

New and Emerging Pharmacological Targets for Neuropathic Pain

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Increasing knowledge of the molecular consequences of nerve injury and the availability of genome databases has greatly increased the range of potential targets for the pharmacological management of neuropathic pain. Controlling neuronal sensitization and the associated alterations in gene expression, protein modification, and neuronal excitability is the key to managing neuropathic pain. Control of neuronal sensitization can occur through inhibition of nerve injury-associated production of cytokines, activation of glial cells, modulation of potassium channel subtypes, mitogen-activated protein kinases, the ubiquitin-proteasome system, or the protection and amplification of spinal cord dorsal horn inhibitory systems. These new and already established targets promise unparalleled opportunities for the prevention, management, and resolution of persistent pain states following nerve injury.

Introduction

This review highlights new and emerging targets for the treatment of neuropathic pain and, as such, is not comprehensive and does not include a discussion of inflammatory pain targets. Pharmacological targeting of the pain processing system has been focused on small (< 500 MW) molecules with good oral availability, predominantly acting on cell-surface receptors. This ignores the other aspects of signal processing within the neuron and associated glial tissue. In light of newly discovered mechanisms for neuropathic pain, it has become clear that the pain system is redundant, at least at the membrane receptor level, and highly specific pharmacological agents directed at these targets have displayed disappointing effectiveness. For these reasons, this article focuses on potential targets central to the process of dorsal horn neuron and primary afferent sensitization. First, the action of glia and cytokines on neuronal sensitization, which opens up the area of neuroimmunology and starts to blur the line between inflammatory and purely neuropathic mechanisms of

pain, is discussed. Second, the emerging area of potassium channel modulation as a means of regulating neuronal excitability without precipitating conduction block is reviewed. Activation of neurons after injury leads to intracellular signaling cascades and altered gene expression, leading to long-term alteration in neuronal function. Highly specific inhibitors target these kinase cascades. Once genes are triggered by injury, new proteins must be made and inserted into neuronal membranes; existing proteins often have to be recycled and the selective post-translational modification of membrane proteins can be accomplished by the ubiquitin-proteasome system for which new inhibitors recently have become available. Third, new targets to minimize the loss of dorsal horn inhibitory interneurons caused by excessive primary afferent excitation and glutamate release seen in nerve injury are addressed. The loss of inhibitory systems can lead to sensitization as a result of unopposed excitatory signals.

Glial Cell Modulation

Glial cells (microglia, astrocytes, and Schwann cells) constitute more than 70% of the total cell population in the nervous system. These glial cells have a central role in processing nociceptive information and the development of thermal and mechanical hyperalgesia as well as allodynia after injury to the nervous system [1••].

Involvement of glia, with their extensive network of connections to one another and with distant neurons, helps explain the expansion of the region from which pain is perceived, leading to regional pain distribution that does not respect classical neuronal anatomy. Many functional somatic syndromes and pain states are not limited to known neuronal territories and thus are susceptible to be diagnosed as non-anatomical.

Measures to reduce the activation of neuroglia or recruitment of monocytes or macrophages or inhibiting the release and action of pro-inflammatory cytokines after nerve damage could influence the development and maintenance of neuropathic pain.

Modulation of Cytokines

Shortly after nerve injury, spinal neurons and glia become activated (neuroimmune activation) [2]. The microglia may be the first response system for central nervous system

(CNS) injury and they respond to and produce cytokines including interleukin-1 β (IL-1 β), interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), and interferon- γ [3,4], which in turn can lead to chemotactic cytokine release, infiltration of immune cells, and further activation of glial cells. Some time after the activation of glia and release of pro-inflammatory cytokines, gene expression for anti-inflammatory cytokines such as IL-10 and IL-4 is upregulated to help dampen the injury response [3]. Cytokines can induce the release and expression of cyclooxygenase-2, inducible nitric oxide, and substance P as well as enhance capsaicin sensitivity in the primary afferents [5]. This process will amplify and sustain inflammatory responses affecting neuronal function as observed in animal models of neuropathic hyperalgesia and tactile allodynia [6]. The development of persistent pain states then will be dependent on how effectively this system can be dampened endogenously or by exogenous immunopharmacotherapy [4].

