noxious stimuli, a signal is sent from the peripheral terminals of the primary afferent to the central terminals. Here they enter the dorsal horn and form a small tract, referred to as Lissauer’s tract. They ascend or descend up to two spinal segments and terminate in the dorsal horn (Figure 6). Activity in nociceptive primary afferents triggers the release of glutamate, substance P, CGRP, and BDNF at dorsal horn synapses where they operate on their respective receptors (Figure 7) (Morton, Sandhu, & Jones, 2016).
Figure 5. The peripheral terminals of nociceptive primary afferent neurons express various receptors for noxious stimuli. The neurons are thus sensitive to one or more stimulus modalities. Adapted from “Cellular and Molecular Mechanisms of Pain,” by A. I. Basbaum et al., 2009, Cell, 139, p. 270.
Figure 6. Laminar organization of the dorsal horn of the spinal cord. The nociceptive neurons (C and Aδ) terminate mainly in the superficial laminae, while non-nociceptive neurons (Aβ) terminate in the deeper laminae. Adapted from “Cellular and Molecular Mechanisms of Pain,” by A. I. Basbaum et al., 2009, Cell, 139, p. 268.
Figure 7. Multiple transmitters are released at the dorsal horn synapse in response to stimulation of the nociceptive primary afferent neurons, including glutamate, substance P, CGRP, and BDNF. During peripheral sensitization, transmitter release is increased. Activation leads to several important changes in the post-synaptic neuron, including both immediate posttranslational and gene transcription effects. The activation of NMDARs is especially important, where prolonged depolarization leads to the removal of the Mg$^{2+}$ plug. Adapted from “Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity,” by A. Latremoliere and C. J. Woolf, 2009, *The Journal of Pain, 10*, p. 901.

Aδ-fiber nociceptors are small-bodied and thinly-myelinated, and signal both “first pain” (rapid, highly-localized, with a sharp, pricking, or aching quality) and “second pain” (slow, poorly-localized, with a burning sensation). They can be categorized as type I and type II according to their modality sensitivity and response characteristics. Only type I A fibers are found in the glabrous skin (hand or paw). These fibers conduct signals rapidly (25-55 m/s) and most are mechanically and thermally sensitive, with high mechanical and thermal thresholds. Their response to mechanical stimuli is low-latency and slowly-adapting. Their response to heat stimuli is high-latency and gradually-increasing. These patterns are consistent with first pain and second pain, respectively. The lack of type II A fibers in the glabrous skin is consistent with the lack of rapid first pain for thermal stimuli (Dubin & Patapoutian, 2010).

C-fiber nociceptors are small-bodied and unmyelinated neurons that make up approximately 70% of the primary afferent neurons (Saeed & Ribeiro-da-Silva, 2012). They are slowly-conducting (less than 2 m/s) and signal second pain. Polymodal C fibers are the most common. They are sensitive to mechanical, heat, and in most cases, chemical stimuli. Other combinations of modality sensitivity exist, as well as “silent” C-fiber nociceptors, which are insensitive to mechanical and heat stimuli unless they have been sensitized by inflammatory mediators. C fibers maintain a slow baseline firing rate (less than 1 Hz) that is not consciously experienced, which may be related to homeostatic signaling. Noxious stimulation increases this
firing rate to 1-10 Hz and leads to summation, resulting in pain (Dubin & Patapoutian, 2010). Thermosensitive C fibers have a temperature threshold of about 39-41 °C, and can be separated into two categories based on their response profile to noxious heat. Quick C fibers exhibit high-frequency discharge with a low latency and adapt within 1 second, whereas slow C fibers have a longer latency and relatively uniform discharge throughout the stimulus (Johanek et al., 2008).

A number of heat-sensitive receptors have been identified on the primary afferent neurons (Figure 8). However, these only partially account for heat sensitivity, and there appears to be significant redundancy among these receptors. The transient receptor potential vanilloid 1 (TRPV1) receptor is the most important receptor for noxious heat (> 43 °C) and its activity increases as a function of stimulus intensity. It is a calcium-permeable, non-selective cation channel that was first cloned in 1997 (Caterina et al., 1997). It is a polymodal receptor sensitive to thermal stimuli, acidity, and chemical stimuli. Capsaicin, the active ingredient in chili peppers, is used to study it. TRPV1 is found in 50% of primary afferents overall and 75% of small-medium diameter primary afferents (Dubin & Patapoutian, 2010). Most TRPV1-positive neurons are C fibers, though some are A fibers (Cordero-Erausquin, 2016).
Upon activation, Ca\(^{2+}\), Na\(^{+}\), and Cl\(^{-}\) ions flow into the cell, leading to a net depolarization and activation of voltage-gated N\(^{+}\) channels, while closing the K\(^{+}\) channels that would counteract it. This leads to an action potential and excitation of lamina I projection neurons via monosynaptic connections and increased activity in lamina II neurons. TRPV1 receptors adapt during prolonged heat stimulation (Cordero-Erausquin et al., 2016).

Inflammation produces several changes in TRPV1 channels, including increased activity, decreased desensitization, and increased translocation to the membrane (Dubin & Patapoutian, 2010). Multiple second messenger pathways act on TRPV1 channels, including protein kinase A (PKA), protein kinase C (PKC), PI3K, Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM-KII), and PI3K-AKT-MTOR pathway.
kinase II (CaMKII), and the mitogen-activated protein kinase (MAPK) p38, increasing their contribution to nociceptive signaling (Gold & Gebhart, 2010).

TRPV1’s contribution to heat sensitivity is complex and some results are inconsistent with the typical description of the role of these receptors. Mouse studies employing TRPV1 blockade and gene deletion suggest that heat sensitivity is not mediated, or only partially mediated, by TRPV1 receptors on heat-sensitive C fibers under normal conditions. There may be limitations in methodology and generalizability, but human studies appear to be consistent with these results (Dubin & Patapoutian, 2010).

Heat sensitivity may also mediated by TRPV2 receptors. These receptors were described as thermoreceptors with a much higher threshold (> 52 °C), but most TRPV2-expressing primary afferents do not respond to heat, and TRPV2-deficient mice respond normally to heat (Caterina et al., 1999; Zhang, 2015). Other TRP channels contribute to heat sensitivity, including TRPV3 and TRPV4 (Patapoutian, Tate, & Woolf, 2009). TRPV3 and TRPV4 are found in keratinocytes in addition to neurons. They are primarily receptors for non-noxious heat, but are sensitized by repeated heat stimuli and contribute to peripheral sensitization by releasing pro-inflammatory substances. However, they do not appear to mediate mechano-heat sensitive C-fiber sensitization (Zimmermann et al., 2005).

The transient receptor potential cation channel subfamily M member 3 (TRPM3) and calcium-activated chloride channel anoctamin 1 (ANO1) are additional receptors for noxious heat with thresholds of 40 °C and 44 °C, respectively. Deletion of either decreases responses to noxious heat, but only partially. They are often co-expressed with TRPV1 (Zhang, 2015). Heat sensitivity is also mediated by constitutively-active K⁺ channel (KCNK2), which is inhibited by heat (Alloui et al., 2006). Keratinocytes also play a role in heat transduction. Activation triggers
the release of adenosine triphosphate (ATP), which activates the purinergic receptors P2X2 and P2Y2 on free nerve endings (Dubin & Papapoutian, 2010).

The spinal cord connects the primary afferents to the brain. It consists of a gray matter core made up of cell bodies surrounded by white matter made up of myelinated axons (Figure 9). Rexed (1952) described the spinal cord as being organized into ten distinct layers with different structural and functional characteristics, referred to as laminae I-X (Figure 10).

Figure 9. Gross anatomy of the spinal cord, including the anterior funicle (1) and lateral funicle (2), where the ascending anterolateral system projects to the brain. The lateral portion of the anterolateral spinothalamic tract carries temperature and pain signals. Adapted from “Spinal Cord Anatomy and Clinical Syndromes,” by E. D. Diaz and H. Morales, 2016, Seminars in Ultrasound and CT and MRI, 37(5), p. 361.
Figure 10. Laminae of the spinal gray matter, originally described by Bror Rexed, 1952. Laminae 1 and 2 are involved in the processing of temperature and pain signals. Adapted from “Spinal cord anatomy and clinical syndromes,” by E. D. Diaz and H. Morales, 2016, Seminars in Ultrasound and CT and MRI, 37(5), p. 367.

The synapse connecting the primary afferent and dorsal horn neurons is perhaps the most important segment of the nociceptive pathway. Here, nociceptive signals enter the central nervous system and undergo extensive processing. Peripheral input also shapes the structure and
function of the dorsal horn, especially in the case of persistent input from inflammation or long-lasting injury. Fiber type and termination pattern are important factors in the operation of this system (Cordero-Erausquin et al., 2016). Studies generally focus on only one or a few nociception-related molecules, but numerous factors interact in complex ways in the nervous system. Several categories of spinal nociceptive mediators have been identified, including peptides (Seybold, 2009), cytokines (Schäfers & Sorkin, 2008; Verri et al., 2006), prostanoids (Vanegas & Schaible, 2001), and neurotrophic factors (Kelleher, Tewari, & McMahon, 2017).

Typically, Aβ-fibers are described as non-nociceptive, while Aδ- and C-fibers are described as nociceptive, but this is an oversimplification. There are nociceptive and non-nociceptive neurons in all these fiber types, and the projection patterns of nociceptive and non-nociceptive primary afferents differ (Cordero-Erausquin et al., 2016). Non-nociceptive Aβ fibers terminate in laminae III-VI, as well as II, to a very small degree. Non-nociceptive Aδ and C fibers terminate in laminae II-III and II, respectively. The nociceptive fibers terminate mostly in the superficial laminae. Aβ fibers terminate in lamina II, Aδ fibers terminate in laminae I-II, and V, and C fibers terminate in laminae I-II (Prescott & Ratté, 2012).

The ability to distinguish between fiber types is compromised by several issues. The response properties of primary afferents can change. For example, repeated heat stimulation causes mechanical Aδ fibers to gain sensitivity to heat, and Aδ mechano-heat sensitive fibers to respond to non-noxious heat stimuli. Non-nociceptive neurons also respond to noxious stimuli, such as low-threshold mechanosensitive fibers responding to intense mechanical stimuli. The overall function of the system is also more complex, where specificity in the periphery feeds into integration and processing by convergent central circuits (Prescott & Ratté, 2012).
Laminae I-VI make up the dorsal horn, where primary afferents signals are received, processed in neural circuits, and transmitted to several brain areas via ascending tracts (Figure 11). The laminae are also referred to in functional pairs. Laminae I-II are known as the superficial dorsal horn laminae, also called the marginal zone of Waldeyer and substantia gelatinosa of Rolando, respectively. Laminae III-IV are known as the nucleus proprius. Laminae V-VI are known as deep dorsal horn laminae. Laminae III-IV are sometimes categorized as deep, depending on the study (Cordero-Erausquin et al., 2016).
Figure 11. The pain pathway features multiple fiber types and distinct pathways. The nociceptive primary afferent neurons detect noxious (C fiber and Aδ fiber) and innocuous (Aβ fiber) stimuli and carry the signals to the dorsal horn of the spinal cord. The ascending spinal cord neurons are organized into the spinoparabrachial and spinothalamic tracts. Within the brain, spinoparabrachial tract ascends from the parabrachial nucleus to the insular and cingulate cortices via the amygdala, and the spinothalamic tract ascends from the thalamus to the somatosensory cortex. Adapted from “Central Mechanisms of Pathological Pain,” by R. Kuner, 2010, Nature Medicine, 16, p. 1259.

The superficial laminae are largely dedicated to nociceptive input, whereas the deeper laminae integrate nociceptive and non-nociceptive input. However, this is an oversimplification. There are connections between nociceptive primary afferents and neurons in deeper laminae via dendrites that extend into the superficial laminae (Diaz & Morales, 2016), and there is some degree of nociceptive processing in all laminae (Cordero-Erausquin et al., 2016).

There are different types of neurons in the dorsal horn distinguished by their input and function. The nociception-specific neurons (NS) respond to noxious stimuli only. They are found in laminae I-IIo and V-VI, where they receive input from mechano-heat sensitive Aδ fibers and polymodal C fibers. Punctiform receptive fields in peripheral terminals and somatotopic organization in lamina I allow these neurons to encode the location and quality of the stimulus. The WDR neurons respond to both noxious and non-noxious stimuli. They are found in laminae I-IIo and IV-VI, where they receive input from Aβ, Aδ, and C fibers. They signal the intensity of the stimulus, and modulation of their activity is an important factor in gate control theory. However, sensitization can change the function of these fibers, such as NS neuron activation being driven by input from normally-innocuous fibers (Almeida, Roizenblatt, & Tufik, 2004).

The dorsal horn contains two major categories of neurons distinguished by their connections. Projection neurons make up about 10% of the dorsal horn neurons and carry signals from the dorsal horn to the brain. Interneurons make up about 90% of the dorsal horn and are
arranged in processing circuits that modulate those signals. Excitatory neurons tend to have a transient pattern of activity, whereas inhibitory neurons have a tonic pattern. This correlates with signals punctuating the baseline activity of the spinal circuits (Cordero-Erausquin et al., 2016).

The superficial laminae have been researched far more extensively than the others. They primarily process pain and temperature. Lamina I contains both projection neurons and interneurons and receives input from both peptidergic C fibers and Aδ fibers (Basbaum et al., 2009). Lamina I neurons are also influenced by activity in deeper laminae, as evidenced by intralaminar connections. The dendrites of projection neurons generally stay within lamina I, but the dendrites of some lamina I interneurons extend into lamina II and even laminae III-IV. Multiple morphological classes have been found in lamina I projection neurons, including fusiform, pyramidal, and multipolar neurons (Figure 12), though morphological class does not distinguish projection neurons and interneurons (Cordero-Erausquin et al., 2016).
Lamina II is made up almost entirely of interneurons. These cells have been classified into four categories: islet, central, radial, and vertical cells. They are thought to operate mostly in local circuits, in which excitatory and inhibitory interneurons with small cell bodies process nociceptive signals as they arrive from the periphery and the brain. For example, connections
between these interneurons mediate interactions between inputs from different fiber types. Some lamina II, central neurons that receive unmyelinated C-fiber input directly excite lamina II vertical neurons that receive Aδ-fiber input. Some lamina II vertical neurons that receive Aδ-fiber input directly excite both projection and non-projection lamina I neurons that receive C-fiber input (Lu & Perl, 2005).

These connections among dorsal horn neurons are important for nociceptive processing. While there does not appear to be significant axonal projection from laminae III-IV to the superficial laminae, ventrally projecting dendrites from the superficial laminae may mediate communication from the deeper laminae (Kato et al., 2009). Some laminae I-II neurons have long ventral dendrites that penetrate to laminae III-IV, where they receive excitatory input. There is also a distinct zone ventral to the border between lamina I and II. It is only one cell thick and is unusual in that dendrites from this zone receive significant inhibitory input from laminae III-IV, in addition to the typical excitatory input. Given both excitatory and inhibitory signaling from the deeper laminae, this zone may be uniquely suited to modulate nociceptive processing in the superficial laminae (Kosugi et al., 2013).

Laminae III and IV primarily process vibration and pressure touch. They contain relatively few cell bodies and are made up predominantly of large non-nociceptive projection neurons and some small-bodied interneurons (Cordero-Erausquin et al., 2016).

Lamina V neurons are involved in nociception. Almost all the neurons in this lamina are WDR neurons, which receive innocuous signals from Aβ fibers, as well as nociceptive signals from Aδ and C fibers. Aδ fibers synapse directly onto the WDR neurons, while C fibers connect polysynaptically. They respond proportionally to mechanical heat, and cold stimuli and this range of sensitivity allows WDR neurons to signal stimulus intensity. They are also involved in
gate theory, where nociceptive and non-nociceptive signals compete to influence the activity of
the WDR neurons that project to the brain (Basbaum et al., 2009).

Glutamate was proposed as the primary excitatory neurotransmitter in the 1950s, but a
consensus was not formed until the 1970s. Agonists and antagonists were developed and used to
establish a wide range of glutamatergic mechanisms and identify three ionotropic receptor
subtypes. These are named after their respective selective agonists, N-methyl-D-aspartate
(NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and Kainate.
Subsequent research on these receptors advanced significantly with the identification and
manipulation of subunits, allowing sophisticated genetic, molecular, and pharmacological
experimentation and treatment. Glutamate is involved in many nervous system phenomena, the
most important being synaptic transmission and plasticity (Collingridge et al., 2017).

Nociceptive signaling at the dorsal horn synapse is mediated primarily by glutamate
acting on the ionotropic (AMPA, NMDA, and Kainate) and metabotropic glutamate (mGlu)
receptors. AMPA receptors are activated by glutamate alone, whereas NMDA receptors require
both glutamate and glycine for activation. AMPA receptors can be homomers or heteromers,
made up of subunits GluA1-4, while NMDA receptors are obligate heteromers, made up of
subunits GluN1, GluN2A-D, and GluN3A-B. The composition of these subunits determines the
function of these receptors (Regan & Furukawa, 2016).

AMPA receptors are involved primarily in synaptic transmission and plasticity. They
mediate most of the glutamate-driven depolarization in the dorsal horn. They are also important
in activity-dependent sensitization, where phosphorylation increases their activity, calcium-
permeability, and trafficking to the membrane. These receptors are Ca\(^{2+}\) permeable so long as
they do not express the subunit GluA2 and exhibit activity-dependent receptor insertion during long-term potentiation only if they express the subunits GluA1 or GluA4 (Zhuo, 2017).

NMDA receptors are critical to long-term plasticity, including long-term potentiation (LTP). They have a high permeability to Ca\(^{2+}\), but are normally under substantial inhibition due to Mg\(^{2+}\) blocking their channel. Depolarization removes the Mg\(^{2+}\) block and significantly increases calcium conductance. Increased intracellular calcium activates a number of intracellular signaling pathways, including mechanisms of transcription and protein synthesis that underlie chronic sensitization (Zhuo, 2017).

Kainate receptors are found on small-diameter DRG neurons and can contribute to pre-synaptic depolarization of C fibers, regulating the release of glutamate. They are involved in high-intensity signaling and pre-synaptic LTP. Pre-synaptic actions of kainate may also suppress AMPA and NMDA receptor activity and promote (γ-Aminobutyric acid) GABA and glycine release in the dorsal horn (Zhuo, 2017).

mGlu receptors are found throughout the neuraxis and are critical modulators in both acute nociception and the development of central sensitization. They modulate the activity of AMPA and NMDA receptors, and trigger the release of calcium from intracellular stores (Latremoliere & Woolf, 2009).

A number of molecular markers are used to help classify nociceptive afferents and correlate these classes with receptive properties. Useful features include molecules expressed on the cell surface (e.g., receptors), molecules stored in and released from the cell (e.g., peptides), and enzymes. Aδ- and C-fiber nociceptors are classified by their protein and enzyme expression. The non-peptidergic neurons do not express substance P or CGRP, but do express the purinergic P2X3 and glial cell-derived neurotrophic factor (GDNF) receptors, and bind isolectin B4 (IB4),
which indicates the presence of the enzyme fluoride-resistant acid phosphatase (FRAP).

Peptidergic neurons express substance P and CGRP, but do not bind IB4. The peptidergic neurons also express the high-affinity nerve growth factor (NGF) receptor tyrosine receptor kinase A (trkA) (Saeed & Ribeiro-da-Silva, 2012). However, non-peptidergic Aδ-fibers nociceptors may not bind IB4 (Cordero-Erausquin et al., 2016). These markers also distinguish nociceptive and non-nociceptive cells. Cells that contain SP or CGRP are nociceptive, whereas cells that contain neither are non-nociceptive. However, it should be noted that there are distinct species differences in these molecular markers of peptidergic and non-peptidergic neurons. These categories are very distinct in mice, while there is significant colocalization between peptide-expressing and IB4-binding neurons in rats. In rats, approximately 40% of DRG cells, 50% of C fibers, and 20% of Aδ fibers are peptidergic (Price & Flores, 2007).

