David C. Warltier, M.D., Ph.D., Editor

Anesthesiology 2001; 95:216–40

Gene Therapy for the Management of Pain

Part II: Molecular Targets

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TREATMENT of chronic pain, particularly of neuropathic etiology, is extremely difficult and resistant to many available pharmacologic therapies. Current analgesic agents may be limited with regard to analgesic efficacy or side effects.[1,2] Newer and experimental pharmacologic agents may also have significant limitations.[3] By targeting a specific receptor or other specific protein targets, a gene therapy approach to the treatment of pain may provide greater analgesic efficacy without the limitations associated with current pharmacotherapy. Advances in the field of gene therapy, along with significant increases in our understanding of the neurobiology of nociception and knowledge of the fundamental genetic structure of many nociceptive targets, have made gene therapy for the management of pain a conceivable reality.

In part I of this review,[3A] we introduced the basic concepts of gene therapy with an emphasis on the available tools (*e.g.*, viral vectors and antisense oligonucleotides) and strategies for upregulating antinociceptive or downregulating pronociceptive targets. In part II, we summarize current knowledge regarding the nociceptive role, molecular biology, and antisense and knockout data of several novel nociceptive targets for gene therapy. We base our selection of the targets included in this review on the three aforementioned criteria. The targets selected are the best characterized and, in our opinion, most likely amenable to the gene therapeutic approach. A simple but feasible strategy and potential gene therapy targets for the management of pain are summarized in figure 1. However, the list is admittedly incomplete, and the readers are referred to other recent reviews cited in part I of this review for a broader perspective on potential targets for the management of pain.[3A]

Potential Novel Targets for Gene Therapy in the Management of Pain

Assuming that an ideal gene delivery system is available, viral or otherwise, what would be the therapeutic target? Of the many targets comprising the complex nociceptive cascade, how does one choose the best point for therapeutic intervention? An ideal target should have (1) a well-defined role in the pathogenesis of neuropathic pain, (2) a well-defined pharmacologic profile with specific pharmacologic tools (*i.e.*, agonists and antagonists) available, and (3) little role in normal physiology, thus limiting the chances of side effects associated with pharmacologic manipulation of the target. No consensus exists on such an ideal target for management of pain.

Targets that lend themselves to a genetic approach to pain management should have strong evidence for a role in nociception, a reasonably defined molecular biology and function, and antisense knockdown and knockout data. Space precludes discussion of several traditional and obvious targets, such as opioid and α_2-adrenergic receptors, and some potentially promising targets, including N-type calcium channels, voltage-gated Ca^{2+} channels, adenosine triphosphate–sensing purinergic P_2X_3, acid-sensing ion channel, and neurotrophin receptors. Some of these targets were recently reviewed elsewhere.[4] The opioid receptors as a target for a genetic approach to pain management is clearly well supported by existing literature. We chose not to include opioid receptors in our review for the simple reason that many conventional and experimental drugs highly selective for this receptor already exist. In addition, therapeutic limitations imposed by the development of tolerance appear to be a fundamental property of opioid receptors

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Received from the Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins Hospital, Baltimore, Maryland; the Department of Anesthesiology and Pain Management, University of Texas Southwestern Medical Center, Dallas, Texas; and the Departments of Anesthesiology, Pharmacology, and Physiology, University of Rochester School of Medicine and Dentistry, Rochester, New York. Submitted for publication August 10, 2000. Accepted for publication November 27, 2000. Supported by grant No. BC971561 from the US Army Medical Research and Materiel Command, Fort Detrick, Maryland (to Dr. Yang), grant No. R21 GM58057 from the National Institutes of Health, Bethesda, Maryland (to Dr. Garry), and a research fellowship from the Foundation for Anesthesia Education and Research/Oreganon Inc., Rochester, Minnesota (to Dr. Zollo).

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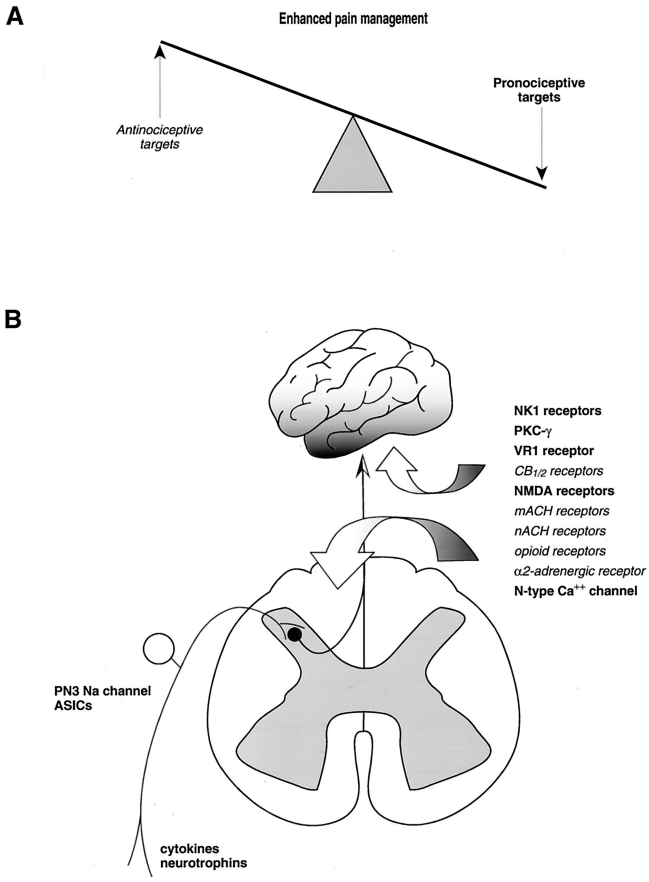

Fig. 1. Potential molecular targets for gene therapy. (*A*) Conceptually, enhanced pain management can be attained by increasing the antinociceptive targets (italics) or decreasing the pronociceptive targets (bold). (*B*) Specific molecular targets. Not all putative targets are discussed in this review. The broad arrows indicate that the targets are present both at the spinal cord and the supraspinal levels. NK1 = neurokinin 1; PKC-γ = protein kinase C-γ; VR1 = vanilloid receptor 1; $CB_{1/2}$ = cannabinoid 1/2; NMDA = *N*-methyl-D-aspartate; mACH = muscarinic acetylcholine; nACH = nicotinic acetylcholine.

most likely irreconcilable even through a genetic approach. Although our review focuses on receptors as the prime target, nonreceptor targets, such as the enzymes responsible for the synthesis of the neurotransmitter that acts on the receptors or signal transduction molecules that mediate the downstream effect of receptor activation, are also potential targets.