Measurement of cytokines (IL-1 β , IL-6, and TNF- α) in sural nerve biopsies from 41 patients showed a strong correlation between cytokine expression and the presence of neuropathic pain [7]. This correlation was strongest for vasculitic neuropathies.

The pro-inflammatory cytokine TNF- α plays an important role in neuropathic pain. TNF- α is produced by neurons, glia, macrophages, and monocytes [8]. Epineurally applied exogenous TNF- α [9] can induce ectopic activity and decrease mechanical thresholds in primary afferent nociceptors [10], which may contribute to the hyperalgesia seen in neuropathic pain. Selective inhibition of TNF- α by etanercept attenuates mechanical allodynia induced by spinal nerve ligation [11].

In nerve ligation models, the uninjured nerve rapidly develops increased sensitivity to TNF- α and responds to previously subthreshold levels of TNF- α , suggesting that it may play a role in widening the receptive field and sustaining the neuropathic pain state [12]. This enhanced sensitivity is accompanied by a rapid increase in TNF- α receptors type 1 and 2 in the injured and uninjured dorsal root ganglia (DRG) [13]. Chronic constriction injury to large fibers of the sciatic nerve stimulates TNF- α gene expression in large fibers and the TNF- α is anterogradely transported [14] to the muscle. It is possible that the deep muscle pain associated with neuropathic pain is caused by TNF- α because direct injections into the muscle can produce hyperalgesia in rats [15].

Inhibition of TNF- α activity may have benefits extending beyond reduction of hyperalgesia in patients with chronic pain. TNF- α has been implicated in illness behavior and it can mediate illness-induced hyperalgesia [1••].

An increase in soluble TNF- α receptors has been noted in the serum of patients with allodynia compared with neuropathy patients who do not report allodynia [16]. TNF- α plays a pivotal role in herniated nucleus pulposus-induced nerve root damage [17]. In a small open-label

study, six of 10 (60%) patients with acute, severe sciatica were pain-free after a single intravenous infusion of the TNF- α monoclonal antibody infliximab compared with a 16% response in a historical control group. This effect lasted for at least 3 months in the study group [18].

Tumor necrosis factor- α -induced activity in DRG cells and subsequent sensitization can develop by several mechanisms such as activation of the mitogen-activated protein kinases p38 and JNK [11], phosphorylation of potassium channel subtypes as seen in retina [19], inhibition of glial glutamate transporters in the dorsal horn leading to inhibitory cell death [20], and translocation of cytoplasmic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor into the DRG and dorsal horn cells as seen in hippocampus [21••, 22].

Reduction of the cytokine release after nerve injury may reduce the development of neuropathic pain, but one must be careful not to reduce the factors responsible for nerve regeneration (*eg*, IL-6) [23] or the anti-inflammatory factors (*eg*, IL-10), which decreases endoneural TNF- α expression, thermal hyperalgesia, and macrophage recruitment associated with chronic sciatic constriction injury neuropathy [24]. Glucocorticoids have been used in neuropathic pain states, but they are inhibitory in a non-selective manner to a wide range of cytokines. This non-selectivity may contribute to their limited usefulness. Selective reduction in cytokines, as in TNF- α blocking agents (etanercept, infliximab, adalimumab), cytokine modulation as seen with thalidomide, or selective glial cell inhibition with propentofylline-like compounds [25], may hold greater promise.

Potassium Channel Modulation

Potassium channels are diverse in structure and function and influence many aspects of excitable tissue function, including action potential shape, resting membrane potential firing rates, and neurotransmitter release. Increased neuronal excitability is a prominent feature of neuropathic pain and has several components, including increased sodium and decreased potassium channel activity. Agents that increase the activity of potassium channels will reduce the excitability of a membrane by stabilizing it at a more negative potential [26]. Traditionally, much attention was given to sodium channel blockers and the study of normal and pathologic sodium fluxes associated with nerve injury. Although this approach has great merit, potassium channel modulation may allow much greater control over neuronal excitability, particularly in the treatment of neuropathic pain, as a result of nerve injury.