The tachykinin neurotransmitters make up a family of neuropeptides distributed widely throughout the body. There are many tachykinins and they influence a wide range of physiological processes. Substance P was the first tachykinin discovered in 1931 and is the most important in nociception. It is encoded by the Tac1 (preprotachykinin) gene, which has several splice variants (α-, β-, γ-, and δTac1). αTac1 encodes substance P, and its deletion has been found to decrease pain and prevent neurogenic inflammation. Substance P acts on three G protein-coupled receptors, NK-1, NK-2, and NK-3, with the highest affinity for the NK-1 receptor (Steinhoff et al., 2014). Substance P signaling can be blocked by the selective NK-1 receptor antagonist CP-96,345 (Snider et al., 1991; Yashpal, Pitcher, & Henry 1995).

Peptidergic primary afferents are glutamatergic, express the TRPV1 channel, and release substance P in response to capsaicin. These primary afferents have small cell bodies and unmyelinated C or lightly myelinated Aδ fibers that conduct signals slowly. Tissue injury or
inflammation leads to peripheral and central substance P and CGRP release (Figure 13). In the periphery, CGRP acts on the calcitonin receptor-like receptor (CLR) on arterioles, triggering dilation and hyperemia. Substance P acts on NK-1 receptors on endothelial cells of post-capillary venules, triggering plasma extravasation and granulocyte infiltration, and further increasing inflammation. The cytokines tumor necrosis factor α (TNFα), interleukin 6 (IL-6), and IL-8 are released to promote activation of the pro-inflammatory transcription factor nuclear factor kappa B (NF-κB) via PKCδ and increase COX-2 expression, resulting in increased prostaglandin production. TRPV1 receptors are also sensitized through PKCε-dependent phosphorylation, which increases nociceptive input to the dorsal horn (Steinhoff et al., 2014).
Primary afferent C fibers terminate on NK-1 receptor-expressing neurons (Figure 14) in laminae I and II, with termination organized by C fiber type (Figure 6). These fibers are divided evenly between peptidergic and non-peptidergic. Peptidergic C fibers terminate in laminae I-IIo, while non-peptidergic C fibers terminate in lamina IIi. Additionally, C fibers have polysynaptic
connections with lamina V. Aδ fibers terminate in laminae I-IIo and V. The NK-1 receptor is expressed only on glutamatergic spinal neurons. It is found in all laminae of the dorsal horn with the highest concentration in lamina I, as well as some laminae III-IV neurons with dendrites that extend to lamina I and synapse with peptidergic primary afferents. Many of these are projection neurons involved in thermal nociception and receive input from TRPV1-expressing primary afferents (Labrakakis & MacDermott, 2003; Todd, 2010). In the lamina I spinothalamic neurons, about 69% are innervated by peptidergic C primary afferents, 28% by Aδ primary afferents, and 14% by both. They receive input from a large rostrocaudal range, compared to NK-1-negative neurons, indicating that they can integrate local input from a larger area (Cordero-Erausquin et al., 2016).
Figure 14. Confocal pseudocolor image of the Substance P receptor distribution in the superficial dorsal horn after unilateral intraplantar capsacin injection. Activated NK-1 receptors internalize and concentrate in endosomes. Scale bar, 0.4 mm. Adapted from “Receptor Endocytosis and Dendrite Reshaping in Spinal Neurons after Somatosensory Stimulation,” by P. W. Mantyh et al., 1995, Science, 268, p. 1629.

Substance P is released at extrasynaptic sites and works through volume transmission, and its release increases in response to sustained or intense stimulation. When it binds to the NK-1 receptor, it activates several intracellular mechanisms, including phospholipase C (PLC), adenylyl cyclase (AC), phospholipase A₂ (PLA₂), and voltage-gated calcium channels. PLC acts on phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce inositol triphosphate (IP₃) and diacylglycerol (DAG). DAG activates PKC directly, while IP₃ triggers the release of calcium from intracellular stores, which also activates PKC. Elevated calcium then activates a number of intracellular mechanisms. AC causes cyclic adenosine monophosphate (cAMP) accumulation, which activates PKA. PLA₂ generates arachidonic acid, which is a precursor to lipid inflammatory mediators, including prostaglandins (Steinhoff et al., 2014).

Substance P causes excitatory post-synaptic potentials that summate over time, originally described as “windup” (Mendell & Wall, 1965; Zhuo, 2017). This sustained depolarization is instrumental to the removal of the Mg²⁺ plug, which allows glutamate to bind to the NMDA receptors, leading to a rapid increase in synaptic efficacy and an increase in intracellular Ca²⁺ levels. These effects help induce central sensitization (Latremoliere & Woolf, 2009).

NK-1 receptors internalize after activation (Figure 15), which is used as a measurement of receptor activation (Mantyh et al., 1995). Capsaicin administration and resulting TRPV1 activation lead to receptor internalization. NK-1-expressing dorsal horn neurons can be selectively destroyed by administration of the cytotoxin saporin. Rats given this treatment have normal acute pain function, but dramatically reduced inflammatory hyperalgesia (Mantyh et al.,
Despite this finding, NK-1 receptor antagonists have proven unsuccessful as analgesics (Steinhoff et al., 2014).
Figure 15. Fluorescent confocal image of substance P receptors of lamina I lumbar spinal cord neuron cell bodies (A, C) and dendrites (B, D). A and B depict neurons from naïve animals, while C and D depict neurons from animals five minutes after capsaicin injection into the paw. Capsaicin treatment triggered NK-1 receptor activation and internalization. Additionally, the dendrites of activated neurons developed swollen varicosities packed with NK-1 containing endosomes. Scale bars, 20 μm (A, C) and 10 μm (B, D). Adapted from “Receptor Endocytosis and Dendrite Reshaping in Spinal neurons after somatosensory stimulation,” by P. W. Mantyh et al., 1995, Science, 268, p. 1630.

Substance P signaling can be regulated through multiple mechanisms (Figure 16). Cell-surface mechanisms include substance P reuptake and substance P degradation by the cell-surface peptidase neprilysin (NEP). β-arrestins control other important mechanisms, including desensitization, endocytosis, and endosomal signaling. Desensitization and resensitization following activation is a normal pattern of receptor function. In desensitization, G protein receptor kinases (GRKs) phosphorylate the receptor when it is occupied by substance P, promoting interaction with β-arrestins. This uncouples the receptor from its G proteins, resulting in desensitization, which is reversible by protein phosphate 2A- (PP2a)-mediated dephosphorylation (Steinhoff et al., 2014).
Figure 16. The regulation and trafficking of NK-1 receptors. NK-1 receptors can be regulated through a number of mechanisms. The cell-surface peptidase neprilysin (NEP) degrades substance P. Activated NK-1 receptors attract β-arrestins, which decouple the receptor’s G protein. β-arrestins can also resensitize the receptor or promote its endocytosis. This leads to endosomal signaling, rapid or slow recycling, as well as degradation in the context of sustained stimulation and high concentrations of substance P. Adapted from “Tachykinins and Their Receptors: Contributions to Physiological Control and the Mechanisms of Disease,” by M. S. Steinhoff et al., 2014, *Physiological Review, 94*, p. 274.
These mechanisms also regulate endosomal signaling. β-arrestin promotes endocytosis of the substance P-containing receptor and recruits Src, mitogen-activated kinase kinase (MEKK), and extracellular signal-regulated kinases 1/2 (ERK1/2) to assemble the signalosome. This consists of the endosome and receptor with an attached complex of Src, MEKK, and extracellular signal-regulated kinase (ERK) 1/2, allowing the receptor to continue signaling in superficial or perinuclear endosomes. Signaling is terminated when the receptor dissociates from the β-arrestin. Acidification of the endosome dissociates substance P from the receptor, and the membrane metalloendopeptidase endothelin-converting enzyme -1 (ECE-1) degrades substance P. This destabilizes the NK-1 receptor/β-arrestin/Src/MEKK/ERK1/2 signaling complex, which attenuates ERK signaling. The characteristics of the triggering stimulation determine the next step. If substance P stimulation was brief, the receptor is recycled back to the membrane and resensitized as a result of dissociation from β-arrestin. If substance P stimulation was sustained, the NK-1 receptor is marked for degradation in a lysosome. This results in downregulation of substance P signaling. NK-1 receptor activation can lead to ERK activation through other mechanisms, including the PKA and PKC pathways, as well as G protein-mediated transactivation of the epidermal growth factor receptor (EGFR). This leads to its dimerization and phosphorylation, and assembly of a SHC/Growth factor receptor-bound protein 2 (Grb2) complex. This complex then activates MAPK and ERK1/2 (Cattaruzza, Poole, & Bunnett, 2013).

Overall, approximately 90% of spinal cord neurons are interneurons. Primary afferent neurons synapse onto a number of spinal circuits made up of excitatory and inhibitory interneurons in laminae I-II of the dorsal horn. These neurons receive input from nociceptive C fibers and can connect to other interneurons or projection neurons (Cordero-Erausquin et al., 2016). Projection neurons make up about 5% of lamina I neurons and are absent from lamina II,
indicating that the vast majority of the superficial neurons are interneurons. These laminae are
made up of approximately one third inhibitory (GABAergic and/or glycinergic) and two thirds
excitatory (glutamatergic) interneurons (Prescott & Ratté, 2012).

Lamina II is made up almost exclusively of interneurons, which have been classified as
islet, central, radial, and vertical cells, based on their dendritic morphology (Figure 17). Islet
cells are GABAergic, radial cells and most vertical cells are glutamatergic, and central cells can
be either. This system leaves about 30% of interneurons unclassified. Lamina I interneurons are
less understood, but have been classified as pyramidal, fusiform, and multipolar (Todd, 2010).
The inhibitory interneurons have several important roles in maintaining normal signaling. They
silence activity in nociceptive neurons in the absence of noxious stimuli, reduce the
responsiveness of nociceptive neurons, prevent crosstalk between stimulus modalities, and
restrict the spread of activity (Sandkühler et al., 2009). Relatively little is known about excitatory
interneurons, despite being the majority of the dorsal horn neurons. They may be involved in
inflammation-induced dorsal horn plasticity (Yasaka et al., 2010).
There are a number of key brain areas in the neuromatrix, including the thalamus, insular cortex (IC), primary and secondary somatosensory cortices I and II (SI and SII), anterior cingulate cortex (ACC), amygdala, and prefrontal cortex (PFC). Studies of brain damage have demonstrated that pain is not localized in a particular region of the brain. Nociceptive processing is distributed across these brain regions (Morton, Sandhu, & Jones, 2016).

The nociceptive projection neurons of the spinal cord carry signals from the primary afferents to the brain and make up approximately 10% of the dorsal horn neurons. Primary afferents connect to these glutamatergic neurons both mono- and poly-synaptically, and they form tracts originating primarily in laminae I or V that ascend to the brain. These ascending fibers make up the anterolateral system, which is found in the anterior and lateral funiculi (Figure 18). The spinothalamic tract is the most important of these pathways and its lateral component carries temperature and pain signals. It decussates via the anterior white commissure at its level of entry into the spinal cord and ascends to the thalamus, with collaterals connecting to the medullary nuclei, lateral parabrachial area, periaqueductal gray matter (PAG), and hypothalamus. Additional tracts ascend directly to various brain areas, such as the spinoreticular, spinoparabrachial, spino-PAG, and spinomesencephalic tracts (Diaz & Morales, 2016; Millan, 1999).
The spinothalamic tract can be separated into medial and lateral pathways based on their termination in the thalamus (Figure 19). The medial pathway ascends to the medial thalamus and subsequently the ACC and IC. This pathway is associated with the affective-emotional aspects of pain. The lateral pathway ascends to the lateral thalamus and subsequently to SI and SII. This pathway is associated with the sensory-discriminative aspects of pain (Morton, Sandhu, & Jones, 2016).
**Figure 19.** The pain matrix. Two major pathways have been identified in the brain. The spinothalamic tract ascends to the thalamus, with collaterals to the RVM and PAG. The medial pathway (dark gray) projects to the ACC and IC, and processes affective-motivational aspects of pain. The lateral pathway (light gray) projects to the primary and secondary somatosensory corticies (SI and SII) and IC, and processes sensory-discriminative aspects of pain. The PFC and amygdala participate in descending modulation of nociceptive processing. Adapted from “Brain Imaging of Pain: State of the Art,” by D. L. Morton, J. S. Sandhu, and A. K. P. Jones, 2016, *Journal of Pain Research, 9*, p. 614.

It should be noted that projection pathways named after their terminations in the brain are misnomers to some degree, as most projection neurons project to multiple areas. Eighty-five percent of lamina I neurons project to both the caudal ventrolateral medulla (CVLM) and the lateral parabrachial area, 90% of lamina I neurons project to both the PAG and the CVLM or lateral parabrachial area, and 80% of lamina I spinothalamic neurons also project to the lateral parabrachial area (Cordero-Erausquin et al., 2016).
Nociception in the brain was difficult to study directly until relatively recently. Research was limited to animal models, external measurements of brain activity, postmortem studies, brain stimulation during surgery, and subjective reports. Technological advances in the last half-century, such as positron emission tomography (PET), magnetoencephalography (MEG), and functional magnetic resonance imaging (fMRI), have provided unprecedented access to the internal workings of the brain. These technologies allow precise temporal and spatial measurement and observation of structural and functional changes in response to pain (Morton, Sandhu, & Jones, 2016).

Nociceptive input to the brain can trigger rapid plastic changes in the cortex. Repeated pain stimulation leads to changes in the gray matter in many areas of the brain, including the thalamus, parabrachial area of the pons, insula, ACC, somatosensory cortices, amygdala, and PFC. Other effects can be observed, including increases or decreases in microstructural integrity, which affects the effectiveness of signaling (Morton, Sandhu, & Jones, 2016; Zhuo, 2017).

The function of the human neuromatrix is influenced by psychological factors, including attention, expectations, and memories of previous pain experiences. These effects can be correlated with the function of particular neuromatrix subsystems. Negative emotions are correlated with greater activity in the ACC and anterior IC. Increased activity can be observed throughout the neuromatrix in anticipation of pain, even without stimulation. Expectations also influence perceived pain intensity. When participants are told that a stimulus will be more or less intense, their reports skew in that direction. Placebo analgesia can also be examined as a neuromatrix phenomenon. Expectations correlate with increased activity in the PFC, leading to decreased activity in medial pathway brain areas, including the ACC, IC, and thalamus, as well as the spinal cord. Increased endogenous opioids have also been observed in the ACC, IC,
dorsolateral PFC, and nucleus accumbens (NA). Placebo analgesia also involves activity in the PAG, which is critical for descending inhibition (Morton, Sandhu, & Jones, 2016).

Axons descend from the brain and exert tonic and transient excitatory and inhibitory influence on nociceptive processing in the dorsal horn (Figure 20). These pathways descend from numerous brain regions, including the PFC, cingulate cortex, PAG, locus coeruleus, parabrachial nucleus, and medullary formation. The PAG is an important site of convergence among descending pathways. It has reciprocal connections with limbic areas and connections to autonomic centers in the parabrachial area and nucleus tractus solitarius (NTS) of the medulla. Critically, the PAG controls the descending neurons of the RVM. These neurons include ON (excitatory) and OFF (inhibitory) cells that influence spinal nociceptive processing. Their cell bodies intermingle in the RVM, but descend in separate pathways. They terminate in the superficial dorsal horn, where they use the monoamine neurotransmitters dopamine, norepinephrine, and serotonin, as well as GABA. They can be activated by ascending nociceptive signals, opioids, and cognitive-attentional processes to produce excitatory or inhibitory effects, which are involved in hyperalgesia and analgesia, respectively. This system exhibits plasticity in the context of central sensitization (Cordero-Erausquin et al., 2016; Prescott & Ratté, 2012).
Figure 20. Ascending and descending nociceptive pathways, including descending modulation of dorsal horn nociceptive processing via the PAG and the ON/OFF cells of the RVM. Adapted from “Computational Functions of Neurons and Circuits Signaling Injury: Relationship to Pain Behavior,” by L. M. Mendell, 2010, Proceedings of the National Academy of Sciences of the United States of America, 108(3), p. 4.

Opioids operate in a critical descending modulatory system. The opioid receptors were discovered before the peptides themselves (Pert & Snyder, 1973), and the endogenous opioids were the first neuropeptides discovered (Hughes et al., 1975). The typical peptides dynorphin and enkephalin are the most studied. Opioids are produced in the CNS, pituitary, and adrenal glands, where they are derived from the precursor proenkephalin. They act on three G protein-coupled receptors classified into three major subtypes, δ, κ, and μ, found in glutamatergic and GABAergic neurons in the superficial dorsal horn and primary afferents (Sardella et al., 2011). They modulate nociception in these neurons by activating inwardly-rectifying potassium channels and inhibiting voltage-gated calcium channels, as well as inhibiting the cAMP pathway and activating MAPK pathways. Opioid synthesis and release may help set pain sensitivity, given that pain thresholds vary with enkephalin levels (Oshita et al., 1990). Acute pain leads to the release of endogenous opioids in particular brain regions of the neuromatrix, including the insula, thalamus, amygdala, cingulate cortex, and NA. This release is compromised in many chronic pain conditions. Prolonged opioid treatment leads to reduced gray matter and compromised functional connectivity in many brain areas of the neuromatrix, including the amygdala, cingulate, insula, and NA. These effects increase as a function of treatment duration and are a significant obstacle to pain management (Morton, Sandhu, & Jones, 2016).

Sensitization. Plasticity is the ability of the nervous system to change in response to experience. It encompasses a wide range of changes, from minute to extensive, local to systemic, and momentary to permanent. Structure and function can be altered by molecular and cellular
changes in the number and function of synaptic, somal, and axonic ion channels, receptors, enzymes, transporter molecules, and transcription factors. These changes can have various effects, including alterations in the synthesis, release, and uptake of neurotransmitters and neuromodulators, induction or maintenance of synaptic or intrinsic plasticity, as well as changes in cell morphology. Plasticity is a fundamental component of sensitization of the nociceptive system (Luo, Kuner, & Kuner, 2014).

Some terminology must be clarified in order to understand the literature on sensitization. The term hyperalgesia refers to “a state of increased intensity of pain sensation induced by either noxious or ordinarily non-noxious stimulation of peripheral tissue” (Hardy, Wolff, & Goodell, 1950). The term allodynia was added later to describe pain elicited by normally-innocuous stimuli. However, these two terms are often used in confusing or inconsistent ways. For example, peripheral inflammation will shift the stimulus-response curve to the left, representing both an increase the intensity of normally painful stimuli and pain in response to normally-innocuous stimuli. Additionally, researchers often use the convention of calling this phenomenon heat hyperalgesia, but mechanical allodynia (Sandkühler et al., 2009). The IASP recommends that the term “hyperalgesia” be used to refer to increased pain sensitivity and the term “allodynia” to refer to pain associated with normally non-nociceptive signaling (e.g., Aβ-fiber input) (IASP Task Force on Taxonomy, 2008).

Sensitization refers to increased nociceptive fiber responses, while hyperalgesia refers to increased pain behavior responses. Hyperalgesia is categorized as primary when the increased pain response results from stimulation at the site of injury, and secondary when the increased pain response results from stimulation of the uninjured area around the site of injury. Primary hyperalgesia occurs with both heat and mechanical stimuli, while secondary hyperalgesia is
indicated by mechanical allodynia, pain triggered by normally-innocuous tactile stimulation outside the area of injury (Lewis, 1935).