In the following sections, we review selected central nervous system (CNS) targets for gene therapy, with particular attention to the evidence of role in nociception, information on the molecular biology, and studies investigating a gene therapeutic approach in altering nociception. Molecular biologic information, including the size of the cDNA encoding the target protein and genomic structure, are critical in designing concrete strategies for gene therapy using viral vectors or oligodeoxyribonucleotides (ODNs). Figure 2 shows the protein topology and table 1 summarizes the pertinent molecular biologic information for the potential therapeutic targets described below. Table 2 lists the conventional agonists and antagonist drugs described in the text for the selected targets.

Tachykinin Neurokinin 1 Receptor

Evidence of Role in Nociception. With substance P (SP) as its most selective endogenous ligand, the tachykinin neurokinin 1 (NK-1) receptor is present throughout the CNS and peripheral nervous system and mediates a variety of physiologic activities.[5] Of the three neurokinin receptor subtypes, NK-1 is the most prevalent at the spinal cord level and important in enhancing action of excitatory amino acids (EAAs) and mediating secondary hyperalgesia and central sensitization.[5,6] The NK-1 receptors have been localized postsynaptically to afferent nerve fibers in dorsal horn laminae I, III, and IV[7–9] and are consistent with the role of SP in nociception.[6] Neurokinin-1 receptors appear to be expressed mostly in excitatory neurons, as NK-1 receptor immunoreactive neurons are minimally γ-aminobutyric acid (GABA)- or glycine-immunoreactive.[8] Chronic inflammatory and sciatic nerve transection models of persistent pain result in upregulation of NK-1 receptor immunoreactivity in the superficial laminae of the dorsal horn.[10] Death of SP receptor-containing lamina I spinal neurons after internalization of a SP-coupled neurotoxin results in significant attenuation to noxious stimuli and inhibition of hyperalgesia.[11]

Data from administration of NK-1 agonists support the role of NK-1 receptors in mediating excitation in noxious stimuli–responsive spinal neurons. Iontophoretic application of SP results in preferential excitation of dorsal horn nociceptive neurons, whereas nonnociceptive neurons are unaffected.[12] Intrathecal injection of NK-1 receptor agonists into awake rats and mice elicit biting, scratching, and licking of forelimbs.[13–15] After surgical deafferentation, there is an increase in dorsal horn NK-1 binding sites, and intrathecal injection of SP produces significantly increased pain-related behaviors.[9,16] Administration of the selective NK-1 agonist, Sar-SP, results in production of c-fos in laminae I, and injection of NK-1 antagonist L-668,169 significantly decreases formalin-induced c-fos expression.[17] In addition, increased expression of NK-1 mRNA in the dorsal horn of rat after peripheral noxious stimuli is blocked by the NK-1 receptor antagonist LY-306,740.[18]

Use of NK-1 antagonists in whole animal behavior models has emphasized the role of NK-1 receptors in mediating nociception after prolonged noxious chemical stimuli as opposed to phasic mechanical stimuli.[19,20] NK-1 receptors appear to be important in development of thermal and mechanical hyperalgesia.[21–23] The NK-1 antagonists L-733,060 and CP-99,994 inhibit late- but not early-phase response after injection of formalin in mice and gerbils.[19,24] Electrophysiologic studies after injec-

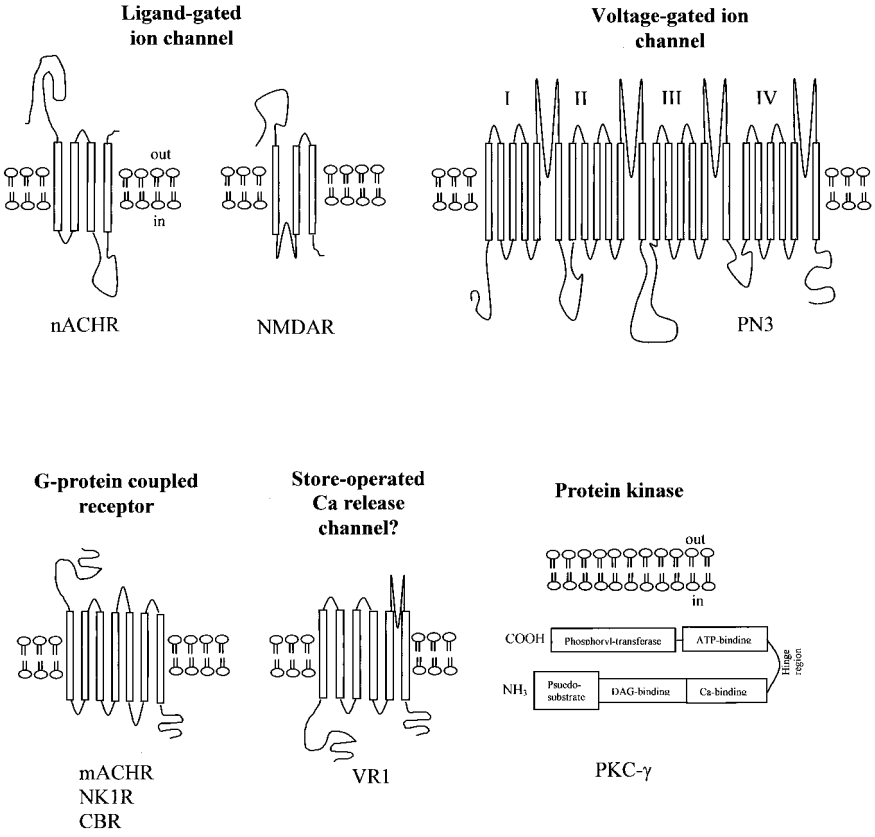

Fig. 2. Heterogeneity of the molecular target protein topology. A schematic of the six potential targets from five classes of proteins discussed in the review. The rod-like structures denote the transmembrane segment of the protein sitting in lipid bilayer. Protein kinase C (PKC)-γ is an intracellular protein, although activation leads to translocation of the protein from the cytoplasm to membrane. The functional domains common to all classical PKCs are denoted. nACHR = nicotinic acetylcholine receptor; NMDAR = *N*-methyl-D-aspartate receptor; mACHR = muscarinic acetylcholine receptor; NK1R = neurokinin-1 receptor; CBR = cannabinoid receptor; VR1 = vanilloid receptor 1; ATP = adenosine triphosphate; DAG = diacylglycerol.