Initiation of neuronal firing is determined by depolarization or the development of a pacemaker current, which allows a slow ramping of depolarization to the threshold for sodium channel activation. An increase in this pacemaker current would lead to increased spontaneous firing,

similar to that seen in injured neurons. Chaplin *et al.* [27] have provided evidence for hyperpolarization-activated cyclic nucleotide-modulated (HCN) pacemaker potassium channels. They propose that these channels are expressed in primary afferent cell bodies and that they play a role in touch-related pain and spontaneous neuronal discharge in damaged DRG. A specific inhibitor of HCN activity, ZD7288 (not selective for HCN subtypes) [27,28], was able to reverse allodynia and decrease the spontaneous firing in A β fibers by 90% in a rat nerve ligation model. This inhibitor did not have activity on tests of acute pain such as hot plate or thermal responses to acute inflammation, such as complete Freund's adjuvant. The effect was expressed in the periphery because intrathecal ZD7288 was ineffective. However, clinical use of ZD7288, in particular, is limited by bradycardia and low potency [28].

Nerve injury induced by spinal nerve ligation results in dramatic reductions in voltage-gated K⁺ (Kv) channel subunit expression in DRG neurons [29,30]. The Kv channels in large non-nociceptive neurons usually are composed of a mixture of α subunits, but the channel is homomeric in nociceptive c fibers, with only the Kv 1.4 subunit expressed. Specifically, up-regulating these channels may be a genetic therapeutic strategy for neuropathic pain, whereas specific agonists to the homomeric Kv1.4 channels may have analgesic activity in non-neuropathic states.

The difficulty in specifically inhibiting certain potassium channels or activating other channels without untoward effects is a great clinical problem. Advances in gene therapy have allowed Johns *et al.* [31•] to demonstrate the ability to insert an inducible gene in superior cervical ganglion cells using an adenoviral vector and thereby control excitability by the inducible expression of a potassium channel Kir2.1. Kir2.1 is an inwardly rectifying potassium channel that influences the baseline or leakage current and inhibits evoked and spontaneous activity. Future challenges would include specifically infecting neurons, not glial cells, and careful selection of the inducer molecule system. Targeted delivery of these vectors could lead to controllable suppression of excitability, allowing the potential for genetic analgesia in a titratable manner [31•].

In a recent paper, Derjean *et al.* [32] found that most (90%) of the deep dorsal horn neurons tested were able to alter their intrinsic firing properties from tonic to plateau or endogenous bursting patterns. The degree of alteration of intrinsic properties was dependent on the balance of inhibitory γ -aminobutyric acid-B receptor (GABA-BR) activity and excitatory group I metabotropic glutamate receptor activity (mGluR). The modulation had a common target: the inwardly rectifying potassium channel Kir 3 [32]. The changes in intrinsic properties fell into three functional states that correlated with pain modalities ranging from acute nociception to long-lasting central sensitization to bursting activity. This bursting activity state could account for the distorted sensory processing seen in neuropathic pain states [32]. Control of this alteration

in intrinsic activity could be accomplished by targeting the mGluR, the GABA-BR, or the potassium channels directly. Further work in this area no doubt will produce more targets, but the mere fact that most of the deep dorsal horn neurons are susceptible to this modulation confirms the dynamic nature of pathologic pain. Many of the deep dorsal horn neurons are wide dynamic-range neurons. The alteration of intrinsic firing patterns to a plateau state by mGluR activation would serve to enhance the responsiveness of these cells to less effective synaptic inputs, presumably from low-threshold afferents, and may account for the increased receptive fields and mechanical hyperalgesia and allodynia observed with pathologic pain [32].

Passmore *et al.* [33] have reported the presence of KCNQ/M currents in sensory neurons. M currents are involved in regulating neuronal excitability and the molecular correlates of this current are the M channel components KCNQ 2 or 3. Mutations in these subunits alter neuronal excitability and are linked to a form of juvenile epilepsy [34]. The anticonvulsant retigabine enhances the M current [35] and application to the spinal cord inhibited c and A- δ primary afferent fiber activity as well as a wind-up discharge resulting from repetitive stimulation [33]. Retigabine also displays antihyperalgesic activity in inflammatory and neuropathic pain models in rats [33,36]. These data strongly suggest the investigation of M-channel activators as therapies for pathologic pain. However, M-channel blockers also have been studied for cognitive enhancement and the use of an M-channel activator may be associated with cognitive compromise.