Sensitization is a key feature of the nociceptive system and is normally adaptive. There are four specific changes that fall under the label "sensitization:" (1) increased responsiveness of nociceptive neurons to normally painful stimuli (hyperalgesia), (2) recruitment of a nociceptive response to normally innocuous stimuli (allodynia), (3) increased spontaneous discharge of nociceptive neurons, (4) and increased size of receptive fields of nociceptive neurons (Bourinet et al., 2014; IASP Task Force on Taxonomy, 2011). Sensitization can occur peripherally, at the peripheral terminal of the primary afferent, or centrally, at the synapse between the primary afferent and the spinal cord. It is normally adaptive. After injury, increased alertness and associated behavioral changes reduce the likelihood of further damage during the period of highest risk. Sensitization can become pathological when central changes result in pain that is uncoupled from noxious stimulation (Latremoliere & Woolf, 2009).

Peripheral sensitization was discovered in 1976 (Perl et al., 1976) and it is characterized by decreased thresholds, increased responses, and increased spontaneous activity. It occurs in three steps. First, injury triggers the release of inflammatory mediators from the peripheral terminal of the neuron and from non-neuronal cells. Second, these mediators act on their respective receptors. Third, activation of these receptors initiates intracellular pathways that sensitize the neuron through post-translational and transcriptional mechanisms (Figure 21) (Mifflin & Kerr, 2014). Peripheral sensitization is particularly important to heat hypersensitivity at the site of injury. Carrageenan and other agents can also be used to induce inflammatory pain. Injection of these agents leads to sensitization of A- and C-fiber nociceptors, as well as central sensitization (Lisi et al., 2015).
Figure 21. Inflammation-induced modulation of the peripheral terminal of the primary afferent. Injury triggers the release of inflammatory mediators, which trigger post-translational and transcriptional changes. These mechanisms modify various channels and receptors, resulting in decreased thresholds, increased responses, and increased spontaneous activity. Adapted from “Pain: Molecular Mechanisms,” by M. Costigan and C. J. Woolf, 2000, *The Journal of Pain, 1*(3), p. 36.

Peripheral sensitization represents hypersensitivity to stimuli, but this hypersensitivity dissipates after the stimulus or injury has subsided. Pain is still due to activation of nociceptors. This will be contrasted to central sensitization later. Peripheral sensitization does contribute to central sensitization by increasing the release of glutamate, substance P, CGRP, and BDNF from the central terminal of the primary afferent neuron (Sandkühler, 2009). Inflammation can also induce NGF-mediated functional changes in large and medium A-fiber primary afferents, such that they begin to express neuropeptides and neuromodulators typical of C fibers (substance P.
and BDNF). Low-threshold Aβ fibers and high-threshold Aδ fibers can undergo a phenotypic switch and function like C fibers, contributing to central sensitization (Costigan & Woolf, 2000).

Tissue injury triggers an immune response from the peripheral terminal of the primary afferent and many non-neuronal cells, including mast cells, basophils, platelets, macrophages, neutrophils, endothelial cells, keratinocytes, fibroblasts, and Schwann cells. These cells release inflammatory mediators, including substance P, bradykinin, prostaglandins, ATP, NGF, serotonin, TNFα, IL-1β, IL-6, chemokines, histamine, nitric oxide (NO), glutamate, and protons, which act on G protein-coupled receptors, cytokine receptors, receptor tyrosine kinases (trk), purinergic receptors, and ion channels. Most of these mediators sensitize the neuron rather than activate it (Basbaum et al., 2009). Many modulate nociceptive signaling through phosphorylation of tetrodotoxin (TTX)-resistant sodium channels and modulation of TRPV1 channels (Ma & Quirion, 2007). Additionally, chemokines recruit immune cells to modulate primary afferent activity, including sensitization of TRPV1 receptors (Wang et al., 2008).

Several inflammatory mediators act on G protein-coupled receptors to activate intracellular pathways, such as bradykinin, serotonin, prostaglandins, and chemokines. Their effects depend on the type of G protein that they activate. $G_s$ activation stimulates AC, which increases cAMP levels and PKA activation, whereas $G_i$ inhibits AC, which reduces cAMP levels and PKA activation. Activation of $G_{q/11}$ activates phospholipases, including PLC, which generate IP$_3$ and DAG from PIP$_2$ in the membrane. DAG activates PKC directly, while IP$_3$ activates it indirectly. IP$_3$ diffuses to the endoplasmic reticulum where it acts on IP$_3$ receptors, triggering the release of calcium into the cytoplasm. Finally, activation of $G_q$ stimulates PLA$_2$, which cleaves membrane phospholipids to produce arachidonic acid. Prostaglandins are then synthesized from
arachidonic acid. G proteins can also affect ion channels and enzymes directly, such as PLC activation by the βγ subunit (Smrcka, 2008; Zylbergold et al., 2010).

Substance P is an important inflammatory mediator at the peripheral terminal of nociceptive primary afferents. It was discussed above.

Bradykinin is released from damaged tissue as well as mast cells and macrophages. It activates and sensitizes myelinated and unmyelinated nociceptors through the G protein-coupled receptors B1 and B2 receptors in both the periphery and spinal cord. It triggers activation of PLC and PKC, production of arachidonic acids, and modulation of TRPV1 channels (Mizumura et al., 2009).

Prostaglandins are cyclooxygenase (COX) products of arachidonic acid and one of the earliest known inflammatory mediators. Upon binding to the G protein-coupled receptor EP2, prostaglandin E2 (PGE2) potentiates AMPA receptor and NMDA receptor currents, activates non-selective cation channels, including reduction of Na\textsubscript{v}1.8, a TTX-resistant voltage-gated Na\textsuperscript{+} channel thresholds, and reduces glycinergic inhibition. PGE2 also leads to increased pre-synaptic transmitter release via its EP4 receptor (Reichling, Green, & Levine, 2013).

ATP and other purines are released from platelets and damaged tissue during inflammation or injury. ATP activates P2X3 receptors on small-diameter primary afferents to produce pain and inflammation. It is also involved in neuronal-glial-immune interactions. Its effects are mediated to some degree by TRPV1-expressing Aδ and C fibers, where the receptors are upregulated and phosphorylated by PKA and PKC during inflammation (Morales-Lazaro, Simon, & Rosenbaum, 2013). ATP and other adenosine molecules also work through the G protein-coupled P2Y receptor to facilitate TRPV1 signaling, increase calcium levels via opening of ion channels and IP\textsubscript{3}-DAG-mediated intracellular release, potentiate TTX-resistant sodium
channel Na\textsubscript{V}1.8 current, and inhibit potassium channel current. This leads to increased neuronal excitability and cAMP response element binding protein (CREB) activation (Molliver et al., 2002; Yousuf et al., 2011).

NGF was the first neurotrophic factor found to affect nociception. Inflammation triggers the release of NGF from several non-neuronal cells, including fibroblasts, keratinocytes, Schwann cells, lymphocytes, macrophages, and mast cells. It binds to the trkA receptor, which is found on approximately half of small-diameter nociceptors, and on non-neuronal cells. NGF induces nociceptor sensitization through multiple mechanisms. TrkA binding on nociceptors leads to both altered gene expression and post-translational regulation of receptors and ion channels. It modulates the activity of ligand- and voltage-gated ion channels, including TRPV1, P2X3, acid sensing ion channel 3 (ASIC3), and TTX-resistant voltage-gated Na\textsubscript{V}1.8 channels, as well as triggering the release of inflammatory mediators from non-neuronal cells. It may also increase the proportion of A\text{	extgreek{d}} fibers that are nociceptive (Chuang et al., 2001). NGF can activate the MAPK, PI3K, and PLC-γ pathways (Cheng & Ji, 2008), and it can increase the central release of substance P, CGRP, and BDNF (Costigan & Woolf, 2000). TrkA receptors are also expressed in laminae I and II\textsubscript{outer} in the spinal cord. Taken together, these findings indicate that NGF affects nociceptive afferents that express substance P and CGRP and terminate in the superficial laminae of the spinal cord (Averill et al., 1995; Molliver et al., 1995).

Serotonin is released from platelets when degranulating mast cells release platelet activating factor (PAF), and it can activate nociceptors (Lang et al., 1990). Serotonin can also interact with bradykinin to enhance its effects. The serotonin receptor 5-HT\textsubscript{2A} is found in 40% of small-to-medium DRG neurons and has significant overlap with TRPV1 channels (van Steenwinckel et al., 2009).
The cytokine TNFα initiates an important cascade that triggers the release of other cytokines. Inflammation triggers its release from mast cells, Schwann cells, neutrophils, and macrophages. TNFα binding to the TNF receptor 1 (TNFR1) triggers IL-1β and IL-6 generation and release. IL-1β then stimulates COX-2, resulting in prostaglandin release and resulting nociceptor sensitization (Moalem & Tracey, 2006). TNFα binding to TNFR1 also activates the p38 MAPK, which enhances excitatory TTX-resistant voltage-gated Na\(^+\) channel current, resulting in potentiated nociceptive processing (Mifflin & Kerr, 2014).

Chemokines are small molecule cytokines that induce pain through direct action on neurons and immune cell recruitment, and many chemokines are up-regulated in response to inflammation or tissue injury. Their effects are mediated by G protein-coupled chemokine receptors (CCR). Chemokine (C-C motif) ligand 2 (CCL2) and CX3CCL1 modulate pain processing in the spinal cord and periphery, and they may also act as peripheral pain mediators. CCL3 also induces pain by sensitizing TRPV1 receptors on DRG neurons (Wang et al., 2008).

NO is a non-receptor-mediated intercellular signaling molecule produced in many cells adjacent to neurons in both the periphery and spinal cord, where it diffuses to its sites of action. NO modifies intracellular processes by acting on guanylate cyclase to produces cyclic guanosine monophosphate (cGMP), which then activates protein kinases, ion channels, phosphodiesterases, and COX enzymes (Tegeder et al., 2011). NO can also activate TRPV1 channels in DRG neurons (Miyamoto et al., 2009).

Excitatory amino acids, especially glutamate, are released from the peripheral terminal of the primary afferent neurons and non-neuronal cells in response to nociceptor stimulation. The non-neuronal sources include plasma, macrophages, and epithelial, dendritic, and Schwann cells. Glutamate acts on ionotropic receptors and mGluRs 1 and 5 to induce hyperalgesia. mGluR1
activation activates PLC, triggering the release of calcium from intracellular stores and activation
of PKC (Davidson et al., 1997). mGluR5 has also been found to overlap with TRPV1 channels
(Walker et al., 2001).

Central sensitization was originally defined as a “central excitatory state” (Hardy, Wolff,
& Goodell, 1950), but today the standard definition is “increased responsiveness of nociceptive
neurons in the central nervous system to their normal or subthreshold afferent input” (IASP Task
Force on Taxonomy, 2008). Note that this does not specify increased pain, though that is
normally the intended meaning. Some changes might occur in analgesic or pain-neutral
mechanisms. Even then, central sensitization could include decreased responsiveness of certain
neurons, such as excitation resulting from decreased activity in inhibitory neurons (Latremoliere
& Woolf, 2009; Sandkühler, 2010).

Central sensitization is characterized by increased pain sensitivity due to changes in the
structure and function of the central nociceptive system, including decreased thresholds,
increased responses to suprathreshold stimuli, crosstalk between pain and touch pathways
(Figures 22 and 23), increased spontaneous signaling, and expanded receptive fields. It is
induced by activity in neuronal, glial, and immune cells, through post-translational and
transcriptional mechanisms. There are multiple types of central sensitization that will be
described below. Typically, central sensitization develops in response to intense, repeated, and
sustained noxious stimulation due to injury or inflammation. Unlike peripheral sensitization, it
can persist long after the original injury (Latremoliere & Woolf, 2009).
Under normal conditions, the sensations of pain and innocuous touch operate separately. Low-intensity stimuli activate only the touch pathway and lead to innocuous sensations, while high-intensity stimuli activate only the pain pathway and lead to painful sensations. The two pathways do not functionally intersect. This separation is maintained by strong synaptic connections within the pathways and inhibitory neurons that help prevent signals from crossing over. Adapted from “Central Sensitization: Implications for the Diagnosis and Treatment of Pain,” by C. J. Woolf, 2011, Pain, 152, p. S4.
Clifford Woolf (1983) discovered central sensitization, which can be induced by intense, repeated, and sustained nociceptive stimulation. He found that injury to one paw facilitated the withdrawal reflex in the non-injured paw, establishing plasticity in the (motor) ventral horn and indicating a central mechanism. This finding was subsequently extended to the (sensory) dorsal horn (McMahon & Wall, 1984). In the decade before Woolf's discovery, an understanding of peripheral sensitization was developing, but central pain processing was generally described as a passive relay system that carries peripheral signals encoded for the onset, duration, intensity, location, and quality of noxious stimuli (Woolf, 2011).
Several important findings preceded the discovery of central sensitization. Lloyd (1949) first described use-dependent synaptic plasticity in the CNS, and Hardy, Wolff, and Goodell (1950) suggested that secondary hyperalgesia could be explained by the activation of interneurons that facilitate pre-existing connections between the areas of primary and secondary hyperalgesia. Mendell and Wall (1965) discovered the phenomenon of windup (temporal summation) in the dorsal horn of the spinal cord, where repeated, low frequency, constant strength C-fiber stimulation led to progressively increasing rate of action potentials. Bliss and Lomo (1973) described LTP in the hippocampus, precipitating extensive research into the mechanisms of synaptic plasticity. LTP was later established in the spinal cord, though the use of this term is disputed (see below) (Randić, Jiang, & Cerne, 1993; Woolf, 2011).

Activity-dependent sensitization refers to short-term plasticity that operates on a time scale of seconds to hours, and it can be observed throughout the nociceptive system. It is the first of two phases of central sensitization, and it is mediated by post-translational mechanisms. Pre- and post-synaptic changes can produce hyperexcitability, such as increased neurotransmitter release or sensitization of receptors, respectively. Short-term depression can also occur, such as pre-synaptic depletion of available neurotransmitter vesicles or post-synaptic receptor desensitization. These depressive effects can work to filter incoming nociceptive signals. Activity-dependent central sensitization is adaptive because it results in increased vigilance around an injury and avoidance of potentially harmful stimuli (including normally-innocuous ones), but reverses after the cessation of injury or noxious input (Luo, Kuner, & Kuner, 2014).

Chronic sensitization is the second phase of central sensitization, where activity-dependent sensitization lasts beyond the original injury. It is established through transcription and synthesis of new proteins on a time scale of minutes to permanent.
operate without some of the mechanisms necessary to induce it. The result is chronic pain, a disease of the nervous system where pain is persistent and uncoupled from noxious stimulation (Latremoliere & Woolf, 2009).

Central sensitization must be distinguished from simpler forms of potentiation and LTP. There are two forms of activity-dependent facilitation, but only one constitutes central sensitization. Homosynaptic facilitation refers to potentiation of signaling at an activated synapse on a dorsal horn neuron, while heterosynaptic facilitation refers to potentiated signaling in a non-activated synapse triggered by activation of a different synapse on that post-synaptic neuron (Figure 24). Windup is mediated by homosynaptic facilitation. Repeated C-fiber activation (0.5-5 Hz) triggers substance P and CGRP release, which produces cumulative post-synaptic activation through temporal summation. The terminology becomes difficult at this point. Windup is often equated with LTP as discovered in the hippocampus (Bliss & Lomo, 1973). They are similar in that hippocampal LTP is also a form of homosynaptic facilitation, but the process of windup itself is brief, not long-term. Central sensitization is also often equated with LTP, but should not be. Heterosynaptic sensitization of the entire neuron, i.e., facilitation of the synaptic connections to other incoming signals, is necessary for the classic features central sensitization. For example, changes in receptive fields explain tactile allodynia in the area of secondary hyperalgesia. Nociception-specific dorsal horn neurons receive significant small-amplitude input from low-threshold Aβ afferents outside its normal receptive field. Heterosynaptic facilitation potentiates this input, such that these fibers can drive nociceptive signaling in the spinal cord, making even light touch painful. Loss of tonic inhibition contributes to tactile allodynia and spontaneous pain. Reduced GABA and glycinergic inhibitory control causes normally-subthreshold input to activate nociception-specific dorsal horn neurons (Latremoliere & Woolf,
2009). Note that this description of LTP and sensitization has been challenged (Latremoliere & Woolf, 2010; Sandkühler, 2010)

Figure 24. Homosynaptic and heterosynaptic facilitation. In homosynaptic facilitation, facilitation occurs only in the activated synapse. In heterosynaptic facilitation, facilitation occurs at additional synapses, which can sensitize the entire neuron. This mechanism underlies allodynia and secondary hyperalgesia. Adapted from “Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity,” by A. Latremoliere and C. J. Woolf, 2009, The Journal of Pain, 10, p. 906.

Numerous descriptions of the roles of neurotransmitters, receptors, channels, and physiological states in nociception involve activation of intracellular signaling pathways. They form a complex web of mechanisms that modifies the structure and function of the cell, its environment, and its interactions with neighboring cells. The following are the most common second messengers relevant to nociception and sensitization, which operate through both post-translational and transcriptional mechanisms (Figures 25 and 26).
Figure 25. Several intracellular pathways contribute to the generation of central sensitization. Most of the cascades converge to activate ERK. Adapted from “Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity,” by A. Latremoliere and C. J. Woolf, 2009, *The Journal of Pain, 10*, p. 903.
A - Phosphorylation

B - Trafficking

C - Transcription

Transcription:
- c-Fos
- NK1
- TrkB
- Cos2...
Figure 26. Multiple mechanisms underlie central sensitization, including (A) immediate posttranslational modulation, (B) receptor trafficking, and (C) gene transcription. (A) AMPA and NMDA receptor phosphorylation leads to changes in their thresholds and activation kinetics. ERK also leads to the phosphorylation of Kv4.2 channels, decreasing K\(^+\) current and increasing excitability. (B) Insertion of GluR1-containing AMPA receptors increases calcium permeability. (C) Changes in gene transcription, activated through multiple (particularly calcium-mediated) intracellular mechanisms, contribute to the maintenance of central sensitization. Adapted from “Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity,” by A. Latremoliere and C. J. Woolf, 2009, *The Journal of Pain, 10*, p. 902.

PLC is activated by group I mGlu receptors and NK-1 receptors. PLC catalyzes the hydrolysis of membrane-bound PIP\(_2\), producing IP\(_3\) and DAG. IP\(_3\) translocates to the endoplasmic reticulum where it binds with its receptor, releasing calcium from intracellular stores. Calcium participates in a number of important mechanisms (Reichling, Green, & Levine, 2013).

cAMP/PKA was the first second messenger system discovered and is involved in many nociceptive mechanisms. It carries out most of its effects through phosphorylation of voltage- and ligand-gated sodium, potassium, and calcium channels, initiation of receptor trafficking to or from the cell membrane, and activation of DNA transcription pathways, which helps induce activity-dependent sensitization and establish chronic sensitization. These increases in cation channel current feed back, triggering greater second messenger activity. G\(_\text{as}\)-protein activation stimulates AC, which in turn triggers cAMP production. cAMP then activates PKA. Elevated calcium levels also drive calmodulin-mediated stimulation of AC 1 and 8, producing cAMP, and activating PKA (Wei et al., 2006).

PKC also contributes to many nociceptive mechanisms. It is activated by the PLC/IP\(_3\) pathway through multiple routes. IP\(_3\)-mediated increases in intracellular calcium activate PKC, while DAG activates it directly. Much like PKA, PKC phosphorylates cation channels and receptors, driving post-translational and transcriptional mechanisms to induce activity-dependent
sensitization and establish chronic sensitization. These increases in cation channel current feedback, triggering greater second messenger activity (Latremoliere & Woolf, 2009).

CaMKII is activated by calcium and, along with PKA, increases receptor activation and insertion into the cell membrane. The resulting increases in cation channel current feedback, triggering greater second messenger activity (Latremoliere & Woolf, 2009).