tion of SP in rats reveal an increase in response to noxious mechanical stimuli that is reduced in a dose-dependent manner by the specific NK-1 receptor antagonist CP-96,345 but not by its inactive enantiomer, CP-96,344.[25] Although SP results in neuronal excitation in response to nonnoxious stimuli, NK-1 antagonists do not inhibit responses to nonnoxious mechanical stimuli.[25] Previous administration of a selective NK-1 antagonist (RP-67,580), but not its inactive isomer (RP-68,651), produced a dose-related reduction in the number of formalin-evoked c-fos–like immunoreactive spinal neurons.[26]

Human clinical studies have not supported analgesic action of NK-1-receptor antagonists thus far. Initial studies demonstrated analgesic action of CP-99,994 equivalent to ibuprofen for dental pain.[27] Subsequent studies in osteoarthritis, neuropathic pain, and migraine have demonstrated no analgesic effect, although access to CNS by the compounds relatively impermeable to the blood–brain barrier may be a problem.[28] In contrast, NK-1 receptor antagonists have demonstrated potent antiemetic effects and offer promising use in treatment of psychiatric disorders.[28,29] The reason for the failure of NK-1 receptor antagonists to demonstrate clinical efficacy in humans is not clear but may be because of the pharmacokinetic limitations of the particular antagonists examined or the discordance between painful behavior measured in animals and human clinical pain.[28,30]

Acting at the NK-1 receptor, tachykinins will facilitate the acute, excitatory effects of EAAs on *N*-methyl-D-aspartate (NMDA) receptors to produce prolonged enhancement of EAA responses, resulting in sensitization of dorsal horn neurons and secondary hyperalgesia.[5,31,32] Co-application of SP and NMDA produces enhancement of responses of primate spinothalamic neurons and increased sensitivity to cutaneous mechanical stimulation.[33] SP and glutamate coexist in the same primary afferent terminals in the dorsal horn, and tachykinins may tonically be released to modulate NMDA-mediated glutamatergic transmission.[34,35] Administration of selective NK-1 antagonists will block the potentiating effect of SP on EAA actions on spinal neurons.[36,37] In addition, co-application of an NMDA receptor and selective NK-1 antagonist produce a supraadditive effect in inhibition of nociception, suggesting an interaction between intracellular signal transduction cascades initiated by the two ligands.[32]

Thus, the NK-1 receptor appears to play an important role in mediating persistent nociception and may contribute to central sensitization. NK-1 receptors are expressed in spinal cord at locations consistent with processing of afferent nociceptive input and are rapidly downregulated after nociceptive afferent input. Physiologic experimental evidence also supports the role of NK-1 receptors in mediating nociception despite the fact that human clinical data with selective NK-1 receptor antagonists have been disappointing.

Molecular Biology and Receptor Function. As with other G protein–coupled receptors, the tachykinin NK-1

Table 1. Potential Targets for Gene Therapy*

Target Protein	cDNA Size	Classification	cDNA Accession No.†	Comments
Tachykinin NK-1 (SP) receptor	1,224 bp	GPCR ($G_{q/11}$)	M84425	5 exons dispersed over 60 kb of genomic DNA
Protein kinase C-γ isoform	2,564 bp	Kinase	M13977, Z15114‡	87 bp upstream of transcription start site confers promoter activity
Vanniloid receptors				
VR1	2,517 bp	Store-operated Ca channel?	AF029310§	
VR-L1	2,295 bp	″	AF103906	
Peripheral sodium channel				
PN3	5,874 bp	Voltage-gated ion channel	X92184§	27 exons spanning ~90 kb of genomic DNA
Cannabinoid receptors				
CB1	1,419 bp	GPCR ($G_{i/o}$)	U73304	Entire open reading frame contained within a single exon (true for all GPCR)
CB2	1,083 bp	″ ($G_{i/o}$)	X74328	
Acetylcholine receptors				
M1	1,383 bp	GPCR ($G_{q/11}$)	X15263	GPCR genomic structure as noted above
M2	1,401 bp	″ ($G_{i/o}$)	X15264	
M3	1,440 bp	″ ($G_{q/11}$)	X15265	
M4	1,773 bp	″ ($G_{i/o}$)	X15266	
M5	1,599 bp	″ ($G_{q/11}$)	NM012125	
Nα7	1,509 bp	Ligand-gated ion channel	U62436	6–10 exons; the first 4 exons of all known nAChR subunits are identical; significant information on the promoter location
Nα4	1,884 bp	″	NM000744	
Nβ2	1,509 bp	″	NM000748	
NMDA subtype of glutamate receptors				
NR1	2,721 bp	Ligand-gated ion channel	AF015730	22 exons of which 3 undergo alternative splicing resulting in multiple splice variants; functional receptors consist of combinations of NR1 and NR2 subunits
NR2A	4,395 bp	″	NM000833	
NR2B	4,455 bp	″	NM000834	
NR2C	3,711 bp	″	NM000835	
NR2D	4,011 bp	″	NM000836	

* All sequences are human unless otherwise stated. † The complementary DNA (cDNA) sequence can be downloaded by entering the accession No. at the URL http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html (National Library of Medicine; accessed August 10, 2000). Primary references and information regarding the genomic structure for the given sequence often can be found in the header section of the data file. ‡ Overlap of these two partial sequences gives the entire coding sequence of protein kinase C-γ gene. § Rat sequence.

NK-1 = neurokinin 1; SP = substance P; bp = base pairs; GPCR = G protein–coupled receptor; nAChR = nicotinic cholinergic receptor; kb = kilobase; NMDA = *N*-methyl-D-aspartate.

receptor has seven hydrophobic transmembrane domains (TM1–TM7) with an extracellular NH_2 and intracellular COOH terminus.[38–40] A functional cDNA encoding rat SP receptor consisted of 407 amino acids with a molecular mass of approximately 46,385 Da.[38,40] There is a section of a GU dinucleotide repeat at the 5' end and CU and CA dinucleotide repeats in the 3' end that may result in a hairpin loop formation, thus potentially affecting translation or stability of receptor mRNA.[40] Like other G protein–coupled receptors, there are potential phosphorylation sites (serine and threonine residues) in the COOH terminus. Mechanisms of interactions between NK-1 receptors and its agonists and antagonists have not been definitively elucidated; however, two popular hypotheses for binding include the volume-exclusion hypothesis (agonist and antagonist binding may overlap) and receptor–ligand interaction, where allosteric alteration in receptor conformation occurs after binding by an agonist or antagonist.[41]