Mitogen-activated Protein Kinase Modulation

Spinal cell plasticity seen in central sensitization and glial stimulation is a consequence of activation of intracellular signaling cascades working on transcriptional and post-transcriptional modification of several proteins. Cells respond to extracellular stimuli in part through G-protein-linked receptor systems, which depend on a cascade of intracellular mediators with kinase activity to phosphorylate many copies of the next member of the cascade, greatly amplifying the original stimulus. Normal neuronal function depends on a tightly controlled balance of phosphorylase and phosphatase activities. A disruption in this system can lead to the abnormal function seen in pathologic pain states. The convergence of many membrane receptors on a limited number of downstream kinases emphasizes the redundancy of the pain system and also suggests that specific inhibition of just one membrane receptor may have limited effectiveness because other mediators still will be allowed to activate the kinase systems.

Membrane receptor activation of kinases, including protein kinase A (PKA), C (PKC), and mitogen-activated protein kinase (MAPK), can lead to critical membrane protein phosphorylation, which can alter neuronal activity for the medium term (minutes to hours). Activation of

MAPK can lead to changes in gene expression and affect the persistent changes in neuronal function that underlie chronic pain-associated sensitization states [37•].

Mitogen-activated protein kinase systems are composed of enzyme cascades containing three enzymes: MAPK, MAPK-kinase, which phosphorylates the MAPK, and MAPK-kinase-kinase, which phosphorylates the MAPK-kinase. This system allows multiple levels of control and amplification. The MAPK family has at least three groups: extracellular signal-related kinases (ERK) [38], p38 kinases [39], and c-Jun, N-terminal, or stress-activated protein kinases (JNK) [40]. ERK is primarily activated by neuronal activity, p38 is mainly activated by cytokines, and JNK is activated by cellular stress or injury. All may contribute to the various responses of a nerve after injury in the short- and long-term.

Activated p38 MAPK is present in activated microglia, but not in neurons or astrocytes in the spinal cord [41]. However, p38 has been localized to small DRG neurons, which were positive for TNF- α [11]. A specific inhibitor of p38 MAPK (SB203580), given intrathecally at the time of nerve injury and for 7 to 14 days after, inhibited the development of tactile allodynia in two separate studies [11,41]. p38 also is activated in DRG neurons after peripheral inflammation, but this is secondary to nerve growth factor produced in the periphery and retrogradely transported to the cell body.

Extracellular signal-related kinases activity is downstream from several post-receptor modulators including PKA and PKC, all of which are activated by membrane receptors. ERK phosphorylation in primary afferent neurons by pain stimuli is intensity- and modality-dependent and most likely plays a major role in peripheral sensitization in response to tissue injury or inflammatory mediator-based noxious stimuli [42].

c-Jun, N-terminal, or stress-activated protein kinase apparently is the MAPK most involved in the response to nerve injury. Very early after sciatic nerve injury an upregulation of the immediate early gene transcription factor c-Jun mRNA and protein appears in DRG cells [43]. c-Jun is responsible for regulating several genes in response to stress and injury and is regulated by TNF- α and IL-1 β among other mediators. Sciatic nerve transection resulted in chronic activation of JNK in the effected DRGs and is accompanied by JNK phosphorylation in neurons and long-lasting transcription factor AP-1-binding activity [43]. The timing of the JNK activation is dependent on the distance from the axotomy to the DRGs, suggesting that a retrograde signal is required for activation. AP-1 binding by JNK and elevated c-Jun levels persist for 30 days after axotomy if regeneration is blocked, but degrade rapidly if reinnervation is allowed. JNK activation may play a role in the neuronal sprouting after nerve crush, accounting for the abnormal innervation seen in the dorsal horn (and possibly in peripheral neuromas) after nerve injury and possibly for the abnormal sensory patterns reported after nerve injury [44,45]. JNK