Calcium plays a major role in both peripheral and central intracellular mechanisms (Figure 27). Pre-synaptic voltage-gated calcium channels mediate neurotransmitter release, and appear to be an effective target for analgesics (Waxman & Zamponi, 2014). Intracellular calcium in post-synaptic neurons represents a major convergence point of signaling, and it activates several other intracellular mechanisms that contribute to activity-dependent and chronic sensitization. Glutamate, substance P, CGRP, and BDNF act on their respective receptors (ionotropic, in the case of glutamate) to depolarize the post-synaptic neuron and open voltage-gated calcium channels, while group I metabotropic glutamate receptors and NK-1 receptors trigger the release calcium from intracellular stores via the PLC/IP₃ pathway. Mitochondria also influence intracellular calcium levels. These increases activate calcium-dependent intracellular mechanisms, including PKA, PLC, PKC, and CaMKII, nitric oxide synthase (NOS), and ERK. These mechanisms increase the synaptic efficacy of AMPA and NMDA receptors, increase AMPA receptor calcium permeability and trafficking to the membrane, and increase NMDA receptor reactivity to glutamate by reducing Mg²⁺ blockade of the ion channel (Luo, Kuner, & Kuner, 2014; Mifflin & Kerr, 2014; Reichling, Green, & Levine, 2013).
Figure 27. Sources of calcium in the dorsal horn synapse. (A) Control, non-activated conditions. (B) Nociceptor input leads to NMDA and mGlu receptor activation, resulting in a rapid increase in calcium concentration. Calcium influx leads to PKC and CaMKII activation, which contributes to central sensitization. (C) Inflammation-induced central sensitization features a shift from GluR2/3- to GluR1-containing AMPA receptors, as well as voltage-dependent calcium channel and NMDA receptor activation, and additional entry of calcium. Combined with the action of G protein-coupled mGlu, NK-1, B2, and CGRP1 receptors, these mechanisms lead to the release of calcium from intracellular stores, recruiting PKC and CaMKII, resulting in a strengthened excitatory synapse. Adapted from “Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity,” by A. Latremoliere and C. J. Woolf, 2009, *The Journal of Pain, 10*, p. 901.

NO is produced through multiple types of NOS, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). It acts both peripherally and centrally, and contributes to activity-dependent and chronic sensitization. nNOS is found in the dorsal horn and is the most important type in nociception. NOS activation is driven by NMDA receptor-mediated increases in intracellular calcium. When NO acts on its soluble guanylyl cyclase receptor, it triggers the conversion of guanosine triphosphate into cyclic guanosine monophosphate (cGMP). Diffusion of NO from the post-synaptic neuron allows it to act as a neurotransmitter. It increases glutamate, substance P, and CGRP release from the pre-synaptic terminal and triggers astrocyte- and microglia-mediated production of NO through iNOS. Combined with NMDA receptor-mediated increases pre-synaptic glutamate release, NO participates in a feedback loop that increases excitability (Cury et al., 2011; Mifflin & Kerr, 2014).

PKA, PKC, and ephrinB receptor tyrosine kinase signaling converge on the Src family kinases (SFK), which significantly upregulate NMDA receptors. Src can also mediate IL-1β upregulation of NMDA receptors (Salter & Kalia, 2004; Viviani et al., 2003).

All nociceptors use glutamate as their principal signaling molecule, which acts on three types of ionotropic receptors and a number of metabotropic receptors. Glutamate drives most
post-synaptic excitation. The ionotropic receptor types are AMPA, NMDA, and Kainate.

Modification of glutamate signaling in dorsal horn neurons is critical to sensitization.

The AMPA receptors mediate much of the excitatory post-synaptic potential at glutamatergic synapses. They are tetramers found in almost every synapse. Under normal conditions, they are Na\(^+\)-permeable, and Ca\(^{2+}\)-permeable as well, so long as they express the GluR1 or GluR3 subunits and not the GluR2 subunit. Under normal conditions, signaling is influenced by the arrangement of these receptors. Inhibitory interneurons preferentially express the calcium-permeable GluR1 subunit, while excitatory neurons express preferentially express the calcium-impermeable GluR2 subunit, resulting in relatively strong inhibition and weak excitation. GluR1/GluR2 heteromers exhibit GluR2-like properties. Intense or sustained input leads to AMPA receptor phosphorylation by PKA, PKC, CaMKII and ERK, which increases excitatory activity through increased probability of receptor opening, conductance, and trafficking, as well as a shift from calcium-impermeable GluR2/3-expressing receptors to calcium-permeable GluR1-expressing receptors at the membrane. During inflammatory pain, calcium-permeable AMPA receptors account for a significant portion of the calcium influx, comparable to NMDA receptors. This influx drives calcium-dependent intracellular signaling pathways (Luo, Kuner, & Kuner, 2014; Qin et al., 2005).

The NMDA receptors are also tetramers found in nearly every synapse, and their activation is essential to activity-dependent central sensitization and the establishment of chronic sensitization. They contain two low-affinity glycine binding NR1 subunits and two subunits from six others, NR2\(_{A,D}\) and NR3\(_{A/B}\). They are non-selective cation channels activated by simultaneous binding of glutamate and glycine, but first require depolarization of the neuron to remove a Mg\(^{2+}\) block from the channel. Sustained release of glutamate, substance P, and CGRP
into the dorsal horn synapse drives the necessary depolarization. NMDA receptor activation
increases both synaptic efficacy and intracellular calcium levels, which drive several important
intracellular mechanisms that contribute to sensitization. NDMA receptor-mediated calcium
influx may be influenced by inflammatory mediators as well. There is some evidence of the
cytokine IL-1β causing Src kinase-mediated phosphorylation of NMDA receptor subunits NR2A
and NR2B, influencing the number of NMDA receptors at the membrane. NMDA receptor
function is increased by PKA-, PKC-, ERK-, CaMKII-, and Src-mediated phosphorylation,
resulting in increased cation flow through the channel and reduced endocytosis of activated
receptors (Luo, Kuner, & Kuner, 2014; Salter & Kalia, 2004; Slack et al., 2004; Viviani et al.,
2003). The ionotropic Kainate receptors are expressed both pre- and post-synaptically. They are
activated by strong noxious stimuli (Latremoliere & Woolf, 2009).

mGlu receptors can be homo- or hetero-dimers, and their activation can have pro-
nociceptive or anti-nociceptive effects. They are found throughout the nociceptive system
(Figure 28) and mediate slower modulatory signaling compared to the ionotropic receptors.
There are eight types broken down into three families. Group I (mGluR1 and 5) couple with the
stimulatory Gq-protein and activate PLC, resulting in release of calcium from intracellular stores.
Group II (mGluR2 and 3) and Group III (mGluR 4, 6, 7, and 8) couple with the inhibitory Gqi/o-
protein, and their activation inhibits AC, activates G protein-coupled inwardly-rectifying
potassium channels, and inhibits voltage-gated calcium channels. Group I mGlu receptors are
found mostly post-synaptically, where they increase neuronal excitability. Group II and III mGlu
receptors are mostly found pre-synaptically, where they act as autoregulators to decrease
neurotransmitter release (Montana & Gereau, 2011). The group I mGlu receptors are expressed
in specific dorsal horn laminae. mGluR5 are found in laminae I-II, whereas mGluR1 are found in
lamina V. Group I mGluRs are necessary for C fiber-driven activity-dependent central sensitization, while group II and III reduce it (Chiechio & Nicoletti, 2012). Additionally, peripheral inflammation promotes mGluR1 trafficking to the synapse and mGluR5 insertion into the membrane (Pitcher, Ribeiro-da-Silva, & Coderre, 2007).
Synaptic transmission and central sensitization involve several important mechanisms in addition to glutamate, especially substance P, CGRP, and BDNF. Substance P contributes to central sensitization through temporal summation and long-lasting depolarization in the context of inflammation. NK-1 receptor activation increases intracellular calcium levels in the post-synaptic neuron by opening voltage-gated channels and activating the PLC/PKC pathways, activating post-translational and transcriptional mechanisms that contribute to sensitization. NK-1 receptor activation can also influence other G protein-coupled receptors, such as inhibiting or facilitating μ-opioid receptor endocytosis and desensitization (Steinhoff et al., 2014). NK-1 receptors are upregulated through an ERK-dependent mechanism during inflammation, increasing their contribution to central sensitization (Ji et al., 2002). CGRP is co-released with substance P. It binds to the CGRP1 receptor and potentiates the effects of substance P, increases BDNF release, and activates intracellular signaling pathways, including PKA and PKC (Latremoliere & Woolf, 2009). Substance P signaling can be blocked by the selective NK-1 receptor antagonist CP-96,345 (Snider et al., 1991; Yashpal, Pitcher, & Henry 1995).

BDNF is a neurotrophic factor and synaptic modulator that binds to the trkB receptor. It is released by primary afferents in response to intense noxious stimulation, and from microglia in response to ATP, and it contributes to central sensitization through pre-synaptic and post-synaptic mechanisms. Post-synaptically, it acts on lamina I-II receptors, causing them to autophosphorylate, and activates intracellular mechanisms, including PLC/PKC and MEK/ERK. It also increases Kv4.2 potassium channel phosphorylation, leading to increased membrane
excitability. BDNF inhibits the K⁺/Cl⁻ co-transporter (KCC2), which raises the intracellular Cl⁻ concentration and decreases or even reverses GABAₐ⁻ and glycine-mediated inhibition. When reversed, GABA and glycine signaling can contribute to depolarization (Coull et al., 2005). BDNF drives transcriptional mechanisms as well, including ERK/CREB and PI3K (Mifflin & Kerr, 2014; Pezet et al., 2008).

Bradykinin is produced in the spinal cord after intense peripheral stimulation. It contributes to central sensitization when it binds to the Gq protein-coupled B2 receptor in the dorsal horn to increase synaptic strength and activate intracellular signaling pathways, including PKA, PKC, and ERK (Latremoliere & Woolf, 2009).

Serotonin contributes to central sensitization when it binds to the ionotropic 5-HT₃ and possibly the 5-HT₇ Gs protein-coupled receptor (Latremoliere & Woolf, 2009). In addition to peripheral input, descending modulation by the 5-HT system may also contribute to ERK activation (Ji et al., 2009).

GABA and glycine mediate fast inhibitory synaptic transmission through the ionotropic GABAₐ and glycine receptors. GABA also mediates slow, long-lasting, mostly pre-synaptic inhibition through the G protein-coupled GABAₐ receptor (Figure 29). Inhibitory interneurons have several critical functions: mute activity in excitatory neurons through tonic GABA-/glycinergic inhibition, which prevents spontaneous pain; attenuate excitation through tonic, feedback, and feedforward mechanisms, where disinhibition leads to hyperalgesia; limit the spread of excitation within the spinal cord, which prevents radiating and referred pain; prevent crosstalk between nociceptive and non-nociceptive pathways by inhibiting pre-existing connections, which prevents allodynia; and prevent plasticity in nociceptive pathways by inhibiting pre-synaptic neurotransmitter and neuromodulator release, as well as inhibiting post-
synaptic voltage-gated AMPA- and NMDA-mediated \( \text{Ca}^{2+} \) conductance, which prevents chronic pain. Inhibitory interneurons require excitatory drive in order to exert their effects. This input comes from descending axons, excitatory interneurons, and primary afferent neurons. Decreased excitatory drive compromises inhibitory control (Figure 30). Glycinergic inhibition can also be inhibited by a prostaglandin-PKA mechanism in response to peripheral inflammation. When inflammation induces COX-2 and microsomal prostaglandin E synthase, PGE2 is produced in the dorsal horn. PGE2 then raises cAMP levels when it binds to the EP\(_2\) receptor. In turn, elevated cAMP levels activate PKA, and PKA phosphorylates \( \alpha 3 \) subunit-containing glycinergic receptors. This phosphorylation suppresses the inhibitory function of these receptors, resulting in disinhibition (Ahmadi et al., 2002; Luo, Kuner, & Kuner, 2014).
Figure 29. GABA-/glycinergic inhibition in the spinal dorsal horn. Several populations of neurons are found here, including GABAergic and glycinergic interneurons, and descending GABAergic and glycinergic neurons. Adapted from “Spinal Dis-inhibition in Inflammatory Pain,” by H. U. Zeilhofer and U. B. Zeilhofer, 2008, Neuroscience Letters, 437, p. 172.

Intracellular Cl\textsuperscript{−} concentration determines whether GABA and glycine are excitatory or inhibitory. Inhibitory interneurons normally inhibit nociceptive neurons post-synaptically by releasing GABA\textsubscript{A} and glycine, which bind to their receptors and open Cl\textsuperscript{−} channels. The KCC2 maintains a low Cl\textsuperscript{−} concentration in the nociceptive neuron, resulting in a more negative Cl\textsuperscript{−} equilibrium potential ($E_{Cl^{-}}$) compared to the resting potential of the neuron ($V_{Rest}$). This promotes inward flow of Cl\textsuperscript{−} into the nociceptive neuron, leading to hyperpolarization and inhibition. This post-synaptic inhibition can be reduced or even reversed by BDNF-mediated reduction in KCC2 function. In this case, the Cl\textsuperscript{−} concentration in the neuron increases and the $E_{Cl^{-}}$ becomes less negative than $V_{Rest}$. GABA\textsubscript{A} and glycine receptor activation then leads to outward flow of Cl\textsuperscript{−}, such that GABA and glycine have excitatory effects (Coull et al., 2005).

Inhibitory interneurons also exert pre-synaptic inhibition, but through a different mechanism. The sodium-potassium-chloride co-transporter-1 (NKCC1) brings Cl\textsuperscript{−} into the primary afferent terminal. This increased Cl\textsuperscript{−} concentration makes the $E_{Cl^{-}}$ less negative than $V_{Rest}$ and therefore Cl\textsuperscript{−} flows out of the neuron when GABA\textsubscript{A} receptors are activated. This has an inhibitory effect because moderate depolarization of the terminal inactivates voltage-gated calcium channels, which are necessary for neurotransmitter release. It also shunts the current of incoming action potentials (Rudomin & Schmidt, 1999). It is possible that GABA\textsubscript{A} receptor-mediated depolarization becomes larger during inflammation such that it leads to voltage-gated calcium channel activation and action potentials, which would produce excitatory pre-synaptic effects (Cervero et al., 2003; Pitcher & Cervero, 2010).

The MAP kinases are an important family of messengers that translate extracellular signals into a wide variety of intracellular responses via transcriptional and non-transcriptional mechanisms. The ERK and p38 pathways are the most important for nociception. They are
activated by noxious stimulation and inflammation, and mediate several mechanisms for the induction and maintenance of sensitization.

ERK was the first MAP kinase discovered and is the most important in inflammatory pain. pERK levels in the dorsal horn are low under normal circumstances, and ERK activation correlates with neuronal plasticity, making pERK an important marker of inflammation. C-fiber activation leads to ERK activation, which involves AMPA, NMDA, mGlu, NK-1, and trk receptors, likely through converging actions of PKA and PKC (Kawasaki et al., 2004).

Administration of inflammatory agents activates C fibers, leading to rapid (less than 1 minute), intensity-dependent ERK phosphorylation. ERK’s role in sensitization has been demonstrated by inhibition of the ERK activator MEK. This prevents second-phase hyperalgesia in the formalin test, which is associated with central sensitization, as well as LTP induced by tetanic C-fiber stimulation (Ji et al., 1999; Xin et al., 2006).

ERK is found in two isoforms, ERK1 and ERK2, and several important pain pathways converge on it (Figure 31). First, NMDA receptor activation leads to increased intracellular Ca\(^{2+}\) levels. This activates PKC and subsequently Src, which activates the Ras-Raf-1-MEK-ERK pathway at the level of Raf-1. Second, BDNF trkB receptors act directly on Ras and indirectly on Raf-1 via PI3K-Rac. Both pathways activate ERK. Third, mGlu receptors and NK-1 receptors activate an AC-dependent cascade composed of Ca\(^{2+}\)-AC1/8-cAMP-PKA-RAP-1-B-Raf-MEK-ERK. mGlu receptors and NK-1 receptors activate PLC, which cleaves membrane-bound PIP\(_2\) into DAG and IP\(_3\). IP\(_3\) binds to receptors on the smooth endoplasmic reticulum and triggers calcium release, which activates AC 1 and 8, as well as PKC. Additionally, descending serotonin input may contribute to ERK activation. ERK then activates both post-translational and transcriptional mechanisms. This leads to receptor and channel modulation, resulting in
decreased Kv4.2 conductance and increased AMPA and NMDA receptor conductance and trafficking. Once phosphorylated, ERK can enter the nucleus and activate ribosomal s6 kinases. This results in phosphorylation of CREB at the serine 133 residue. CREB binds to the cAMP response element (CRE) and initiates transcription. ERK also promotes gene expression of NK-1, COX-2, c-fos, Zif268, pro-Dyn, and trkB. These post-translational and transcriptional effects lead to induction and maintenance of central sensitization (Ji et al., 2009; Latremoliere & Woolf, 2009; Mifflin & Kerr, 2014).
**Figure 31.** ERK is activated in superficial dorsal horn neurons by multiple receptors through multiple intracellular mechanisms. ERK contributes to the induction of central sensitization through post-translational mechanisms and maintenance of central sensitization through transcriptional mechanisms. Adapted from “MAP Kinase and Pain,” by J. R. R. Ji et al., 2009, *Brain Research Reviews, 60*, p. 140.

There are four isoforms of the MAP kinase p38 (p38α, p38β, p38γ, and p38δ), but p38α and p38β are the most important for pain. p38α is the most abundant form in the DRG and spinal cord (Ji et al., 2002), and p38β is found in spinal microglia. Basal p-p38 levels are moderate and increase robustly in response to nerve injury. Many mediators and receptors have been found to activate p38, including TNFα, IL-1β, and the NK-1 receptor. When it is activated by the MAPK kinase MKK3/6, it translocates to the nucleus, where it phosphorylates transcription factors, such as activating transcription factor 2 (ATF-2). Once activated, p38 increases the synthesis of many inflammatory mediators, including TNFα, IL-1β, COX-2, and iNOS. These may be the result of transcriptional regulation, but p38 activation also triggers IL-1β release from microglia through a post-translational mechanism (Ji et al., 2009). p38 activation also leads to the production of prostaglandins (Svensson et al., 2003a,b).

The PI3K pathway is important for plasticity and the establishment of chronic sensitization. It will be discussed in detail later.

**Estrogen.** Sex differences in pain perception and the prevalence of chronic pain conditions have been well documented. Women have lower pain thresholds in response to thermal, mechanical, chemical, and inflammatory stimulation (Fillingim et al., 2009), and are at greater risk of chronic pain (Carmichael, Charlton, & Dostrovsky, 2009). However, the literature describing sex differences in pain is complex. There are many contradictory findings, making consensus difficult to reach. However, the existence of sex differences in the experience and frequency of pain is strongly supported (Bartley & Fillingim, 2013; Mogil, 2012).
There are three operationally defined types of sex differences: (1) sexual dimorphism, where a feature exists in only one sex (e.g., nursing) or is expressed differently in each sex (e.g., mating behaviors); (2) sex differences, where a feature differs quantitatively between males and females; and (3) sex convergence and/or divergence, where the sexes share a feature, but the underlying neural mechanisms are qualitatively different (McCarthy et al., 2012). Quantitative differences have received more attention in the literature, but research into qualitative findings is increasing. Qualitative differences are predicted to be more important in the long run (Mogil, 2012).

One issue is determining whether observed sex differences are organizational (due to developmentally-determined, sex-specific physiologies) or activational (due to reversibly-modulated, sex-shared physiologies, such as levels of gonadal steroids). Evidence has been found supporting both categories (Mogil, 2012). Two approaches are used to study the effects of hormones on pain. The role of endogenous hormones is studied in pre- versus post-menopausal women or intact versus OVX female animals. The individual role of estrogens is studied using hormone replacement (Amandusson & Blomqvist, 2013).