The NK-1 receptor is encoded by a five-exon and four-intron gene structure.[42,43] The NK-1 receptor is approximately 45 kilobases in length, and the five exons consist of 965, 195, 151, 197, and 2,010 base pairs.[43] Exon 1 encodes for the NH_2 terminus to TM3, which is important for SP binding.[44] Exons 2 and 3 encode for TM4 and TM5. The genetic code for the COOH terminus and TM6–TM7 is included in exons 6 and 7.[45] Putative NK-1 receptor promoter regions include a proximal promoter consisting of six to eight bases located immediately upstream from the conventional TATA sequence and several conserved sequences, including an adenylate cyclase (cAMP)-responsive, phorbol ester, and calcium-activated transcription sites.[42] A proposed structure for the NK-1 receptor has been described.[46–48] Certain structural areas are important for various NK-1 receptor functions. The third intracellular loop is crucial to downstream second messenger activation, as substitution in this area prevents cAMP production and phosphoinosi-

Table 2. Conventional Pharmacologic Agonists and Antagonists for the Targets Described in the Text

	Agonist	Antagonist
Tachykinin NK-1 receptor	Sar-SP	L-668,169, LY-306,740, L-733,060, and CP-99,994 RP-67,580 and RP-68,651 (inactive isomer) CP-96,345 and CP-96,344 (inactive enantiomer)
Protein kinase C	Phorbol esters	NPC-15437
Vanniloid receptor	Capsaicin Resinferatoxin Heat, H^+ ions	Capsazepine
Sodium channels	Veratridine	Tetrodotoxin and saxitoxin
Cannabinoid receptors	Δ-9-Tetrahydrocanabinol WIN 55,212-2 and 55,212-3 (inactive enantiomer) CP-55,940, anandemide	SR144528 (CB2 specific) SR141716A (CB1 specific)
Acetylcholine		
Muscarininc	Depends on subtype*	Depends on subtype
Nicotonic	Depends on subtype	Depends on subtype
NMDA	NMDA	MK-801

* A comprehensive list of agonists and antagonists and their vendors can be found in *2000 Receptor and Ion Channel Nomenclature Supplement*.[371]

NK-1 = neurokinin 1; NMDA = *N*-methyl-D-aspartate.

tol turnover after NK-1 agonist application.[49] Sections of TM2 (Asn-85, Asn-89, Tyr-92, Asn-96) and TM7 (Tyr-287) are required for high-affinity binding of peptides.[48] Other regions, such as Glu-78 in TM2 and Tyr-205 and Tyr-216 in TM5, may be important for G-protein coupling and activation.[48,50] Tyrosine-containing sequences in TM7 and COOH terminus interact with the endocytic apparatus and may be important for the process of NK-1 receptor endocytosis.[51]

The NK-1 receptor is coupled to G proteins that are sensitive (G_o, G_i) and insensitive (G_q, G_{11}) to pertussis toxin.[39] Several independent second messengers mediate some of the biologic effects after NK-1 receptor activation.[39,52] NK-1 agonists may differentially stimulate second messenger pathways, and targeting downstream mediators of NK-1 receptor activation to attain antinociception may be difficult.[53,54] Other effects may not require participation of second messengers, such as those mediated through direct modulation of channel activity and SP-induced stimulation of a nonselective inward current.[39]

Once activated by SP, the NK-1 receptor undergoes receptor internalization in the cell bodies and dendrites of nociceptive spinal neurons as marked by a loss of SP receptor immunoreactivity on cell membranes and increase in SP receptor–positive endosomes.[55,56] Within 1 min of SP injection, 60% of SP receptor–immunoreactive neurons show receptor internalization with return to baseline in approximately 60 min.[55] Once internalized, the receptor undergoes morphologic reorganization such that the uniform tubular structure of dendrites is transformed into "swollen varicosities" with eventual endosomal SP degradation and possibly receptor and recycling or resynthesis of NK-1 receptor back to the cell membrane.[56–58] Shape transformation of the dendrites actually may result in transformation of neural impulses, which may be relevant to development of pain states.[59] The number of neurons internalizing NK-1 receptors corresponds to concentration of SP used.[55] NK-1 internalization is inhibited by the NK-1 antagonist RP-67,580.[55] Thus, internalization of NK-1 receptors may functionally downregulate the receptor response to subsequent stimulation by SP. Enhancement of tachyphylaxis to SP by attenuation of receptor turnover may be a novel approach to antinociception. Alternatively, enhancement of receptor internalization by SP fragments without activation of downstream signal transduction[60] may provide analgesia. Viral transduction and expression of an SP fragment and subsequent diffusion of the peptide into the spinal cord proper is an attractive strategy for attaining antinociception. Similar enhancement of receptor internalization could possibly be induced by an overexpression of the intracellular chaperone protein β-arrestin.[61] Quartara and Maggi[5,39] provided an excellent review of the tachykinin NK-1 receptor. Thus, the genomic structure and receptor function of the NK-1 receptor is well described and will facilitate gene-based expression and regulation for analgesia.

Antisense Knockdown and Knockout Data. Two knockouts against the gene encoding SP and neurokinin A and one knockout against the gene encoding the NK-1 receptor were recently described.[62–64] Cao *et al.*[62] disrupted the preprotachykinin A gene, thus producing mice deficient in SP and neurokinin A. No SP or neurokinin A immunoreactivity was noted, and normal levels of all three neurokinin receptor subtypes were present. There was no difference between mutant and wild-type mice in response to low and high intensities of thermal stimuli; however, mutant mice showed a reduced sensitivity at an intermediate temperature. There was no difference between groups in response to the late phase of the formalin test. In tests for mechanical nociception, mutant mice showed reduced response to tail clip, but there was no difference between groups in response to

von Frey hairs. When compared with wild-type mice, mutant mice showed a significant reduction in response to chemical and visceral pain. Mutant mice failed to develop neurogenic inflammation after peripheral capsaicin injection.[62]

Zimmer *et al.*[63] also produced mutant mice deficient in SP but with a different phenotype than that produced by Cao *et al.*[62] Expression of NK-1 receptor was significantly higher in mutant mice, possibly resulting from receptor upregulation in absence of endogenous ligands. There was no difference between mutant and wild-type mice with respect to number and distribution of small-diameter, calcitonin gene-related peptide (CGRP)-immunoreactive dorsal root ganglia (DRG) neurons, suggesting that there was not a significant decrease in primary sensory neurons. There were no differences in response between wild-type and mutant mice after tail-flick assay and acetic acid–induced writhing tests; however, mutant mice were hypoalgesic in hot-plate and formalin tests.[63]

De Felipe *et al.*[64] developed a mouse deficient in NK-1 receptors. Mutant mice were healthy and fertile and showed normal behavior except for a decrease in aggressive behavior. Absence of NK-1 receptors was confirmed by receptor autoradiography, and normal SP and CGRP distribution were noted. There were no differences in response between wild-type and mutant mice after tail-pinch, paw-withdrawal, tail-flick, or hot-plate assays (acute nociception); however, mutant mice did not develop spinal neuron sensitization.