is coded for by three distinct genes (*JNK1*, *JNK2*, *JNK3*) and 10 isoforms are possible by variable mRNA splicing [46]. Although *JNK1* and *JNK2* have wide tissue distribution, *JNK3* is expressed predominantly in neurons with some expression in cardiac smooth muscle and testes [46]. *JNK3* knockout mice show resistance to neuronal apoptosis. Specific inhibitors of JNK [46] and a JNK kinase [47] have been described and have been employed to assess the role of the JNK system in Parkinson's disease [48] and hippocampal function [49]. Application in neuropathic pain states is an obvious next step. There is cross-talk between glucocorticoid receptors and AP-1 in regulating gene expression [50]. Specific modification of the upstream regulators of AP-1 such as JNK holds the promise of providing the beneficial effects of glucocorticoids without the long-term adverse events of joint necrosis, osteoporosis, and metabolic effects [51].

Ubiquitin-Proteasome System Modulation

In a manner similar to phosphorylation, ubiquitination is a rapid and reversible mechanism to covalently modify target proteins. Unlike phosphorylation, the ubiquitin system assigns proteins for sequestration or degradation and thus can regulate protein levels and gene expression without a change in genotype. Ubiquitin tagging is regulated by a system composed of three enzymes: the E1 enzyme, which activates ubiquitin (a small, 76-amino acid protein), the E2 family of carrier enzymes, and the highly target-specific E3 ligase enzymes, which attach ubiquitin to the target protein. Monoubiquitination influences protein trafficking between cellular compartments while polyubiquitination targets the protein for degradation in the proteasome [52]. The proteasome degrades a polyubiquitinated substrate to small peptides. The substrates and enzymes of the ubiquitin pathway can be regulated by phosphorylation and a complementary system of deubiquitinating enzymes can reverse the process, adding an additional layer of control [53].

Ubiquitin has crucial roles in the nervous system including synapse formation and long-term synaptic plasticity through the regulation of the amount and function of neurotransmitter receptors and ion channels in the neuronal membrane [54,55]. Rapid and long-lasting effects on synaptic strength can be produced by cell-surface receptor endocytosis mediated by monoubiquitination, which has been reported for glutamate receptors [56]. Polyubiquitination and subsequent degradation of the regulatory subunit of PKA persistently activates PKA and pushes the gene expression cascade forward, thereby inducing synaptic long-term facilitation [57]. By modulating target proteins in the synapse, the ubiquitin system can influence the pattern, activity, and plasticity of synaptic connections [58•].

The functional and molecular organization of the postsynaptic membrane is regulated in an activity-dependent manner through ubiquitin-proteasome-mediated protein

turnover, providing a mechanistic link between synaptic activity, protein turnover, and the functional reorganization of synapses [59]. The coupling of *N*-methyl-D-aspartate (NMDA) receptors to intracellular pathways is influenced by ubiquitin-proteasome-mediated remodeling of the post-synaptic densities such that NMDA receptors at active synapses elicited greater activation of gene expression while inactive receptors were selectively coupled with ERK-MAPK activity [59]. Intervention in this system could be accomplished by inhibition of the proteasome, which would globally effect proteins targeted for destruction during the period of drug exposure or one could target the specific E3 ligases to inhibit the application of ubiquitin to specific proteins.

Intrathecal administration of specific proteasome inhibitors, in a model of neuropathic pain, attenuated hyperalgesia and allodynia with some reduction in normal nociceptive, but not non-nociceptive responses [60]. Iontophoretic application of the inhibitors attenuated sensitized dorsal horn neuron responses to innocuous brush or cold stimuli. The recent approval of a proteasome inhibitor for treatment of cancer brings this therapeutic approach within reach for neuropathic pain [61]. Identification of a unique family of E3 ligases (ZNR proteins) located in the presynaptic membrane and induced by peripheral nerve injury brings us closer to selective targeting of neuronal-specific E3 ligases and control over ubiquitination [62]. Through their ubiquitin ligase activity, ZNR proteins play a role in the establishment and maintenance of neuronal transmission and synaptic plasticity. Selective use of specific E3 or deubiquitination enzymes could up- or down-regulate selected protein levels and provide unprecedented control over sensitization processes involved in neuropathic pain. Drug targeting for E3 ligases may benefit from work on the human genome. Thus far, there are 391 genes for E3 ligases, with 631 known isoforms coding for distinct proteins and 86 genes for deubiquitination enzymes with 136 isoforms [63].