The OVX hormone model has limitations. It does not completely eliminate estrogens from the system, as it is synthesized from androstenedione in the adrenal cortex. They are also stored in fatty tissue and may be released for weeks following ovariectomy (Deslypere, Verdonck, & Vermeulen, 1985). The reduction of estrogens itself has limitations, as findings based on non-cycling female animals may not extrapolate to women, especially pre-menopausal women. Finally, although the OVX model allows for precise hormone manipulations, similar treatments can produce very different outcomes, e.g., short-term and long-term estradiol treatment have opposite effects on trkA mRNA in the DRG (Liuzzi, Scoville, & Bufton, 1999).
Together, the difficulties in hormone and nociception research represent significant obstacles to a comprehensive understanding of pain.

A connection between estrogen and pain in humans was first suggested by Herren (1933). Research since then has shown that women generally have greater sensitivity to pain, less tolerance of pain, and greater somatization. They are 2-6 times more likely to suffer from certain chronic pain conditions, including irritable bowel syndrome (IBS), temporomandibular disorder (TMD), fibromyalgia-like conditions, and chronic headaches. However, other conditions are more likely in men. The incidence of disorders appears to be related to hormones. Fibromyalgia-like conditions in women peak around menopause, and post-menopausal women suffer from joint pain and stiffness twice as frequently as pre-menopausal women. Pain sensitivity also appears to vary across the menstrual cycle, but this finding is less conclusive (Amandusson & Blomqvist, 2013).

The complexity of the hormonal and nociceptive systems precludes simple categorization into pro- vs. anti-nociceptive. There are a variety of estrogens, receptors, mechanisms, cell types and locations, as well as potential indirect effects, such as hormone-immune interactions. These systems may also be modified in different states, such as inflammation, potentiation, etc. All these factors may interact with genetics. Design issues exist as well. Typically, only one or a few factors are assessed in a given study, and the designs of these studies can vary in important ways. Insufficient samples sizes are also common (Craft, 2007; Mogil, 2012; Mogil et al., 2000).

Lack of research on females and inadequate consideration of sex differences are critical issues. Although these shortcomings have received more attention in recent decades, females remain seriously underrepresented (Fillingim et al., 2009), particularly in the preclinical pain literature (Mogil & Chanda, 2005). Avoidance of female subjects may be due in part to concerns
over estrous cycle effects, as well as inertia within the research community. Given that qualitative sex differences have been established and women are disproportionately impacted by pain conditions, researchers must do more to study female subjects and address sex differences (Mogil, 2012).

There are significant methodological issues in the investigation of sex differences. In humans, imperfect assessment of cycle stage, individual variability in hormone levels, underpowered studies are obstacles (Sherman & LeResche, 2006). In animals, a lack of time course assessments are common, limiting the detail that can be achieved. Waxing and waning estrogens are rarely studied, but could be critical for understanding a given pain condition, as is the case for migraine headaches (Craft, 2007). Short-term vs. long-term assessment is also lacking, despite the fact that fluctuating estrogens are known to have an effect on nociception (Craft et al., 2008).

Estrogens are one of the earliest known hormones at around 500 million years old (Lange, Hartel, & Meyer, 2003), and they are found throughout the animal kingdom, in fish, reptiles, amphibians, birds, and mammals. The physiological actions of estrogens display an unusual diversity in different species. Estrogens’ relationship to nociception and its diverse actions may be explained by the early developmental history of the hormones and their receptors. The estrogen receptors (ER) may have developed before the estrogen ligands and operated as a transcription factor for sensory processing, hence its connection to nociception and a variety of other cellular mechanisms (Thornton, 2001).

Ovarian hormones were discovered in 1923 (Allen & Doisy, 1923) and have been established as fundamental factors in basic cellular mechanisms in both males and females. They are involved in numerous physiological processes, especially control of reproduction and
development. Estrogens are synthesized from cholesterol in several steps (Figure 32).

Cholesterol is converted into pregnenolone, which is then converted into both progesterone and dehydroepiandrosterone. These converge and become androstenedione, which is the source of the important hormone testosterone, as well as the less potent estrogen estrone. Testosterone is aromatized into 17β-estradiol, which is the most potent and biologically active estrogen. It is primarily produced in the ovaries, but the aromatase is present in other tissues, including the brain. Estrogen is released in response to a hormonal cascade. Gonadotropin-releasing hormone (GnRH) is released from the hypothalamus. This triggers the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). These two hormones trigger the secretion of estrogen (Amandusson & Blomqvist, 2013).

Figure 32. Estrogen is synthesized from cholesterol through a number of steps and multiple mechanisms. Adapted from “Estrogenic influences in pain processing,” by Å. Amandusson & A. Blomqvist, 2013, *Frontiers in Neuroendocrinology, 34*, p. 330.
Until the 1950s, it was widely thought that estrogens exert their intracellular effects by a metabolic process. This consensus was overturned by the discovery of an estrogen receptor mechanism. The ERs can be divided into the nuclear receptors and membrane receptors, which are found in neurons and glia. Estrogens are lipid soluble and can diffuse into the cell and activate either receptor type (Amandusson & Blomqvist, 2013). Their action may or may not be dose-dependent. Both have been observed (Craft, 2007).

Estrogens work through multiple mechanisms (Figure 33). The nuclear ERs are part of the genomic (or classic) pathway. They are categorized as α or β type, which were cloned in 1985 and 1996, respectively. Both 17α-estradiol and 17β-estradiol bind more strongly to the ERα type. However, the ERβ is more associated with inflammatory and hyperalgesic processes. When they bind to their ligand, nuclear ERs are transported to the nucleus and bind to estrogen response elements (EREs) found in the promoters of target genes. Nuclear ERs can also activate other transcription factors rather than binding to DNA (Björnström & Sjöberg, 2005). This transcriptional mechanism takes minutes to hours to exert an effect. Additionally, as phosphoproteins, ERs can be activated by phosphorylation in the absence of a ligand. In the non-genomic (or novel or alternative) pathway, estrogens bind to ionotropic and G protein-coupled membrane receptors and activate multiple second messenger pathways. These include the MAPK, CREB, PI3K, and PKC pathways, as well as direct Ca^{2+} channel modulation (Ho & Liao, 2002; McEwen, 2001). These mechanisms can also influence the estrogen receptors. The non-genomic pathway does not depend on protein synthesis and therefore can work very rapidly (within seconds). It can also influence second messenger pathways that promote transcription (Amandusson & Blomqvist, 2013; Spooner et al., 2007). These pathways are not independent.
Rapid signaling via membrane-bound ERs can enhance nuclear ER-mediated transcription (Gintzler & Liu, 2012).

Figure 33. Estrogens operate through multiple intracellular mechanisms. The genomic pathway involves estrogen receptors in the cytoplasm and nucleus. Binding to these receptors initiates transcription. The non-genomic pathway involves membrane receptors, which modify ion channels and receptors, as well as intracellular mechanisms that can lead to transcription. Adapted from “Estrogenic Influences in Pain Processing,” by Å. Amandusson and A. Blomqvist, 2013, Frontiers in Neuroendocrinology, 34, p. 331.

ERs are found both post-synaptically and pre-synaptically at the primary afferent to dorsal horn synapse, allowing both direct and indirect modulation of nociception through
multiple mechanisms. ERs in the DRG have been shown to inhibit ATP-induced increases in intracellular calcium (Chaban & Micevych, 2005). Estradiol may also affect nociception mediated by prostaglandins or cytokines (Kuba et al., 2010a,b; Hunter et al., 2011b).

In the spinal cord, ERs are found in the superficial dorsal horn, with heavy concentration in lamina II. Activation inhibits A\(\delta\) and C fiber-mediated nociceptive transmission at the dorsal horn synapse (Zhong, Li, & Zhang, 2010). Lamina II is a major site of nociceptive processing, where incoming signals from primary afferents and descending signals from descending axons meet and are heavily modulated by interneurons. Lamina II circuits help set the excitability level of the dorsal horn (Amandusson & Blomqvist, 2013).

ERs can directly modulate signaling at the dorsal horn synapse to produce a number of important effects: increased pre-synaptic glutamate release; increased NMDA receptor currents via a non-genomic, likely ER\(\beta\)-mediated, mechanism; enhanced sensitization, involving the calcium-PKA-ERK signaling pathway; interactions with NMDA and AMPA receptor subunits, suggesting modulation of both signaling and plasticity; and enhanced steady-state depolarization. Estrogens can have a profound effect on nociception, given this combination of effects at such a critical juncture in the nervous system (Zhang et al., 2012).

Studies of thermal and inflammatory pain have yielded mixed results. Many show anti-nociceptive and anti-inflammatory effects. In rat studies comparing OVX and hormone-replacement females, a 20% concentration of 17-\(\beta\)-estradiol 3-benzoate in a cholesterol vehicle has been found most effective at reducing inflammatory responses (compared to 10% and 30%) (Hunter et al., 2011b; Mannino et al., 2005). Estradiol has been shown to attenuate formalin-induced nociception associated with peripheral and central inflammation (Hunter et al., 2011a; Kuba, Kemen, & Quinones-Jenab, 2005; Kuba et al., 2006; Kuba et al., 2010b; Mannino et al.,
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2007), as well as carrageenan-induced hyperalgesia (Hunter et al., 2011b). Formalin-induced corticosterone (an anti-inflammatory steroid hormone) is higher in estradiol-treated rats compared to OVX (Mannino et al., 2007). Estradiol decreases peripheral PGE2 release (Kuba et al., 2010a). Estradiol deprivation has been shown to increase inflammatory responses, whereas its replacement blocks them (Ghisletti et al., 2005). Estradiol has also been shown to decrease pro-inflammatory cytokines and increase anti-inflammatory cytokines (Shivers et al., 2015), though estradiol-mediated antinociception has been observed in both the presence and absence of inflammation (Hunter et al., 2011b; Li et al., 2009). However, other studies show pro-inflammatory or pro-nociceptive estrogen effects. Female rats and mice exhibit greater sensitivity during phase II of the formalin test, which is associated with central sensitization (Mifflin & Kerr, 2014). TRPV1 findings have been diametrically opposed. Lu et al. (2009) found that 17β-estradiol administration increases capsaicin-induced pain responses via the E2 receptor through a PKA- and PKC-independent mechanism, whereas Xu et al. (2008) found that it inhibits capsaicin-induced signaling. A coherent, comprehensive account of the role of estrogens in nociception remains distant.

Estrogen receptors and aromatase are present in several brain areas associated with nociception, including the parabrachial nucleus, nucleus of the solitary tract, PAG, raphe nucleus, locus coeruleus, and limbic system. ERs are widely distributed in the brain. PAG-RVM receptors influence the anti-nociceptive effects of opioids in connection with the ON- and OFF-cell systems (Bernal, Morgan, & Craft, 2007). Estrogens may also influence the emotional-aversive qualities of pain by modulating activity in the ACC. Pain-related conditioned place preference is prevented in rats treated with ER inhibitors (Xiao et al., 2012). Estrogens and opioids will be described below.
Unbound ERs are found in the cytoplasm and nucleus, held in an inactive state by heat-shock proteins. Upon ligand binding, these proteins dissociate and expose the DNA-binding domain. The receptor is transported to the nucleus where it binds to the ERE DNA sequences and activates transcription. Estrogens can trigger the synthesis of a variety of proteins, depending on the co-regulators and transcription factors involved. After transcription, the ERs dissociate from the DNA and become inactive by binding to heat-shock proteins (Amandusson & Blomqvist, 2013).

Estrogens influence the opioid system. Gonadal hormones can modulate formalin-induced pain, and this effect appears to be mediated by opioids in the spinal cord (Gaumond, Spooner, & Marschand, 2007). There is evidence of hormonal influence in humans, such as sex differences in analgesia, including those evoked by descending monoaminergic pathways, opioids, and stress (Fillingim et al., 2009). Women exhibit analgesia during pregnancy (Gupta et al., 2007), μ-opioid activation in response to sustained pain is estradiol-dependent (Smith & McMahon, 2006), and women in a low-estradiol state also have less capacity for μ-opioid receptor activation compared to men (Zubieta et al., 2002). The opioid system in females is considered to be less robust and effective than in males (Gupta et al., 2007; Mitrovic et al., 2003). This may be explained in part by complexity. Anti-nociception in female rats requires activation of both μ- and κ-opioid receptors in the spinal cord, whereas in male rats it requires activation of only μ-opioid receptors (Liu, von Gizycki, & Gintzler, 2007). The female system may be more prone to malfunction, given that more components have to function properly (Gintzler & Liu, 2001).

Estrogen-opioid interaction occurs in the spinal cord, among other areas. ERα, the G protein-coupled estrogen receptor 30 (GPR30), and opioid receptors are frequently co-localized
in the superficial dorsal horn in female rats (Liu et al., 2011), and up to 70% of lamina II enkephalinergic superficial dorsal horn neurons express ERα (Amundusson et al., 1996). The preproenkephalin gene has EREs that bind ERα in its promoter region (Zhu & Pfaff, 1995), and acute subcutaneous estradiol administration can induce enkephalin gene expression in the dorsal horn of OVX rats (Amandusson et al., 1999). Enkephalin expression in the brain occurs within 2-4 hours and lasts longer than 8 hours, indicating a genomic ER mechanism, but may not last as long in the spinal cord (Priest, Eckersell, & Micevych, 1995). This is important in nociception because these enkephalinergic neurons are thought to be inhibitory interneurons (Fields & Basbaum, 2005; Ma et al., 1997). Estrogens can affect the opioid system through other mechanisms as well, such as modulation of opioid receptor expression and activity. μ-opioid receptor expression in female rats varies across the estrous cycle and during estrus it is significantly lower than male expression in the parabrachial area (Murphy et al., 2009) and the PAG (Loyd, Wang, & Murphy, 2008), areas associated with ascending and descending nociceptive pathways. ERs in the PAG and RVM mediate sex differences in morphine-induced anti-nociception, such that opioids have a smaller effect in females. However, this is cycle-dependent. Smaller nociceptive effects are found in the estrus, but not diestrus phase (Bernal, Morgan, & Craft, 2007).

There are several complications that make interpreting the above findings difficult. Estrogen administration does not always induce enkephalin production in neurons expressing both estrogens and opioids. The cells affected by estrogens may also fall into subcategories, where some have estrogen-dependent preproenkephalin gene expression and others have estrogen-independent expression. Other categories may be controlled by different interactions between ERs and other steroid receptors. These findings may also be complicated by
methodology. Estrogen levels are often brought to supraphysiological levels in gene expression studies and this might decrease generalizability of the results. However, lower estrogen levels are sufficient to induce gene expression and influence nociception. Interpreting results in these studies can also be complicated by disparities in systemic and local estrogen levels. Estrogen levels in the brain can be elevated beyond the systemic level by accumulation and local synthesis (Amandusson & Blomqvist, 2013).

Extrapolation from rodent research to human applications is difficult. They have similarities, including the distribution of ERα. It is comparable, but less abundant and widespread in humans (Vanderhorst, Terasawa, & Ralston, 2009). There are common genetic, molecular, and chemical features, but these produce diverse physiological outcomes. This diversity may stem from differences in transcription factors (Odom et al., 2007). Humans and rodents have similar hormone cycles, but they are not directly comparable. These difficulties are not limited to translational research. Pain and opioid studies produce various outcomes even among subpopulations of rodents (Craft, 2003; Mogil et al., 2000).

Longer life expectancies mean that women spend more time in a post-menopausal, estrogen-depleted state. Estrogens are lipophilic, therefore they can enter cells easily and are involved in numerous mechanisms. This raises questions regarding the risks of side effects in hormone therapy. Current research suggests that it has a positive impact on health, including coronary heart disease and mortality (Lobo, 2016).

Estrogens affect substance P signaling, and vice versa. Nazarian et al. (2014) found that estradiol increased substance P signaling. They suggest several possible explanations for the sex difference in substance P signaling, depending on the location. Estradiol could influence primary afferent release of substance P by acting on receptors on the primary afferents, modulating...
descending inhibition, or controlling local circuits in the dorsal horn. Gonadal hormones appear to regulate substance P levels in the spinal cord (Duval et al., 1996), and direct estradiol control of neuropeptides has been demonstrated in DRG neurons. OVX rats have increased pain sensitivity compared to hormone-treated rats, and estradiol administration influences neuropeptide levels in DRG neurons. Substance P is downregulated, while CGRP is upregulated (Sarajari & Oblinger, 2010). Alternatively, substance P signaling may modulate aromatase activity and estradiol concentration in the dorsal horn by increasing intracellular calcium. More generally, estrogens overlap with substance P in major intracellular pathways, including calcium, PKA, PKC, and ERK (Amandusson & Blomqvist, 2013).

The PI3K-Akt-mTOR pathway. The PI3K-Akt-mTOR signaling pathway regulates synaptic plasticity in the CNS and plays an important role in central sensitization. The PI3K family is highly conserved in evolution. A single PI3K gene is found in yeast, whereas there are at least eight PI3K genes in mammals (Engelman, Luo, & Cantley, 2006). The pathway is activated by nociceptive stimulation, inflammation, and intracellular mechanisms, including elevated calcium (Pezet et al., 2008). Upon activation, PI3K acts on PIP_2 to produce the lipid second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), which regulates the phosphorylation of Akt, locking it in its active conformation (pAkt) (Hirsch et al., 2008). mTOR (specifically mTORC1) operates downstream of Akt and is found in sensory fibers and dorsal horn neurons (Xu et al., 2011). mTOR is the most important factor in the PI3K-Akt-mTOR cascade. The term mTOR has been updated from “mammalian” to “mechanistic” target of rapamycin, but the former remains widely used. It is a master regulator of protein synthesis and synaptic plasticity (Hoeffer & Klann, 2010). It promotes formalin pain behaviors, ERK
activation, and c-Fos expression (Yu et al., 2012), and influences the release of pro-inflammatory cytokines by regulating the activity of astrocytes and microglia (Dello Russo et al., 2013).

mTOR is encoded by a single gene. It combines with other proteins to form two complexes, mTORC1 and mTORC2, which participate in different mechanisms and have different functions. mTORC1 can be activated by a variety of molecules and conditions, including growth factors, cytokines, energy status, oxygen, and amino acids (Laplante & Sabatini, 2012). It is an important factor in protein synthesis mediated by the eukaryotic initiation factor-4E-binding protein (4E-BP1) and S6 kinase 1 (S6K1) pathways. 4E-BP1 is normally bound in an inactive state, but once activated by mTORC1, it can promote the translation of specific mRNAs by loading ribosomes onto them. mTORC1 also phosphorylates S6K1, which contributes to protein synthesis mediated by several effectors, including ribosomal S6 protein. These proteins help regulate peripheral and central sensory neuron activity. The ribosomal S6 protein also reduces the activity of trk receptors by phosphorylation, which reduces the activity of Akt and the Ras-Raf-MEK-ERK pathway, thus forming an inhibitory feedback loop (Lisi et al., 2015).