The differences observed between the three knockouts highlight the problem of interpretation of data obtained from conventional knockout mice. Any observed phenotypic alteration may or may not be the result of targeted gene disruption.[65] In this case, Zimmer *et al.*[63] and Cao *et al.*[62] both produced a knockout mouse deficient in the gene encoding SP; however, significant differences in the phenotype were demonstrated and possibly reflected differences in either genetic background or experimental parameters. In general, other issues that may contribute to difficulty in data interpretation include pleiotropy (multiple functions of genes), epistasis (genes and their products produce biologic phenomena), and compensation (genetic redundancy).[65] Although a detailed review of issues involved in transgenic studies of nociception are beyond the scope of this review, a recent article provides greater depth on this subject.[65]

Antisense ODNs directed against the NK-1 receptor have been described in two studies. Ogo *et al.*[66] developed an antisense ODN directed against the NK-1 receptor NH_2 terminus. By the second day of treatment with target antisense ODN, a reduction of 31% in SP/NK-1 receptor binding and 35% in calcium ion influx induced by SP were noted *in vitro*. Control ODN did not result in any change of either measurement. The investigators then injected antisense ODN–encapsulated liposomes in rat cerebral cortex. When compared with control animals, those with antisense ODNs had a reduction in cortical SP binding sites by approximately 40% 7 days after initial injection. Animals in this study did not show behavioral or neurologic abnormalities.[66]

Hua *et al.*[57] intrathecally injected several antisense ODNs against NK-1 receptor mRNA of the rat. Administration of antisense ODNs alone did not result in a reduction of pain behavior or spinal NK-1 receptors, as marked by immunostaining, and there was no significant difference between groups with respect to levels of NK-1 receptor mRNA. However, intrathecal injection of SP in rats treated with antisense ODNs resulted in a reduction in pain behavior and spinal NK-1 receptor immunoreactivity. Although the duration of antisense ODN administration (2 days) may not have been enough to diminish functional expression or synthesis of the NK-1 receptor in rats treated with antisense alone, those treated with antisense and SP did demonstrate a significant reduction in surface receptor protein, possibly suggesting that receptors, once depleted, are not replaced by *de novo* synthesis. Consequently, behavioral data obtained from ODN treatment potentially may not manifest until turnover of existing receptors occurs. There were no motor dysfunction or other behavioral abnormalities noted in rats treated with antisense ODNs.[57] Like the previous antisense ODN study, it was difficult to conclude definitively whether inhibitory effects of antisense ODN were a result of mRNA degradation (after internalization) or arrest of mRNA translation.[57,66] The long turnover of NK-1 receptors in steady state (> 7 days) may require prolonged administration of antisense ODN to effect NK-1 receptor downregulation.[57,66] A thorough knowledge of the target protein turnover is essential in designing a rational antisense ODN–based gene therapy strategy, unfortunately unavailable for the NK-1 receptor.

Several approaches to gene-based modification of the NK-1 receptor have been described. Initial antisense ODN studies appear promising; however, further *in vivo* studies with appropriate controls and a critical evaluation of the duration of ODN treatment are required .

Protein Kinase C-γ Isoform

Evidence of Role in Nociception. Central sensitization is mediated in part by action of EAAs on NMDA receptors. Although NMDA receptor activation alone is insufficient for development of central sensitization, influx of calcium through NMDA receptor channels may induce prolonged changes by way of intracellular second messengers and protein kinases, including protein kinase C (PKC).[67] PKC appears to mediate CNS neuronal plasticity after tissue injury.[67–69] Rats pretreated with inhibitors of phospholipase C and PKC demonstrate significant reductions in nociceptive behavior.[67] Those treated with PKC activators show significantly enhanced

persistent nociceptive behavior; however, this behavior is not present in early phase of the formalin response.[70] Spinal infusion of the PKC inhibitor NPC15437 prevents sensitization of wide-dynamic-range spinothalamic neurons to mechanical stimuli after intradermal injection of capsaicin.[68] Intrathecal injection of GM1 ganglioside, an inhibitor of PKC activation, reduces spontaneous pain behavior and thermal hyperalgesia in rats after chronic constrictive sciatic nerve ligation.[69] Thus, the role of PKC in nociception is well supported by classical pharmacologic data.

Protein kinase C consists of a number of isoforms that vary in function and distribution. One of these isoenzymes, PKC-γ, is found only in brain and spinal cord. Long-term potentiation or changes in neuronal plasticity, learning, and memory are mediated by the PKC-γ isoform, which appears postnatally and is restricted to CNS.[71–73] In the spinal cord, PKC-γ is found in the cytoplasm and restricted to neurons and interneurons in the substantia gelatinosa and axons of the dorsal corticospinal tract.[74,75] PKC-γ immunoreactivity in the spinal cord dorsal horn increases in rats after chronic constriction injury but does not increase with intrathecal administration of the NMDA antagonist MK-801.[76]

Protein kinase C-γ does not appear to mediate acute nociception but is central to regulation and development of neuropathic pain. Although there are no specific agonists or antagonists of PKC-γ that may be used to investigate the role of PKC-γ in nociception, a mutant mouse lacking PKC-γ has been bred.[75] PKC-γ–deficient mice show a normal response to the first phase of the formalin test but demonstrate significant reductions to the second phase. Unlike wild-type mice, PKC-γ–deficient mice completely fail to develop neuropathic pain after partial sciatic nerve ligation.[75] Furthermore, mutant mice do not develop the typical pattern of neurochemical reorganization in ipsilateral dorsal horn observed after partial nerve injury and complete nerve transection in normal mice.[75] Peripheral nerve injury in mutant mice produces an unaltered response of primary afferents, and effects of PKC-γ deletion are manifested postsynaptically.[75] Thus, it appears that PKC-γ is essential to the development of neuropathic pain. Further studies are necessary to define the downstream events leading to the development of neuropathic pain subsequent to PKC-γ activation.

Molecular Biology and Protein Function. Protein kinase Cs are a family of phospholipid-dependent, serine-threonine kinase isoenzymes that play a critical role in intracellular signal transduction. Conventional PKCs are activated by second messengers such as diacylglycerol, inositol triphosphate, and calcium. Activated PKC is associated with modulation of ion channels, desensitization of receptors, enhancement of neurotransmitter release, and modulation of synaptic transmission.[77] PKC interactions with its substrates are described elsewhere.[78] Prolonged activation of PKC may be central in mediating long-term cellular events such as learning, nociception, tumor genesis, or cellular proliferation and differentiation.[75,79,80] The precise functions of specific isoenzymes are not clear at this time.