Preservation and Amplification of Inhibitory Circuits

Central modification of neuronal function after injury or inflammation can be expressed as increased excitability or sensitization as discussed in the preceding several sections. An alternative mechanism for altered neuronal functions is a loss of inhibition through the loss of inhibitory interneurons. Interneuronal death in the superficial laminae of the spinal cord has been documented after nerve injury [64]. The loss of these cells in effect would produce a disinhibition of incoming nociceptive signals. One of the mechanisms of cell death is overstimulation by excessive glutamate release. The dorsal horn astrocytes and microglia normally provide a buffering function by taking up excess glutamate through a specific glutamate transporter. Microglia activated by injury will produce TNF- α and, in

turn, TNF- α will inhibit glutamate uptake [20]. TNF- α most likely exerts its delayed effect by inhibiting the expression of the glutamate transporter gene [20]. Sung *et al.* [65] recently observed a reduction in glutamate transporter levels after nerve injury in rats. The ERK inhibitor PD98059 increased the down-regulation of the glutamate transporter and worsened the thermal and mechanical hyperalgesia. This strongly suggests that ERK is involved in glutamate transporter gene expression and its inhibition further reduces transporter levels [65]. Therefore, ERK inhibitors may not be as effective for neuropathic pain as they may be for inflammatory pain. In this same system, riluzole, a stimulator of glutamate uptake and indicated for the treatment of amyotrophic lateral sclerosis, was able to reverse much of the hyperalgesia resulting from nerve injury. These results must be viewed with caution because riluzole so far has not been helpful for clinical neuropathic pain [66]. Selective enhancement of glutamate transport has potential as a new treatment for neuropathic pain.

Conclusions

Neuropathic pain represents a large unmet clinical need and despite many potential pharmacological therapies, few have demonstrated more than moderate efficacy. Many new, highly focused therapies have become victims of the mechanistic redundancy present in neuropathic pain. The most important targets may not be present before injury; therefore, normal or acute injury models may not reflect clinical reality. Attention now is focused on the unique long-term consequences of nerve injury involving glial cell activation, spreading sensory nerve receptive fields as well as alteration in neuronal phenotype, and protein content by kinase-facilitated gene expression or ubiquitin-proteasome system modulation.

Greater control over abnormal excitability states in neurons may be available with potassium channel stimulants. These may be more effective and more titratable than the available sodium channel inhibitors such as the local anesthetics and classic antiepileptic or antiarrhythmic drugs. Advancements in gene therapy may allow inducible mechanisms for up- and down-regulation of the potential targets discussed in this review as exemplified by the introduction of Kir 2.1 channels by adenoviral vectors [31•]. Manipulation of the ubiquitin-proteasome system and, consequently, the cellular proteome will allow “molecular surgery” to alter neuronal phenotypes.

Nociceptive neuronal sensitization as a basis of persistent pain is similar to hippocampal long-term potentiation as a basis for memory. Interference with these basic mechanisms may have untoward effects, such as altering memory or cognition. In other situations, the same mediators causing persistent pain in the dorsal horn may impair memory consolidation in the CNS [1••]. Interference may improve both conditions. As we learn more about DRG and peripheral nerve mechanisms of neuropathic pain,

selective pharmacological therapies restricted to the periphery can be fashioned and thereby reduce CNS adverse events.

In the near future, neuroimmunologic therapies affecting cytokines and other glial cell products may blur the distinction between neuropathic and inflammatory pain. The molecular events following tissue or nerve injury may not be limited to the site of injury. Release of cytokines, activation of glia, and stimulation of remote neural pathways may have profound effects on behavioral and cognitive functions [1••]. An exciting time for neuropathic pain pharmacotherapeutics lies ahead.

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