Several important cascades regulate mTORC1 activity, including the PI3K and Ras-Raf-MEK-ERK pathways (Figure 34). These pathways converge on tuberous sclerosis 1/2 (TSC1/2), a heterodimer protein complex that controls mTORC1 activation by inhibiting Ras homolog enriched in brain (Rheb), a GTP-binding protein. When it is active, Rheb binds to mTORC1 and activates it. However, TSC1/2 holds Rheb in its inactive GDP-bound state. When one of the upstream signaling pathways phosphorylates TSC1/2, it degrades and allows Rheb to activate mTORC1, promoting protein synthesis. mTORC1 can be activated by additional pathways, including cytokines, intracellular amino acids, and PI3K via Akt (Laplante & Sabatini, 2012).
mTORC2 is not as well understood as mTORC1. Growth factors can activate it via PI3K, and mTORC2 activates Akt and a number of other intracellular messengers (Figure 35). mTORC2 has a complex relationship with mTORC1 featuring multiple interacting mechanisms (Figure 36). Several important components can be highlighted within this system, which are important to mTORC1-regulated protein synthesis. TSC1/2 inhibits mTORC1 and activates...
Therefore when PI3K, Akt, and ERK degrade TSC1/2, it simultaneously activates mTORC1 and inhibits mTORC2. mTORC1 also inhibits mTORC2 by activating the S6 kinase 1 and increasing its inhibitory influence on mTORC2. However, there is a negative feedback loop between mTORC1 and mTORC2. mTORC2 activates Akt, which in turn activates mTORC1. Therefore inhibition of mTORC2 reduces Akt’s degradation of TSC1/2 and direct excitatory input into mTORC1. Also, PI3K directly activates mTORC2 by phosphorylating it, meaning that PI3K activates Akt both directly and via mTORC2. As stated above, mTORC1 activation of the S6 kinase 1 also activates ribosomal S6 protein, which inhibits the activity of PI3K and ERK1/2 via trk, therefore reducing the excitation of mTORC1. This system allows for very complex regulation of protein synthesis (Lisi et al., 2015).
Figure 35. mTORC2 regulation. mTORC2 can be activated by growth factors via phosphatidylinositol 3-kinase (PI3K). mTORC2 then activates multiple intracellular mechanisms. Adapted from “mTOR Kinase: A Possible Pharmacological Target in the Management of Chronic Pain,” by L. Lisi et al., 2015, *BioMed Research International*, 2015, p. 4.

![mTORC1 and mTORC2 relationship diagram](image)

*Figure 36. mTORC1 and mTORC2 have a complex, interactive relationship. Adapted from “mTOR Kinase: A Possible Pharmacological Target in the Management of Chronic Pain,” by L. Lisi et al., 2015, *BioMed Research International*, 2015, p. 4.*

mTOR inhibitors are useful in investigations of protein synthesis, rapamycin being the most commonly used. It crosses the blood-brain barrier, but does not affect all mTOR-related functions. It is mostly selective for mTORC1, where it disrupts the association of raptor from the complex. Wortmannin, another inhibitor, was originally described as a PI3K inhibitor, but works on mTOR as well. It blocks both mTORC1 phosphorylation of S6K1 and mTORC2 phosphorylation of Akt (Lisi et al., 2015).

mTOR is found peripherally and centrally in normal and inflammatory states. It and its downstream effectors are found in the bodies of peptidergic and non-peptidergic C fibers, the axons of non-peptidergic A fibers, and non-neuronal cells. The effectors are found in the dorsal horn as well, and their phosphorylation is associated with secondary hyperalgesia and allodynia.
The mTOR effector 4E-BP1 has also been found in glial fibrillary acidic protein- (GFAP-) positive satellite glial cells, which surround the cell bodies of sensory neurons in the DRG. They contribute to the development of chronic pain, likely through CGRP receptor-mediated increases in IL-1β, COX-2, and iNOS expression and activity (Lisi et al., 2015).

The mTOR pathway does not appear to be involved in acute nociception. mRNA for mTOR, 4E-BP1, and S6 kinase have been found in the DRG and dorsal horn, but their active forms are found at very low or undetectable levels. mTOR, 4E-BP1, and S6 kinase are found throughout the dorsal horn and are concentrated in the superficial laminae, but intrathecal rapamycin administration does not affect nociceptive behavior (Xu et al., 2010).

The mTOR pathway is involved in persistent pain states, where it has both peripheral and central actions. In the periphery, activated mTOR is present in Aδ fibers, but not C fibers, and it modulates the sensitivity and signaling of these fibers at the dorsal horn. In the dorsal horn, high levels of activated mTOR are present in projection neurons in the superficial laminae, which are associated with the induction and maintenance of chronic pain. mTOR also contributes to central sensitization through plasticity at the level of the WDR neurons in lamina V. Intrathecal rapamycin administration decreases mTOR activation in both Aδ fibers and dorsal horn projection neurons, and decreases pain behavior during phase II of the formalin test. Despite its absence from C fibers, mTOR has an important role in inflammatory hyperalgesia mediated by C fibers. Intraplantar injection of capsaicin and carrageenan activates C fibers and triggers thermal hyperalgesia. This hyperalgesia is inhibited by intrathecal rapamycin, indicating central mTOR involvement. Capsaicin and carrageenan also trigger significant S6 phosphorylation in the dorsal horn, which reliably indicates mTOR activation, and therefore plasticity. This effect is inhibited by intrathecal rapamycin. Complete Freund’s adjuvant (CFA) has been found to activate mTOR
in the DRG and, to a smaller degree, the dorsal horn (Géranton et al., 2009; Lisi et al., 2015). Carrageenan injection triggers substance P-dependent PI3K-Akt activation, which promotes glutamate receptor trafficking. However, it has been shown that these mechanisms work separately. Destruction of NK-1 receptor-expressing superficial dorsal horn neurons by saporin conjugated to substance P blocks Akt phosphorylation, but not glutamate trafficking (Choi, Koehrn, & Sorkin, 2012).

Xu et al. (2011) demonstrated that central sensitization induced by carrageenan inflammation is mediated by the PI3K-Akt-mTOR pathway. This was found especially in the superficial laminae of the dorsal horn, where we might expect nociceptive plasticity to occur. Intrathecal administration of PI3K and mTOR inhibitors (wortmannin and rapamycin, respectively) was shown to attenuate nociception in phase II of the formalin test, as well as thermal hyperalgesia and tactile allodynia. Substance P administration and inflammation triggered hyperalgesia and activation of the PI3K pathway in NK-1 receptor-expressing nociceptive projection neurons (Figure 37). These effects were blocked by PI3K and mTOR inhibition. PI3K inhibitors can even attenuate established hyperalgesia. Wortmannin treatment 130 minutes after carrageenan injection produced a dose-dependent anti-hyperalgesic effect. Like many other findings in basic pain research, mTOR inhibitors have failed to translate into an effective therapeutic treatment (Lisi et al., 2015).
Figure 37. C fiber input to the superficial dorsal horn may increase nociceptive projection neuron excitability. Substance P operates on the NK-1 receptor, initiating the PI3K-Akt-mTOR signaling pathway, which is involved in synaptic plasticity. Adapted from “Spinal Phosphoinositide 3-kinase-Akt-mammalian Target of Rapamycin Signaling Cascades in Inflammation-induced Hyperalgesia,” by Q. Xu, 2011, The Journal of Neuroscience, 31(6), p. 2122.

Interactions between estrogens and the PI3K pathway have been found in a number of contexts. Estradiol activates PI3K through genomic ERα-, but not ERβ-mediated signaling in
breast cancer cells (Lee et al., 2005). This is consistent with findings in mice, where estradiol exposure activates PI3K in mouse endothelial cells, but not ERα/β-knockout mouse endothelial cells (Pedram, Razandi, & Levin, 2006). In some cases, estradiol works in combination with intracellular messengers. Estradiol and ERK have been found to induce Akt phosphorylation synergistically in the hippocampus (Cardona-Gomez, Mendez, & Garcia-Segura, 2002). The estradiol-PI3K link is directly relevant to inflammation. Estradiol inhibits inflammatory gene transcription by inhibiting NF-κB intracellular transport through a PI3K-dependent ERα mechanism (Ghisletti et al., 2005). A pro-inflammatory effect was found in the context of cross-organ pain and sensitization between the bowel and urethra. PI3K-mediated Akt phosphorylation and resulting NMDA receptor phosphorylation were dependent on genomic estradiol signaling (Peng et al., 2010).

Estradiol can interact with PI3K through non-genomic mechanisms as well, including rapid PI3K activation via membrane-bound receptors (Vasudevan & Pfaff, 2007), and the G protein-coupled estrogen receptor GPR30 found on the endoplasmic reticulum, which triggers calcium release and PI3K activation (Revankar et al., 2005). Additionally, non-genomic ER signaling can modulate nuclear ER-mediated transcription via PI3K (Gintzler et al., 2012). PI3K-Akt can activate ERα through estrogen-dependent and estrogen-independent mechanisms in breast cancer cells (Campbell et al., 2001).

**Pain assay.** Carrageenan injection into the plantar aspect of the rodent paw was first used by Winter, Risley, and Nuss (1962) and has since become a common method used to study inflammatory pain. Carrageenans are high molecular weight sulfated polygalactans derived from several species of red seaweeds, and work through toll-like receptor 4. They possess strong inflammatory properties and cause heightened sensitivity to thermal and mechanical stimuli
Previous research has established behavioral hypersensitivity by 4 hours following an injection of carrageenan, lasting up to 96 hours (Kayser & Guilbaud, 1987). Carrageenan administration triggers sensitization of A- and C-fiber nociceptors, and central sensitization (Lisi et al., 2015). It involves ERK activation and potentiation of TRPV1 thermal nociception (Dubin & Patapoutian, 2010; Galan et al., 2002). Females exhibit greater thermal hyperalgesia in response to carrageenan (Tall & Crisp, 2004).

**Study.** Chronic pain is a pervasive medical problem that affects over 50 million Americans, costs over $70 billion annually, and contributes to psychiatric disorders and emotional suffering (Gatchel et al., 2007). Sex differences in pain perception and prevalence of chronic pain conditions have been well documented. Women report more intense pain, more frequently than men, as well as longer lasting pain. Although the underlying mechanisms for sex differences in pain perception are not fully understood, sex hormones play an important role in influencing pain sensitivity (Fillingim et al., 2009). Greater understanding of the nociceptive system and the role of sex hormones might lead to more effective treatment and prevention of chronic pain, particularly in women.

Long-term peripheral nociception leads to reorganization of spinal cord circuitry, resulting in central sensitization. This process involves a cascade of events, including release of inflammatory mediators, increased proinflammatory peptides in the DRG, and long-term potentiation and windup in the spinal cord (Mendell, 2010). Pain signals from the periphery trigger the release of substance P, which acts on the NK-1 receptor in the dorsal horn of the spinal cord. NK-1 receptor activation stimulates the PI3K-Akt-mTor signaling pathway, which is involved in spinal sensitization (Xu et al., 2011). Estradiol affects substance P (Sarajari & Oblinger, 2010). This is a possible mechanism for estradiol's effects on central sensitization in
the context of inflammation (Figure 38). Though the literature is mixed, many studies report anti-nociceptive and anti-inflammatory effects of estradiol. Therefore, we predicted that estradiol would reduce pain responses by decreasing substance P signaling and PI3K-Akt-mTOR pathway activation.

Figure 38. Proposed model. Inflammation-induced increase in substance P release from the central terminals of the peripheral nociceptive neurons is thought to drive central sensitization via the PI3K cascade. We hypothesize that estradiol exerts its anti-nociceptive effects on nociception and central sensitization in part by inhibiting substance P-mediated activation of the PI3K cascade. Adapted from “Spinal Phosphoinositide 3-kinase-Akt-mammalian Target of Rapamycin Signaling Cascades in Inflammation-induced Hyperalgesia,” by Q. Xu, 2011, The Journal of Neuroscience, 31(6), p. 2122.
Five aims were established to test these hypotheses:

Aim 1: Test for an estradiol effect on nociception as indicated by a behavioral pain assay

Aim 2: Test for an estradiol effect on PI3K-Akt-mTOR pathway activation as indicated by protein phosphorylation

Aim 3: Test for an estradiol effect on substance P signaling as indicated by NK-1 receptor internalization

Aim 4: Determine whether estradiol’s effect on nociception is mediated by the PI3K pathway by inhibiting PI3K with wortmannin

Aim 5: Determine whether estradiol’s effect on nociception is mediated by substance P by inhibiting the NK-1 receptor with CP-96,345

Method

Experiment 1 used a 2 (hormone condition) X 2 (pain condition) X 2 (delay condition) design, with n = 4 per group. Behavioral data from a related study were included, yielding n = 10 per group in that analysis. Subjects were implanted with either a cholesterol or estradiol capsule, given either an inflammation-inducing or benign injection, and administered a pain assay 5 or 24 hours later. Sacrifice and tissue dissection followed.

Experiment 2 employed a simplified 2 (hormone condition) X 2 (delay condition) design, yielding n = 12 per group. Subjects were implanted with either a cholesterol or estradiol capsule, given an inflammation-inducing injection, and administered a pain assay 1 or 4 hours later. Sacrifice and tissue dissection followed.
**Subjects.** Experiment 1 (N = 32 for proteins, N = 80 for behavior) and Experiment 2 (N = 48) used OVX female Sprague-Dawley rats purchased from Taconic (Germantown, NY). They were double-housed with free access to food and water, and maintained on a 12-hour light/dark photoperiod (lights on at 8am EST). Rats were allowed 1 week of acclimation after delivery before the start of the study. NIH guidelines for care of laboratory animals were followed. All procedures were approved by the Hunter College Institutional Animal Care and Use Committee.

**Hormone Replacement.** SILASTIC capsules were surgically implanted under the skin at the nape of the neck 1 week prior to testing. The capsules were 1 cm long x 0.058 in ID/0.077 in OD (Dow Corning). The capsules contained Cholesterol (5-Cholesten-3 Beta-ol; Sigma-Aldrich, St. Louis, MO) or Beta-Estradiol (1, 3, 5 [10]-Estratriene-3, 17 Beta-diol; Sigma Aldrich) in the cholesterol vehicle.

Rats were randomly assigned to conditions in pairs, so that cagemates had the same type of implant (and, in Experiment 1, the same type of injection). They were anesthetized with inhaled Isoflurane (2.5% Isoflurane to 0.5-1.0L O₂). Half of the rats received capsules filled with a mix of 20% Beta-Estradiol and 80% Cholesterol. The other half received capsules filled with 100% cholesterol. They were allowed 1 week recovery time before the next phase of the study.

**Behavioral Testing.** Pain thresholds were assessed by measuring paw withdrawal latencies (PWL) using a Hargreaves Box Paw Thermal Stimulator (Department of Anesthesiology, University of California, San Diego) (Hargreaves et al., 1988). The apparatus consisted of a box with a 65x30 cm heated glass surface maintained at 30 °C +/- 0.1 °C. Underneath this surface, a moveable 9v halogen projection bulb was used to apply heat to the intraplantar surface of the paw. Three heat intensities were used, calibrated to 4.5, 4.9, and 5.3mV, respectively. These correspond to 5.0, 5.5 and 6.0 amps. With these settings, the surface
of the glass reached 62.6 °C on the low, 79.6 °C on medium, and 88.9 °C on high (Dirig et al., 1997). Rats were placed in adjoining Plexiglas chambers measuring 9x22x25cm and were free to withdraw their paw from the heat stimulus. The apparatus automatically recorded PWL in seconds. The stimulus immediately turned off when the withdrawal reflex occurred, i.e., at the pain threshold. If a rat failed to withdraw its paw, the stimulus turned off automatically after 20.48 seconds to avoid tissue damage.

Subjects were placed into the Hargreaves apparatus and allowed 30 minutes acclimation prior to baseline testing. In Experiment 1, all 4 subjects were placed into the apparatus simultaneously, whereas in Experiment 2 they were run on a staggered pattern, with up to 4 rats in the apparatus simultaneously. Baseline pain thresholds were assessed on low, medium, and high heat intensities. Thresholds were calculated for both left and right hind paws. Low-intensity thresholds were collected, then medium, then high, such that there was a delay of 3-5 minutes between testing on each intensity level.

Immediately after baseline testing, the rats were injected in the intraplantar region of the right hind paw. In Experiment 1, injection was either 100 µl carrageenan (1% carrageenan; Sigma) or 100µl saline (100% Saline; Braun Medical, Irving, CA). In Experiment 2, all rats were injected with 100 µl carrageenan. They were then returned to their cages. Rats were then retested after the duration indicated in their delay condition (incorporating reacclimation time into this duration). In Experiment 1, rats in the 5-hour and 24-hour groups were placed back in the testing apparatus and allowed 60 and 30 minutes, respectively, to reacclimate. In Experiment 2, rats in the 1-hour and 4-hour groups were placed back in the testing apparatus and allowed 15 minutes to reacclimate. Pain thresholds were then reevaluated using the same procedures as described above.
**Tissue Collection and Sample Preparation.** After behavioral testing, rats were placed individually in a 30x23x28cm Plexiglas chamber with dry ice for approximately 30 seconds. After exposure to CO$_2$, rats were decapitated using a guillotine (Kent Scientific Corporation, Torrington, CT) and the brain and lumbar region of the spinal cord were dissected. The tissue was flash frozen in 2-methylbutane (-40 °C) and stored at -80 °C. The delay between behavioral testing and tissue dissection was highly variable in Experiment 1, ranging between a few minutes and 2 hours. The delay was consistently a few minutes in Experiment 2.

Tissue samples were manually homogenized in a Lysis buffer (50mM Tris-HCl (pH 7.5), 150 nM NaCl, 2mM EDTA, 50ml 10% glycerol, 5ml 1% triton x100, 5ml 1% NP40, and 5g sodium deoxycholate). The following protease inhibitors were included in the buffer: leupeptin, pepstatin, aprotinin, DTT, PMSF, NaF, and NaVO$_3$. Total protein count was measured using a Bradford kit from Bio-Rad Laboratories (Hercules, CA).

For Western blot analysis, equal amounts of protein extracts (50µg) were boiled for 5 minutes in Laemmli buffer containing 1% β-mercaptoethanol and loaded onto Bio-Rad 10% mini-PROTEAN TGX precast polyacrylamide gels (Hercules, CA). Gels were electrophoresed and transferred to Bio-Rad Immun-Blot PVDF membranes. The membranes were blocked for 60 minutes using non-fat dry milk in tris-buffer-saline-tween (TBST) at room temperature. The membranes were then washed three times in TBST and probed overnight at 4 °C with various primary antibodies (1:3000) in a non-fat dry milk and TBST solution. The next day, membranes were washed four times in TBST and probed for 60 minutes at room temperature with the appropriate secondary antibodies (1:1000) in a non-fat dry milk and TBST solution. Finally, the membranes were washed five additional times.
Antibody binding was detected using a GE Healthcare enhanced chemiluminescence kit and high performance chemiluminescence film (ECL Plus and Amersham Hyperfilm ECL; Little Chalfont, United Kingdom). The resulting films were scanned and quantified with a computer densitometer and Image Quant Program (Molecular Dynamics). In order to normalize protein levels, the membranes were reprobed with α-tubulin (1:1000) for 60 minutes. The ratio of phosphorylated or total proteins over α-tubulin was used to calculate protein levels.

**Data Analysis and Figures.** Protein levels were quantified using ImageJ, measuring the area under the peaks. The scores produced by ImageJ were normalized relative to α-tubulin. Behavioral and protein data were analyzed using Microsoft Excel and IBM SPSS. Baseline behavioral data were analyzed using independent-measures t-tests. In Experiment 1, both behavioral and protein data were analyzed using 3-factor ANOVA procedures. PWL data were normalized to mean baseline values within each heat intensity level and expressed as percentages thereof. Behavioral and protein data were analyzed using correlation. Protein scores were normalized relative to the ratio of 24-hour and 5-hour protein scores, and the ratio of estradiol and cholesterol protein scores. These normalized protein scores were then correlated with PWL. In Experiment 2, behavioral data were analyzed using 2-factor ANOVA. Unless otherwise indicated, data from only the injected (right) paw were analyzed. In all conditions, values that fell beyond two standard deviations above or below the mean were considered outliers and excluded.
Results

Experiment 1

**Paw weight.** Levene's test for equality of variances was significant $[F(7, 27) = 3.42, p = .010]$. As shown in Figure 39, significant interactions were observed between pain treatment and time $[F(1, 27) = 21.45, p < .001, \text{partial } \eta^2 = .44]$. Using pairwise comparisons based on the Least Significant Difference (LSD), significant differences were found in the following conditions: for 5-hour, mean paw weight in the carrageenan conditions was significantly higher than in the saline conditions ($p < .001$). Similarly, for 24-hour, mean paw weight in the carrageenan conditions was significantly higher than in the saline conditions ($p = .002$). For carrageenan, mean paw weight in the 5-hour conditions was significantly higher than in the 24-hour conditions ($p < .001$). Significant main effects were observed for pain condition $[F(1, 27) = 85.82, p < .001, \text{partial } \eta^2 = .76]$ and time $[F(1, 27) = 20.59, p < .001, \eta^2 = .43]$. See Table 1.