Protein kinase C-γ is classified as a calcium-dependent or conventional PKC as it is activated by calcium, diacylglycerol, and phosphatidylserine.[81] In general, all PKC isoenzymes, including PKC-γ, consist of four constant regions (C1–C4) separated by five variables regions (V1–V5).[72] The V3 region separates the regulatory domains (V1–V2, C1–C2) from catalytic domains (V4–V5, C3–C4). The C1 region contains a sequence motif in the phosphorylation site that appears to be a pseudosubstrate site with autoregulatory characteristics.[72,82] In addition, there is a cysteine-rich region in C1 that is necessary for diacylglycerol and phorbol ester binding.[83] C2 is important for participation in calcium binding, and C3 contains an adenosine triphosphate binding site. The substrate binding site and phosphate transfer region are located on C4.[72] The V3 region is also known as the hinge region because it is sensitive to proteolytic cleavage. Of all the PKC isozymes, the γ isoform is the most proteolytic and may have a shorter half-life, thus potentially allowing for a more rapid turnover of cellular signaling and increased phosphorylation when compared with other PKC isozymes.[83]

In general, there are several steps in which PKC may be regulated.[81] Before activation, PKC must be posttranslationally phosphorylated, which results in a functional form. PKC is initially transphosphorylated, which allows it to undergo autophosphorylation with Thr-641 and become catalytically competent. PKC release into the cytosol occurs after phosphorylation of Ser-660.[81] PKC may also be regulated by the cofactors diacylglycerol, phosphatidylserine, and calcium, all of which need to bind appropriately to activate PKC. Once activated, PKC translocates and binds to membranes. Maximal membrane binding and stabilization require the presence of phosphatidylserine, diacylglycerol, and calcium. Finally, inhibitory second messengers, such as sphingolipids, may alter diacylglycerol-mediated activation of PKC.[81]

Although the genomic structure of PKC-γ has not yet been completely elucidated, the general genomic structure for PKC has been established.[84] The first exon and intron of the gene sequence data for PKC-γ is available.[85] Promoters and the promoter region for the PKC-γ gene have been identified and may provide information on regulation of PKC-γ expression.[86,87] Functional characterization of the PKC-γ receptor promoter region reveals that it is 87 base pairs upstream from the transcriptional initiation site.[86] Within the promoter region, several sequence segments of transcriptional factor binding sites that may mediate transcription have been identified.[87] Multiple DNA-binding proteins may act in conjunction to modulate PKC-γ expression. There may be positive control of PKC-γ expression by itself or other PKC

isozymes.[72,87] Further elucidation of the genomic structure and function of PKC-γ will allow new gene-based approaches to provide antinociception through regulation of expression levels.

Antisense Knockdown and Knockout Data. Some of the characteristics of PKC-γ–deficient mice have been described previously. PKC-γ–deficient mice are anatomically normal, which is consistent with postnatal appearance of PKC-γ.[71,75] Immunoprecipitable PKC-γ activity and PKC-γ mRNA transcripts are absent in mutant mice. In addition, there is no evidence of upregulation of other PKC isoenzymes. These mice show mild ataxia and deficits in memory and learning but have normal baseline synaptic transmission.[71,75] PKC-γ–deficient mice show abnormal long-term potentiation, and responses to nociceptive input are described in Protein Kinase C-γ Isoform: Evidence of Role in Nociception.[88] PKC-γ appears to be a key regulatory component of synaptic plasticity.[71]

Specific inhibitors of PKC isoenzymes are not widely available nor available for the γ isoenyzme. Experiments in our own laboratory indicate successful antisense ODN–mediated knockdown of PKC-γ *in vitro* and *in vivo* with a parallel decrease in the phase II formalin response. No downregulation of PKC-α and -β isoenzymes was induced by the PKC-γ–specific ODN.[89] The restricted distribution of PKC-γ and implication of PKC-γ in development of neuropathic pain lends itself to an ODN-based approach to antinociception. Direct subarachnoid administration of antisense ODN for a selective knockdown of spinal cord PKC-γ is an attractive strategy for preempting central sensitization most likely responsible for the development of neuropathic pain.

Vanilloid Receptors

Evidence of Role in Nociception. Vanilloid agonists, such as capsaicin, act at vanilloid receptors (VRs) to cause excitation (short-term effect) followed by functional desensitization (long-term effect). There are several subtypes of VRs, and one (VR1) has been cloned.[90,91] Anatomically, VRs are distributed in peripheral projections of sensory neurons and spinal cord, with intense staining observed in afferent fiber terminals projecting to laminae I and II.[81] Additional staining is noted in the sensory nucleus of the trigeminal nerve and caudal brainstem (central projection of primary sensory neurons from the dorsal root and trigeminal ganglia).[92,93] VRs are present predominantly in presynaptic terminals of DRG sensory neurons, and intraaxonal VR transport to the periphery has been demonstrated.[90,94] Thus, VRs appear to be a specific molecular marker for nociceptive neurons; however, there is evidence that VRs may also be expressed in peripheral, nonneuronal locations.[90,95,96]

Capsaicin will interact with C-fiber polymodal nociceptors with subsequent receptor activation, resulting in membrane ion channel excitation followed by calcium-dependent desensitization.[94,97] In animal studies, depletion of neuropeptides, including SP, somatostatin, vasoactive intestinal peptide, and CGRP, from the dorsal spinal cord occurs after capsaicin administration and results in functional desensitization and analgesia to noxious mechanical, chemical, or thermal stimuli.[94] VR1 immunoreactivity does not substantially overlap SP or CGRP on nerve terminals, suggesting a complex mechanism of neuropeptide release.[98] Cumulative effects of resiniferatoxin, a potent analog of capsaicin, on cutaneous nociceptors and release of neuropeptides are similar to that observed with capsaicin.[94] In addition, capsazepine, a competitive antagonist to capsaicin, reversibly antagonizes the excitatory action of capsaicin and resiniferatoxin *in vitro* and responses to capsaicin *in vivo*.[90,99,100] Electrophysiologic data demonstrate that capsaicin facilitates excitatory but not inhibitory synaptic transmissions.[101] Although the data referenced above does not specifically link VR inhibition with subsequent analgesia, a recently isolated functional cDNA coding for VR1 is directly activated by increases in temperature in the noxious range and hydrogen ions, supporting animal and human data that VRs may be activated by multiple noxious stimuli *in vivo*.[90,92] No endogenous ligand for the VR has been identified at this time; however, hydrogen ion and heat remain likely candidates.