**Baseline PWL.** As shown in Figure 40, no differences in mean baseline PWL were found between the estradiol and cholesterol conditions at any temperature level; low heat intensity: $t(84) = -1.27, p = .208$; medium heat intensity: $t(84) = 0.41, p = .681$; high heat intensity: $t(85) = -0.96, p = .337$. Baseline data include both left and right paw measurements. See Table 2.
Table 1

*Means, Standard Deviations, Sample Sizes, and Standard Errors of the Mean for Paw Weight*

*Data by, Time, Pain, and Hormone Condition*

<table>
<thead>
<tr>
<th>Time</th>
<th>Pain</th>
<th>Hormone</th>
<th>Mean</th>
<th>Std Dev</th>
<th>n</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-hour</td>
<td>Saline</td>
<td>Vehicle</td>
<td>1.53</td>
<td>0.10</td>
<td>4</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Estradiol</td>
<td>1.43</td>
<td>0.15</td>
<td>4</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Carrageenan</td>
<td>Vehicle</td>
<td>2.73</td>
<td>0.43</td>
<td>4</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Estradiol</td>
<td>2.68</td>
<td>0.22</td>
<td>4</td>
<td>0.11</td>
</tr>
<tr>
<td>24-hour</td>
<td>Saline</td>
<td>Vehicle</td>
<td>1.50</td>
<td>0.12</td>
<td>4</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Estradiol</td>
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<td>0.15</td>
<td>4</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Carrageenan</td>
<td>Vehicle</td>
<td>2.00</td>
<td>0.26</td>
<td>4</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Estradiol</td>
<td>2.00</td>
<td>0.17</td>
<td>3</td>
<td>0.10</td>
</tr>
</tbody>
</table>
Figure 39. A significant interaction was observed between injection and time (p < .001). Significant differences were found among the following conditions: for 5-hour, mean paw weight in the carrageenan conditions was significantly higher than in the saline conditions (p < .001). For 24-hour, mean paw weight in the carrageenan conditions was significantly higher than in the saline conditions (p < .01). For carrageenan, mean paw weight in the 5-hour conditions was significantly higher than in the 24-hour conditions (p < .001). * p < .05. ** p < .01. *** p < .001.
Table 2

*Means, Standard Deviations, Sample Sizes, and Standard Errors of the Mean for Experiment 1*

**Baseline Paw Withdrawal Latency Data**

<table>
<thead>
<tr>
<th>Heat Intensity</th>
<th>Hormone</th>
<th>Mean</th>
<th>Std Dev</th>
<th>n</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Vehicle</td>
<td>102.07</td>
<td>10.63</td>
<td>84</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>Estradiol</td>
<td>103.95</td>
<td>9.84</td>
<td>81</td>
<td>1.09</td>
</tr>
<tr>
<td>Medium</td>
<td>Vehicle</td>
<td>97.06</td>
<td>19.19</td>
<td>86</td>
<td>2.07</td>
</tr>
<tr>
<td></td>
<td>Estradiol</td>
<td>94.61</td>
<td>18.67</td>
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<td>2.09</td>
</tr>
<tr>
<td>High</td>
<td>Vehicle</td>
<td>96.46</td>
<td>24.13</td>
<td>86</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>Estradiol</td>
<td>97.65</td>
<td>26.42</td>
<td>83</td>
<td>2.90</td>
</tr>
</tbody>
</table>
Figure 40. Mean baseline paw withdrawal latency (PWL) normalized as percentage of baseline for estradiol- and cholesterol-treated rats. No significant differences were observed on low (A), medium (B), or high (C) heat intensity.
Estradiol effects with low intensity stimuli. Levene’s test for equality of variances was significant \( F(7, 53) = 6.08, p < .001 \). As shown in Figure 41, after low intensity stimuli a significant interaction between hormone, pain treatment and time was observed \( F(1, 53) = 5.25, p = .026 \). Using pairwise comparisons based on the LSD, significant differences were found in the following conditions: for carrageenan+5-hour, mean PWL in the vehicle condition was significantly higher than in the estradiol condition \( (p = .006) \); for estradiol+5-hour, mean PWL in the saline condition was significantly higher than in the carrageenan condition \( (p < .001) \); for estradiol+carrageenan, mean PWL in the 24-hour condition was significantly higher than in the 5-hour condition \( (p < .001) \). A significant interaction between pain treatment and time was also observed \( F(1, 53) = 4.98, p = .030 \). Pairwise comparisons yielded significant differences in the following conditions: for 5-hour, mean PWL in the saline condition was significantly higher than in the carrageenan condition \( (p = .001) \); for carrageenan, mean PWL in the 24-hour condition was significantly higher than in the 5-hour condition \( (p = .004) \). Moreover, significant main effects were observed for pain condition \( F(1, 53) = 6.23, p = .016 \) and time \( F(1, 53) = 4.36, p = .042 \). See Table 3.

Estradiol effects with medium intensity stimuli. Levene’s test for equality of variances was significant \( F(7, 54) = 2.42, p = .032 \). As shown in Figure 41, after medium intensity stimuli a significant main effect of time was observed \( F(1, 54) = 5.08, p = .028 \). Mean PWL in the 24-hour condition was significantly higher than in the 5-hour condition. See Table 3.

Estradiol effects with high intensity stimuli. As shown in Figure 41, after high intensity stimuli no significant interactions or main effects were observed. See Table 3.
Table 3

Means, Standard Deviations, Sample Sizes, and Standard Errors of the Mean for Paw Withdrawal Latency Data by Heat Intensity, Time, Pain, and Hormone Condition

<table>
<thead>
<tr>
<th>Heat Intensity</th>
<th>Time</th>
<th>Pain</th>
<th>Hormone</th>
<th>Mean</th>
<th>Std Dev</th>
<th>n</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>5-hour</td>
<td>Saline</td>
<td>Vehicle</td>
<td>102.61</td>
<td>10.04</td>
<td>8</td>
<td>3.55</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>Estradiol</td>
<td>104.53</td>
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</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
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<td>12.98</td>
</tr>
<tr>
<td></td>
<td>24-hour</td>
<td>Saline</td>
<td>Vehicle</td>
<td>108.35</td>
<td>8.08</td>
<td>8</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>Estradiol</td>
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<td>9.85</td>
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<td>3.49</td>
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<td>Vehicle</td>
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<td>Carrageenan</td>
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<td>12.90</td>
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<tr>
<td></td>
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<td>Estradiol</td>
<td>60.67</td>
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<td>10.41</td>
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<tr>
<td></td>
<td>24-hour</td>
<td>Saline</td>
<td>Vehicle</td>
<td>75.02</td>
<td>20.55</td>
<td>8</td>
<td>7.27</td>
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<td>Estradiol</td>
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<td>8.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Estradiol</td>
<td>88.85</td>
<td>18.87</td>
<td>8</td>
<td>6.67</td>
</tr>
</tbody>
</table>
Figure 41. For low heat intensity (A), a significant interaction between hormone, pain treatment and time was observed (p = .026). Significant differences were found among the following conditions: for carrageenan+5-hour, mean PWL in the vehicle condition was significantly higher than in the estradiol condition (p = .006); for estradiol+5-hour, mean PWL in the saline condition was significantly higher than in the carrageenan condition (p < .001); for estradiol+carrageenan, mean PWL in the 24-hour condition was significantly higher than in the 5-hour condition (p < .001). A significant interaction between pain treatment and time was also observed (p = .030). Significant differences were found among the following conditions: for 5-hour, mean PWL in the saline conditions was significantly higher than in the carrageenan conditions (p = .001); for carrageenan, mean PWL in the 24-hour conditions was significantly higher than in the 5-hour conditions (p = .04). Moreover, a significant main effect was observed for pain condition (p = .016). Mean PWL in the saline conditions was higher than in the carrageenan conditions (not marked). A significant main effect was observed for time (p = .042). Mean PWL in the 24-hour conditions was significantly higher than in the 5-hour conditions (not marked). For medium heat intensity (B), a significant main effect of time was observed (p = .028). Mean PWL in the 24-hour conditions was significantly higher than in the 5-hour conditions. For the high heat intensity (C), no significant outcomes were found. * p < .05. ** p < .01. *** p < .001.

Protein analysis. Mean phosphorylation ratio of some proteins within the PI3K pathway were significantly correlated for all subjects (Figures 42-48). The pAkt/Akt and pmTOR/mTOR ratios were significantly correlated, r(27) = .52, p = .005. No other significant correlations were found between the pAkt/Akt, pmTOR/mTOR, and pS6/S6 protein ratios. When the results were analyzed by injection and hormone conditions, the correlation between pAkt/Akt and pmTOR/mTOR was found only in the saline+cholesterol condition, r(7) = .88, p = .010.
Figure 42. Scatterplot of pAkt/Akt and pmTOR/mTOR ratios. The correlation was significant ($p = .005$).
Figure 43. Scatterplot of pAkt/Akt and pS6/S6 ratios. The correlation was not significant (p = .925).
Figure 44. Scatterplot of pmTOR/mTOR and pS6/S6 ratios. The correlation was not significant (p = .734).
Figure 45. Scatterplot of pAkt/Akt and pmTOR/mTOR ratios for saline+cholesterol conditions. The correlation was significant (p = .010).
Figure 46. Scatterplot of pAkt/Akt and pmTOR/mTOR ratios for carrageenan+cholesterol conditions. The correlation was not significant (p = .420).
Figure 47. Scatterplot of pAkt/Akt and pmTOR/mTOR ratios for saline+E2 conditions. The correlation was significant (p = .562).
Figure 48. Scatterplot of pAkt/Akt and pmTOR/mTOR ratios for carrageenan+E2 conditions. The correlation was not significant (p = .690).
As shown in Figure 49, analyses of the Akt/α-tubulin protein levels and pAkt/α-tubulin protein levels yielded no significant main effects or interactions. See Table 4.

As shown in Figure 50, analysis of the mTOR/α-tubulin protein levels yielded no significant main effects or interactions. See Table 5. In the analysis of pmTOR/α-tubulin, a significant interaction was observed between hormone, pain, and time condition [F(1, 18) = 14.51, p = .001, partial η² = .45]. Using pairwise comparisons based on the LSD, significant differences were found in the following conditions: for saline+24-hour, pmTOR levels in the cholesterol condition were significantly higher than in the estradiol condition (p = .005); for carrageenan+24-hour, pmTOR levels in the estradiol condition were significantly higher than in the cholesterol condition (p = .001); for cholesterol+24-hour, pmTOR levels in the saline condition were significantly higher than in the carrageenan condition (p = .006); for estradiol+24-hour, pmTOR levels in the carrageenan condition were significantly higher than in the saline condition (p = .001); for estradiol+saline, pmTOR levels in the 5-hour condition were significantly higher than in the 24-hour condition (p = .040); for estradiol+carrageenan, pmTOR levels in the 24-hour condition were significantly higher than in the 5-hour condition (p = .013). A significant as interaction was also observed between hormone and injection [F(1, 18) = 11.17, p = .004, partial η² = .38]. No significant main effects were observed.

As shown in Figure 51, analyses of the S6/α-tubulin protein levels and pS6/α-tubulin protein levels yielded no significant main effects or interactions. See Table 6.
Table 4

*Mean Protein Levels for Akt and pAkt, Normalized to α-tubulin, by Time, Pain, and Hormone*

**Condition**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Akt/α-tubulin</th>
<th>pAkt/α-tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Std Dev</td>
</tr>
<tr>
<td>5-hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
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<td>Estradiol</td>
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<tr>
<td>Carrageenan</td>
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<td>0.60</td>
</tr>
<tr>
<td>Estradiol</td>
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<td>1.21</td>
</tr>
<tr>
<td>24-hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
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<td>Estradiol</td>
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<td>Carrageenan</td>
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<td>0.18</td>
</tr>
<tr>
<td>Estradiol</td>
<td>1.54</td>
<td>1.31</td>
</tr>
</tbody>
</table>
Figure 49. Mean Akt (A) and pAkt (B) protein levels normalized to α-tubulin and sample Western blots. No significant main effects or interactions were observed.
Table 5

*Mean Protein Levels for mTOR and pmTOR, Normalized to α-tubulin, by Time, Pain, and Hormone Condition*

<table>
<thead>
<tr>
<th></th>
<th>mTOR/α-tubulin</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time</td>
<td>Pain</td>
<td>Hormone</td>
<td>Mean</td>
<td>Std Dev</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-hour</td>
<td>Saline</td>
<td>Vehicle</td>
<td>3.10</td>
<td>4.29</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Estradiol</td>
<td>0.91</td>
<td>0.26</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carrageenan</td>
<td>1.11</td>
<td>0.23</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>Estradiol</td>
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<td>0.44</td>
<td>4</td>
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<tr>
<td></td>
<td>24-hour</td>
<td>Saline</td>
<td>Vehicle</td>
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<td>0.23</td>
<td>4</td>
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<td></td>
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<td>1.16</td>
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<tr>
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<td></td>
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<td>Carrageenan</td>
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<td>0.33</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Estradiol</td>
<td>2.03</td>
<td>1.75</td>
<td>4</td>
</tr>
</tbody>
</table>

|                  | pmTOR/α-tubulin |                      |                      |                  |                  |                  |
|                  | Time           | Pain                 | Hormone              | Mean             | Std Dev          | n                |
|                  |                |                      |                      |                  |                  |                  |
|                  | 5-hour         | Saline               | Vehicle              | 1.19             | 0.32             | 4                |
|                  |                |                      | Estradiol            | 1.15             | 0.18             | 4                |
|                  |                |                      | Carrageenan          | 0.94             | 0.46             | 3                |
|                  |                |                      | Estradiol            | 0.69             | 0.43             | 3                |
|                  | 24-hour        | Saline               | Vehicle              | 1.67             | 1.04             | 3                |
|                  |                |                      | Estradiol            | 0.19             | 0.06             | 3                |
|                  |                |                      | Carrageenan          | 0.23             | 0.12             | 3                |
|                  |                |                      | Estradiol            | 1.98             | 1.10             | 3                |
Figure 50. Mean mTOR (A) and pmTOR (B) protein levels normalized to α-tubulin and sample Western blots. For mTOR/α-tubulin (A), no significant main effects or interactions were found. For pmTOR/α-tubulin, a significant interaction was observed between hormone, pain, and time conditions (p = .001). Significant differences were found among the following conditions: for saline+24-hour, pmTOR levels in the cholesterol condition were significantly higher than in the estradiol condition (p = .005). For carrageenan+24-hour, pmTOR levels in the estradiol condition were significantly higher than in the cholesterol condition (p = .001). For cholesterol+24-hour, pmTOR levels in the saline condition were significantly higher than in the carrageenan condition (p = .006). For estradiol+24-hour, pmTOR levels in the carrageenan condition were significantly higher than in the saline condition (p = .001). For the estradiol+saline, pmTOR levels in the 5-hour condition were significantly higher than in the 24-hour condition (p = .040). For estradiol+carrageenan, pmTOR levels in the 24-hour condition were significantly higher than in the 5-hour condition (p = .013). A significant as interaction was also observed between hormone and injection (p = .004). No significant main effects were observed. * p < .05. ** p < .01. *** p < .001.
Table 6

Mean Protein Levels for S6 and p-S6, Normalized to α-tubulin, by Time, Pain, and Hormone

<table>
<thead>
<tr>
<th>Condition</th>
<th>S6/α-tubulin</th>
<th>p-S6/α-tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time</td>
<td>Pain</td>
</tr>
<tr>
<td>5-hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<td>0.34</td>
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<tr>
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<td>0.17</td>
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Figure 51. Mean S6 (A) and pS6 (B) protein levels normalized to α-tubulin and sample Western blots. No significant main effects or interactions were observed.
As described above, there were significant differences in pmTOR levels in comparisons of delay and hormone conditions in both saline- and carrageenan-treated animals (Figure 50). Two additional analyses were conducted to examine the relationship between protein levels and low heat intensity PWL using normalized pmTOR levels (npmTOR). First, given that pmTOR levels in the estradiol conditions differed significantly between 5-hour and 24-hour delays, pain measurements were examined relative to these trends. Individual pmTOR scores of estradiol-treated animals were normalized to a ratio of pmTOR scores of the 24-hour and 5-hour conditions, separately for the saline and carrageenan conditions. See Table 7. The ratio of pmTOR levels was found to be 0.16 in the saline-treated condition and 2.87 in the carrageenan-treated condition. pmTOR scores of each estradiol-treated animal were multiplied by the appropriate ratio to produce npmTOR scores. A correlation was then run on PWL and npmTOR scores (Figure 52), but yielded no significant relationship, \( r(9) = .014, p = .967 \).