Molecular Biology and Receptor Function. Vanilloid receptors are traditionally considered a heterogeneous group of ligand-gated, calcium-permeable, nonselective cation channels. However, more recent evidence indicates that there are at least two pharmacologically distinct vanilloid subtypes.[91,102,103] The C(apsaicin)-type VR has characteristics typically associated with vanilloid-induced pharmacologic behavior in DRG (ligand-gated, calcium-permeable, cation-nonselective).[95] The R(esiniferatoxin)-type VR shows greater selective binding for resiniferatoxin and compared with the C type and differs with respect to calcium uptake and inhibition by ruthenium red.[91] Presence of several vanilloid subclasses may correlate with different pathways of desensitization and has implications for development of therapeutic agents.[91]

The predicted amino acid sequence, membrane topology, and domain structure of at least one VR clone (VR1) functionally similar to endogenous VRs has been published.[90] The VR1 ion channel consists of six transmembrane domains with an amino-terminal section containing a high density of proline and three ankyrin repeat domains probably essential for cytoskeletal anchoring of this receptor.[90] The NH_2 terminus consists of 432 amino acids, and the COOH terminus contains 154 amino acids with no recognizable motifs.[81] Two other splice variants of the VR1 gene, VR5'sv and stretch-inhibitable channel, were recently reported.[104,105] A VR-like protein 1 (VRL-1), a distinct gene product with 49–66% amino acid identity to rat and human VR1, has been cloned.[90] Unlike the VR1, VRL-1 does not respond to capsaicin, acid, or

moderate heat. Rather, high temperatures with a threshold near 52°C optimally activate VRL-1; however, VRL-1 transcripts are found in nonneural tissues, indicating a nonnociceptive role for this receptor. How the VR1 and other related gene products interact with each other to define the functional properties of the VRs is not known. The full genomic structure and receptor promoter regions of the VRs have not yet been reported.

Because of their lipophilic nature, it was believed that vanilloids were able to act on either side of the cell membrane as confirmed by identical patch clamp responses to capsaicin; however, more recent data suggest that vanilloids bind to the intracellular domain of the receptor.[90,106] VRs are activated by a wide variety of chemical stimuli, and polymodal activation of VR1 most likely occurs *in vivo*.[92] Binding of more than one agonist molecule is needed for full receptor activation, and there is probable cooperative interaction among subunits.[90,107] Although different agonists may not interact with VRs in the same fashion, there is probably some convergence of channel opening pathways as capsazepine blocks capsaicin-, heat-, and proton-evoked currents.[92]

Vanilloid agonists cause a continuum of effects, from neuronal excitation to desensitization and eventually neurotoxicity.[91,94,97] Multiple modulators on channel function have been reported. Excitation results in influx of 10 calcium ions for every sodium ion, and increase in cytoplasmic calcium may cause desensitization through activation of calmodulin-dependent cytosolic enzymes and calcineurin.[97,108,109] Activated VRs on afferent nerve terminals may release CGRP and play a role in transmission of nociception.[110,111] In addition, capsaicin may promote desensitization by inhibiting voltage-gated calcium channels that mediate release of neurotransmitters.[97]

Electrophysiologic desensitization may also be dependent on the presence of extracellular calcium as VR1-transfected HEK293 cells demonstrate minimal desensitization and capsaicin-induced depolarization is reduced in the absence of extracellular calcium.[90,94]

Depending on the dose and other factors, capsaicin may cause sensory neuron cell death. The mechanism of cell death is not clear but may involve osmotic, intracellular calcium-mediated or NADH–plasma membrane electron transport inhibitory mechanism.[97,112] The sensory deficit (analgesia) resulting from cell death is essentially permanent, and neuropeptide depletion is similar to that observed after axotomy.[97] Genetic regulation of VR expression may be possible in the future; however, additional data, including genomic structure and promoter regions, are needed before any type of genetic approach can be seriously attempted. A note of caution in interpreting the role of VR1 in nociception is the recent suggestion that VR1 and the cannabinoid receptors could interact in a yet undefined manner.[113] VR1 and cannabinoid-1 (CB-1) receptors show overlapping ligand recognition properties and, along with their putative endogenous ligand anandamide, coexist in brain nuclei and some sensory neurons. Our current understanding of the role of a given target in nociception *in vivo* is clearly incomplete.

Agonist, Antagonist, and Knockout Data. Although administration of vanilloid agonists will ultimately produce analgesia secondary to desensitization, initial application of these agonists will result in agonist-induced nociception because of release of glutamate.[114,115] Clinically, topical administration of capsaicin results in an initial burning sensation followed by desensitization and analgesia. Desensitization and relapse of pain occur after discontinuation of topical capsaicin.[116] Although resiniferatoxin and capsaicin are both vanilloid agonists, resiniferatoxin appears to have a more favorable clinical profile, partly as a result of its much higher potency, and has been shown to provide analgesia when administered epidurally.[117] When compared with capsaicin, resiniferatoxin will induce desensitization with less toxicity and irritation, diminish noxious thermal nociception, may be used in lower concentrations, and has a wider therapeutic window.[107,118] A single dose of resiniferatoxin will produce full and prolonged desensitization, resulting in thermal hypoalgesia and reduced hyperexcitability with only partial recovery 4 weeks after resiniferatoxin administration.[118,119]

Two capsaicin antagonists, capsazepine and ruthenium red, are commonly used in studies of VRs. Ruthenium red is a noncompetitive VR antagonist and is not specific for VRs as it has been reported to inhibit several types of ion channels.[120,121] Ruthenium red produces several undesirable effects on the CNS when injected systemically (paralysis) or intrathecally (convulsive activity, neuronal hyperexcitability).[122] Capsazepine is generally considered a competitive antagonist at the VR. In addition to its antagonism of VRs, capsazepine has a nonspecific blocking action on voltage-activated calcium channels.[123]

When administered systemically or intrathecally before or in conjunction with subcutaneous capsaicin, capsazepine will reduce immediate pain and prevent behavioral antinociception induced by capsaicin.[100,124–126] Capsazepine significantly decreases numbers of immunoreactive fos-like cells but does not reduce formalin-induced inflammation and edema.[124,125] Capsazepine has no antinociceptive actions when administered by itself.[126] With the well-supported analgesic function of chemical VR antagonists and the role of VRs in mediating multiple noxious stimuli, targeting VR1 may be a particularly promising avenue for a novel approach to pain management.