The second analysis examined another pattern of effects observed in the pmTOR levels (Figure 50). Significant differences were found in the estradiol vs. cholesterol conditions for both saline- and carrageenan-treated animals in the 24-hour, but not 5-hour conditions. These trends moved in opposite directions, i.e. pmTOR levels in the 24-hour conditions were significantly lower in the saline+E2 condition compared to the saline+cholesterol condition, but significantly higher in the carrageenan+E2 condition compared to carrageenan+cholesterol. Pain measurements were examined relative to these trends. Individual pmTOR scores were normalized relative to the ratios of estradiol- and cholesterol-treated pmTOR levels. See Table 8. Given that the estradiol vs. carrageenan comparisons were not significant in the 5-hour conditions, the ratios of pmTOR scores were calculated using the 24-hour conditions, separately for saline- and carrageenan-treated animals. The ratio of the saline-treated estradiol and
cholesterol conditions was 0.11, and the ratio of the carrageenan-treated estradiol and cholesterol conditions was 8.49. pmTOR scores of each animal were multiplied by the appropriate ratio to produce npmTOR scores. A correlation was then run on PWL and npmTOR scores (Figure 53), but yielded no significant relationship, $r(21) = -0.076$, $p = 0.732$. 
### Table 7

*Paw Withdrawal Latency and pmTOR Levels Normalized to the Ratio of 24-hour and 5-hour pmTOR Levels in Estradiol-treated Animals*

<table>
<thead>
<tr>
<th>ID</th>
<th>Pain</th>
<th>pmTOR/α-tub</th>
<th>pmTOR 24:5</th>
<th>npmTOR</th>
<th>PWL</th>
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</thead>
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<td>0.03</td>
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<td>2.87</td>
<td>9.34</td>
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<td>Z17</td>
<td>Saline</td>
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<td>0.16</td>
<td>0.17</td>
<td>115.90</td>
</tr>
<tr>
<td>Z18</td>
<td>Saline</td>
<td>1.05</td>
<td>0.16</td>
<td>0.17</td>
<td>115.90</td>
</tr>
<tr>
<td>Z19</td>
<td>Carrageenan</td>
<td>0.39</td>
<td>2.87</td>
<td>1.13</td>
<td>39.22</td>
</tr>
<tr>
<td>Z20</td>
<td>Carrageenan</td>
<td>0.49</td>
<td>2.87</td>
<td>1.41</td>
<td>23.60</td>
</tr>
<tr>
<td>Z29</td>
<td>Saline</td>
<td>0.24</td>
<td>0.16</td>
<td>0.04</td>
<td>115.90</td>
</tr>
<tr>
<td>Z33</td>
<td>Saline</td>
<td>1.43</td>
<td>0.16</td>
<td>0.23</td>
<td>85.40</td>
</tr>
<tr>
<td>Z34</td>
<td>Saline</td>
<td>1.08</td>
<td>0.16</td>
<td>0.17</td>
<td>115.90</td>
</tr>
<tr>
<td>Z41</td>
<td>Carrageenan</td>
<td>1.33</td>
<td>2.87</td>
<td>3.83</td>
<td>104.75</td>
</tr>
<tr>
<td>Z45</td>
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<td>1.19</td>
<td>2.87</td>
<td>3.41</td>
<td>14.43</td>
</tr>
</tbody>
</table>
Figure 52. Scatterplot of PWL and pmTOR levels normalized to the ratio of 24-hour and 5-hour pmTOR levels in estradiol-treated animals. The correlation was not significant ($p = .967$).
Table 8

**Paw Withdrawal Latency and pmTOR Levels Normalized to the Ratio of Estradiol-treated and Cholesterol-treated pmTOR Levels in the 24-hour Condition**

<table>
<thead>
<tr>
<th>ID</th>
<th>Pain</th>
<th>Hormone</th>
<th>Time</th>
<th>pmTOR/α-tub</th>
<th>pmTOR E2: Sal 24-hour</th>
<th>npmTOR</th>
<th>PWL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z01</td>
<td>Carrageenan</td>
<td>Vehicle</td>
<td>24-hour</td>
<td>0.22</td>
<td>0.11</td>
<td>0.02</td>
<td>43.52</td>
</tr>
<tr>
<td>Z02</td>
<td>Carrageenan</td>
<td>Vehicle</td>
<td>24-hour</td>
<td>0.12</td>
<td>0.11</td>
<td>0.01</td>
<td>115.90</td>
</tr>
<tr>
<td>Z03</td>
<td>Saline</td>
<td>Estradiol</td>
<td>24-hour</td>
<td>0.21</td>
<td>0.11</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>Z04</td>
<td>Saline</td>
<td>Estradiol</td>
<td>24-hour</td>
<td>0.12</td>
<td>0.11</td>
<td>0.15</td>
<td>80.70</td>
</tr>
<tr>
<td>Z05</td>
<td>Carrageenan</td>
<td>Vehicle</td>
<td>5-hour</td>
<td>1.46</td>
<td>0.11</td>
<td>0.29</td>
<td>115.90</td>
</tr>
<tr>
<td>Z06</td>
<td>Carrageenan</td>
<td>Vehicle</td>
<td>5-hour</td>
<td>0.77</td>
<td>0.11</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Z07</td>
<td>Saline</td>
<td>Vehicle</td>
<td>5-hour</td>
<td>1.56</td>
<td>0.11</td>
<td>0.12</td>
<td>84.21</td>
</tr>
<tr>
<td>Z08</td>
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<td>Vehicle</td>
<td>5-hour</td>
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<td>0.11</td>
<td>0.12</td>
<td>101.70</td>
</tr>
<tr>
<td>Z13</td>
<td>Saline</td>
<td>Vehicle</td>
<td>24-hour</td>
<td>2.65</td>
<td>0.11</td>
<td>0.03</td>
<td>115.90</td>
</tr>
<tr>
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<td>Saline</td>
<td>Vehicle</td>
<td>24-hour</td>
<td>0.58</td>
<td>0.11</td>
<td></td>
<td>115.90</td>
</tr>
<tr>
<td>Z15</td>
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<td>Estradiol</td>
<td>24-hour</td>
<td>1.35</td>
<td>0.11</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Z16</td>
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<td>Estradiol</td>
<td>24-hour</td>
<td>3.25</td>
<td>0.11</td>
<td>0.12</td>
<td>115.90</td>
</tr>
<tr>
<td>Z17</td>
<td>Saline</td>
<td>Estradiol</td>
<td>5-hour</td>
<td>1.05</td>
<td>0.11</td>
<td>0.20</td>
<td>115.90</td>
</tr>
<tr>
<td>Z18</td>
<td>Saline</td>
<td>Estradiol</td>
<td>5-hour</td>
<td>1.05</td>
<td>0.11</td>
<td></td>
<td>115.90</td>
</tr>
<tr>
<td>Z19</td>
<td>Carrageenan</td>
<td>Estradiol</td>
<td>5-hour</td>
<td>0.39</td>
<td>0.11</td>
<td>0.12</td>
<td>39.22</td>
</tr>
<tr>
<td>Z20</td>
<td>Carrageenan</td>
<td>Estradiol</td>
<td>5-hour</td>
<td>0.49</td>
<td>0.11</td>
<td>0.09</td>
<td>23.60</td>
</tr>
<tr>
<td>Z27</td>
<td>Carrageenan</td>
<td>Vehicle</td>
<td>24-hour</td>
<td>0.36</td>
<td>8.49</td>
<td>1.90</td>
<td>115.90</td>
</tr>
<tr>
<td>Z28</td>
<td>Carrageenan</td>
<td>Vehicle</td>
<td>24-hour</td>
<td>8.49</td>
<td>0.98</td>
<td></td>
<td>115.90</td>
</tr>
<tr>
<td>Z29</td>
<td>Saline</td>
<td>Estradiol</td>
<td>24-hour</td>
<td>0.24</td>
<td>8.49</td>
<td>12.37</td>
<td>115.90</td>
</tr>
<tr>
<td>Z30</td>
<td>Saline</td>
<td>Estradiol</td>
<td>24-hour</td>
<td>8.49</td>
<td>6.54</td>
<td></td>
<td>115.90</td>
</tr>
<tr>
<td>Z31</td>
<td>Carrageenan</td>
<td>Vehicle</td>
<td>5-hour</td>
<td>0.59</td>
<td>8.49</td>
<td>11.50</td>
<td>70.12</td>
</tr>
<tr>
<td>Z32</td>
<td>Carrageenan</td>
<td>Vehicle</td>
<td>5-hour</td>
<td>8.49</td>
<td>27.62</td>
<td>62.03</td>
<td></td>
</tr>
<tr>
<td>Z33</td>
<td>Saline</td>
<td>Estradiol</td>
<td>5-hour</td>
<td>1.43</td>
<td>8.49</td>
<td>3.33</td>
<td>85.40</td>
</tr>
<tr>
<td>Z34</td>
<td>Saline</td>
<td>Estradiol</td>
<td>5-hour</td>
<td>1.08</td>
<td>8.49</td>
<td>4.18</td>
<td>115.90</td>
</tr>
<tr>
<td>Z39</td>
<td>Saline</td>
<td>Vehicle</td>
<td>24-hour</td>
<td>1.77</td>
<td>8.49</td>
<td>3.06</td>
<td>115.90</td>
</tr>
<tr>
<td>Z40</td>
<td>Saline</td>
<td>Vehicle</td>
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<td></td>
<td></td>
<td>115.90</td>
</tr>
<tr>
<td>Z41</td>
<td>Carrageenan</td>
<td>Estradiol</td>
<td>24-hour</td>
<td>1.33</td>
<td>8.49</td>
<td>4.98</td>
<td>104.75</td>
</tr>
<tr>
<td>Z42</td>
<td>Carrageenan</td>
<td>Estradiol</td>
<td>24-hour</td>
<td>8.49</td>
<td></td>
<td></td>
<td>115.90</td>
</tr>
<tr>
<td>Z43</td>
<td>Saline</td>
<td>Vehicle</td>
<td>5-hour</td>
<td>1.05</td>
<td>8.49</td>
<td>11.32</td>
<td>115.90</td>
</tr>
<tr>
<td>Z44</td>
<td>Saline</td>
<td>Vehicle</td>
<td>5-hour</td>
<td>0.83</td>
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<td>115.90</td>
</tr>
<tr>
<td>Z45</td>
<td>Carrageenan</td>
<td>Estradiol</td>
<td>5-hour</td>
<td>1.19</td>
<td>8.49</td>
<td>10.07</td>
<td>14.43</td>
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<td>Z46</td>
<td>Carrageenan</td>
<td>Estradiol</td>
<td>5-hour</td>
<td>8.49</td>
<td></td>
<td></td>
<td>46.29</td>
</tr>
</tbody>
</table>
Figure 53. Scatterplot of PWL and pmTOR normalized to the ratio of estradiol-treated and cholesterol-treated pmTOR levels in the 24-hour condition. The correlation was not significant (p = .732).
Experiment 2

**Baseline PWL.** As shown in Figure 54, no significant differences in mean baseline PWL between estradiol and cholesterol conditions were found at any temperature level; low heat intensity: $t(42.69) = -0.63, p = .532$; medium heat intensity: $t(37.17) = -1.80, p = .080$; high heat intensity: $t(37.30) = 0.17, p = .869$. See Table 9.

**Estradiol effects with low intensity stimuli.** A paired samples t-test indicated that mean PWL in the BL was significantly higher than after carrageenan injection, collapsing across time and hormone [$t(45) = 7.08, p < .001$]. Levene's test for equality of variances was significant [$F(3, 43) = 5.05, p = .004$]. As shown in Figure 55, a significant main effect of delay was observed, such that mean paw withdrawal latency was significantly lower in the 4-hour condition compared to 1-hour [$F(1, 43) = 7.08, p = .011$, partial $\eta^2 = .141$]. There was no significant main effect of hormone condition or hormone X delay interaction. See Table 10.

**Estradiol effects with medium intensity stimuli.** A paired samples t-test indicated that mean PWL in the BL was significantly higher than after carrageenan injection, collapsing across time and hormone [$t(42) = 10.69, p < .001$]. Levene's test for equality of variance was not significant [$F(3, 40) = 2.27, p = .095$]. As shown in Figure 55, a significant main effect of delay was observed, such that mean paw withdrawal latency was significantly lower in the 4-hour condition compared to 1-hour [$F(1, 40) = 12.70, p = .001$, partial $\eta^2 = .241$]. There was no significant main effect of hormone condition or hormone X delay interaction. See Table 10.
Figure 54. Mean baseline paw withdrawal latency (PWL) for estradiol- and cholesterol-treated rats. No significant differences were observed on low (A), medium (B), or high (C) heat intensity.
Table 9

Means, Standard Deviations, Sample Sizes, and Standard Errors of the Mean for Baseline Paw Withdrawal Latency Data

<table>
<thead>
<tr>
<th>Heat Intensity</th>
<th>Hormone</th>
<th>Mean</th>
<th>Std Dev</th>
<th>n</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Vehicle</td>
<td>18.26</td>
<td>2.30</td>
<td>24</td>
<td>0.47</td>
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<td>Estradiol</td>
<td>18.64</td>
<td>1.73</td>
<td>23</td>
<td>0.36</td>
</tr>
<tr>
<td>Medium</td>
<td>Vehicle</td>
<td>10.66</td>
<td>2.24</td>
<td>23</td>
<td>0.47</td>
</tr>
<tr>
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<td>3.54</td>
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<td>0.74</td>
</tr>
<tr>
<td>High</td>
<td>Vehicle</td>
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<td>1.48</td>
<td>23</td>
<td>0.31</td>
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<tr>
<td></td>
<td>Estradiol</td>
<td>7.69</td>
<td>2.33</td>
<td>23</td>
<td>0.48</td>
</tr>
</tbody>
</table>
Table 10

Means, Standard Deviations, Sample Sizes, and Standard Errors of the Mean for Paw Withdrawal Data by Heat Intensity, Delay, and Hormone Condition

<table>
<thead>
<tr>
<th>Heat Intensity</th>
<th>Delay</th>
<th>Hormone</th>
<th>Mean</th>
<th>Std Dev</th>
<th>n</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>1-hour</td>
<td>Vehicle</td>
<td>73.67</td>
<td>29.83</td>
<td>12</td>
<td>8.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Estradiol</td>
<td>75.52</td>
<td>35.85</td>
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<td>10.35</td>
</tr>
<tr>
<td></td>
<td>4-hour</td>
<td>Vehicle</td>
<td>44.35</td>
<td>21.66</td>
<td>11</td>
<td>6.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Estradiol</td>
<td>52.83</td>
<td>42.17</td>
<td>12</td>
<td>12.17</td>
</tr>
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<td>Medium</td>
<td>1-hour</td>
<td>Vehicle</td>
<td>62.47</td>
<td>29.80</td>
<td>11</td>
<td>8.98</td>
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<td>Estradiol</td>
<td>69.16</td>
<td>34.64</td>
<td>11</td>
<td>10.44</td>
</tr>
<tr>
<td></td>
<td>4-hour</td>
<td>Vehicle</td>
<td>37.73</td>
<td>14.54</td>
<td>11</td>
<td>4.38</td>
</tr>
<tr>
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<td>5.77</td>
</tr>
<tr>
<td>High</td>
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<td>Vehicle</td>
<td>75.87</td>
<td>26.99</td>
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<td>7.79</td>
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<tr>
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<td>4-hour</td>
<td>Vehicle</td>
<td>49.09</td>
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<td>12</td>
<td>6.84</td>
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<td>42.48</td>
<td>24.82</td>
<td>11</td>
<td>7.48</td>
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</tbody>
</table>
Figure 55. Mean PWL, organized by heat intensity (A-C), hormone, and delay condition. Mean PWL in the 4-hour conditions were significantly lower than in the 1-hour conditions (A: \( p = .011 \). B: \( p = .001 \). C: \( p < .001 \)). No significant main effects of hormone or hormone X delay interactions were observed. * \( p < .05 \). *** \( p < .001 \).
Estradiol effects with high intensity stimuli. A paired samples t-test indicated that mean PWL in the BL was significantly higher than after carrageenan injection, collapsing across time and hormone [t(44) = 7.02, p < .001]. Levene's test for equality of variances was not significant [F(3, 43) = 1.40, p = .256]. As shown in Figure 55, a significant main effect of delay was observed, such that mean paw withdrawal latency was significantly lower in the 4-hour condition compared to 1-hour [F(1, 43) = 14.58, p < .001, partial η² = .256]. There was no significant main effect of hormone condition or hormone X delay interaction. See Table 10.

Discussion

Experiment 1. Carrageenan produced inflammation in the injected paw, as indicated by increased weight. This result was consistent with previous studies (Amann & Schuligoi, 2000). Estradiol had no effect on baseline nociception, whereas some previous studies have found an anti-nociceptive estradiol effect on baseline nociception (Hunter et al., 2011b; Li et al., 2009).

A number of effects were observed in low-intensity behavioral testing. PWL was lower in the carrageenan+5-hour groups compared to saline+5-hour groups, as expected (Morris, 2003). Estradiol had an additional effect. PWL was lower in the carrageenan+E2 condition compared to saline+E2. Estradiol appears to be pro-nociceptive in this case, which is inconsistent with previous studies. Hunter et al. (2011b) found that estradiol increased post-carrageenan PWL, and a number of studies found anti-nociceptive effects using the formalin test (Kuba, Kemen, & Quinones-Jenab, 2005; Kuba et al., 2006; Kuba et al., 2010b; Hunter et al., 2011a; Mannino et al., 2005, 2007; Shivers et al., 2015). PWL in the carrageenan+E2+24-hour condition was significantly higher than the carrageenan+E2+5-hour condition, such that there was no difference
between it and carrageenan+cholesterol+24-hour or baseline. The pro-nociceptive estradiol effect at 5 hours apparently dissipated or became masked by another effect by 24 hours.

The lack of carrageenan effects in the low-intensity 24-hour groups, as well as the medium- and high-intensity conditions, may be explained by stress-induced analgesia (SIA). In this phenomenon, exposure to aversive-stressor stimulation triggers analgesia to subsequent noxious stimuli. This effect is mediated by multiple mechanisms, including opioid, cannabinoid, and combination opioid/cannabinoid systems. It can be triggered by various stimuli, including electric shock and capsaicin administration (Werner et al., 2015), exposure to odors from stressed rats (Fanselow, 1985), exposure to cats (Lester & Fanselow, 1985), and exposure to males (discussed below). The SIA response increases as a function of stimulus intensity (Fanselow, 1984a,b), consistent with the lack of effects in the higher stimulus intensity conditions.

It is possible that the male investigator influenced the results of this study. Sorge et al. (2014) demonstrated that male experimenters can affect nociception through an olfactory mechanism. They found that rats and mice, especially females, exhibited SIA in response to male experimenters, T-shirts worn by male experimenters, as well as unfamiliar male mice and rats, guinea pigs, cats, and dogs, as well related chemical compounds.

In the low-intensity conditions, carrageenan effects were present at 5 hours, but not 24 hours. In the medium-intensity conditions, PWL was lower in the 5-hour conditions compared to the 24-hour conditions. This pattern may be explained by the male experimenter effect. The SIA induced by a male experimenter abates in 30-60 minutes, but is reinstated the following day. Testing 5 hours after exposure to the male experimenter may have occurred during this refractory period, though this intermediate time course was not examined by Sorge et al. (2014). In the
high-intensity conditions, there were no differences in PWL between 5-hour and 24-hour conditions. Given that it is intensity-dependent (Fanselow, 1984a,b), the SIA produced by the high-intensity stimulus may have masked any male experimenter effect.

A number of effects were observed in the levels of phosphorylated mTOR. pmTOR levels were lower in the carrageenan+E2+5-hour condition compared to the carrageenan+E2+24-hour condition. This could indicate ongoing or increasing PI3K pathway activation, though previous research has shown mTOR phosphorylation peaking 1 hour after carrageenan injection (Xu et al., 2011). The opposite pattern was observed in saline conditions. pmTOR levels were higher in saline+E2-5-hour compared to saline+E2+24-hour. These results might indicate that estradiol exerts opposing effects on mTOR phosphorylation, and therefore plasticity, depending on the presence or absence of inflammation. This estradiol-induced depression of pmTOR at 24 hours is prominent compared to the saline+cholesterol+24-hour condition. This pattern fits some previous findings. Estradiol can reduce cytokine levels in the absence of inflammation and nociception (Shivers et al., 2015), and the PI3K pathway can be activated by cytokines (Lisi et al., 2015). The depressed pmTOR levels observed in the saline+E2+24-hour condition may be due to this combination. Strangely, pmTOR levels were higher in the saline+cholesterol+24-hour condition compared to the carrageenan+cholesterol+24-hour condition. A clear activation of the PI3K pathway and resulting mTOR phosphorylation were expected in response to carrageenan. Finally, pmTOR levels were lower in the carrageenan+cholesterol+24-hour condition compared to the carrageenan+E2+24-hour condition. This is consistent with the finding that estradiol can activate the PI3K pathway (Lee et al., 2005), but inconsistent with an anti-nociceptive effect of estradiol, given the role of PI3K pathway activation in central sensitization (Xu et al., 2011).
Experiment 2. Experiment 2 excluded the saline conditions. This simplification precluded analysis of inflammation versus control, and interactions of hormone or time with inflammation condition, but increased the sample size per condition. Carrageenan injection produced hyperalgesia compared to baseline at all heat intensities, but no estradiol effect was observed. PWL was lower at 4 hours compared to 1 hour in all conditions, which may be due to increasing inflammation, sensitization, and/or abatement of SIA.

Conclusion. Chronic pain is a disease of the nervous system that affects over 50 million American and costs $70 billion annually. It is a particular problem for women, who exhibit greater pain sensitivity and suffer from chronic pain conditions more often than men. It is difficult to identify the sources of these sex differences in an exceedingly complex nociceptive system. This study investigated the role of interactions between estradiol, the peptide neurotransmitter substance P, and intracellular plasticity regulated by the PI3K-Akt-mTOR signaling pathway. Basic research in animal models of nociception is notoriously difficult to translate to human application, and numerous candidate treatments have hit dead-ends. Despite our best efforts, pain remains a fundamental part of the human experience.
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