A recent characterization of a VR1 knockout mouse supports the role of this receptor in mediating thermal hypersensitivity in the presence of inflammation.[127] The VR1 −/− knockout mice created through a deletion of

an exon encoding part of the first and all of the sixth putative transmembrane domains of the ion channel were indistinguishable from the wild-type littermates with respect to general appearance, gross anatomy, body weight, locomotion, and overt behavior. The knockout animals demonstrated normal responses to noxious mechanical stimuli but showed no vanilloid-evoked pain behavior. Thermal sensitivity after mustard oil or complete Freund adjuvant administration was decreased, but thermal sensitivity caused by a partial sciatic nerve ligation remained unchanged by the gene deletion. A separate study of another VR1 knockout mouse created through a distinct strategy for a targeted deletion of the same gene confirms the critical role of this receptor in mediating inflammatory hyperalgesia.[128] Although further studies are needed to fully understand the role of VR1 and related proteins in nociception, a targeted downregulation of VR1 may prove to be a useful adjuvant approach to pain management since clinical pain states during cancer and arthritis coexist with inflammation.

Peripheral Sodium Channels (PN3/SNS/SCN10a)

Evidence of Role in Nociception. Voltage-gated sodium channels (VGSCs) found in DRG neurons show functional heterogeneity and may be pharmacologically characterized according to whether the channel is sensitive or resistant/insensitive to tetrodotoxin.[129–131] Within the DRG, larger-diameter neurons express tetrodotoxin-sensitive VGSCs with fast activation–inactivation kinetics, whereas small-diameter neurons express tetrodotoxin-resistant VGSCs with slow activation–inactivation kinetics.[131,132] Significantly higher levels of tetrodotoxin-resistant currents (*vs.* tetrodotoxin-sensitive currents) are expressed in capsaicin-sensitive DRG neurons.[133] Furthermore, tetrodotoxin-resistant VGSCs are found only in sensory neurons and may contribute to sustained activation of nociceptors and central sensitization in neuropathic pain and mediate inflammatory pain.[134–136]

Injured peripheral neurons have been shown to contain a specific tetrodotoxin-resistant sodium channel known as PN3 or SNS (SCN10a).[134,137] Using *in situ* hybridization and immunohistochemical and electrophysiologic methods, investigators have demonstrated SNS localization intracellularly in small-diameter, capsaicin-sensitive, sensory neurons of the DRG and trigeminal ganglia in normal, uninjured rats.[133,134,137,138] In addition, the presence of SNS has been demonstrated in human peripheral nerves in patients with chronic neurogenic pain.[139] SNS has not been shown to be expressed by central neurons or nonneuronal tissues.[137,140] SNS VGSCs are resistant to tetrodotoxin and demonstrate slow inactivation kinetics.[140] Thus, based on its pharmacology and restricted pattern of expression, SNS appears to be responsible for tetrodotoxin-resistant currents observed in sensory neurons *in vivo*, although a contribution of SNS2/NaN to the tetrodotoxin-resistant current has been demonstrated in a SNS-null mutant mouse.[132,141]

After peripheral nerve injury, there is SNS accumulation at the site of neuronal injury.[134] *De novo* synthesis may be a possible source of axonal SNS; however, investigators have not demonstrated increased levels of PN3/SNS mRNA levels after nerve injury that would support this hypothesis.[134,142,143] Although some investigators have noted downregulation of both tetrodotoxin-resistant current and α-SNS sodium channel mRNA expression, data suggest that redistribution or translocation of presynthesized α subunits of sodium channels from DRG neurons to peripheral axons by rapid axoplasmic transport is the source of axonal SNS after nerve injury.[134,143–145] In the process of continual axonal remodeling, sodium channels normally undergo insertion and recycling, with biosynthesis occurring within 120 min and a cell surface half-life of approximately 18–22 h.[134,146] As anterograde axonal transport is blocked by nerve injury or neuroma formation, SNS accumulation may occur, especially because myelin removal may be a permissive factor in axonal sodium channel insertion.[147]

SNS may also mediate inflammatory pain as there is increased tetrodotoxin-resistant current density and expression of α-SNS sodium channels in small DRG neurons after injection of carrageenan.[148] Inflammatory mediators may cause an increase in activation of or magnitude in tetrodotoxin-resistant current, suggesting that modulation of the tetrodotoxin-resistant sodium current may play a role in sensitization of nociceptors.[36] Bradykinin-evoked release of CGRP and activation of primary afferent fibers are sodium-dependent but resistant to tetrodotoxin, again suggesting tetrodotoxin-resistant sodium channel involvement in inflammatory nociception.[149,150] Thus, SNS appears to be involved in nociception after peripheral nerve injury and may be important in the transmission of nociceptive information in inflammatory and neuropathic pain states.

Another potential target amenable to gene therapy is the voltage-gated sensory channel, SNS2 or NaN, which has been genetically sequenced and located to primary sensory neurons[151–153]; however, key antisense ODN data suggest little role for NaN in some models of chronic pain,[154] and it is therefore not discussed further.

Molecular Biology and Receptor Function. Voltage-gated sodium channels of the peripheral nervous system consist of an α subunit (260 kd), β1 subunit (36 kd), and associated β2 subunit (33 kd).[132] The α subunit is able to synthesize functional VGSCs, and the β subunits are involved in regulating and enhancing functional channel expression.[132] At least 12 VGSCs have been identified, and PN3 (SNS or SCN10a) is the only tetrodotoxin-resistant VGSC found exclusively in sensory neurons. Consisting of 1,956 amino acids, SNS is structurally

similar to other VGSCs in that the α subunit contains four homologous domains (D1–D4), each with six transmembrane segments forming α helices (S1–S6) and intracellular loops connecting the four domains (ID1–ID4).[132,138,155] Functionally critical regions are conserved in all VGSCs and include ID3 (sodium channel inactivation), S4 (voltage sensors), and the S5–S6 extracellular loop of D1, which forms the channel vestibule and is involved in ion selectivity and toxin binding.[37] Unlike other VGSCs, PN3–SNS possesses a hydrophilic serine (Ser-356) residue in the channel vestibule between S5 and S6 that appears to confer tetrodotoxin resistance as substitution of phenylalanine for Ser-356 changes the function of the SNS channel from a tetrodotoxin-resistant to tetrodotoxin-sensitive channel.[37] Unlike other sodium channels, SNS contains an unique consensus protein kinase A phosphorylation sites in D2 between S3 and S4 and another in ID2–ID3.[138] In addition, there is a glutamine insertion between Pro-583 and Ala-584; however, the significance of these unique aspects of SNS are not clear at this time.

SNS has a defined amino acid sequence and domain structure.[138] The genomic structure of SNS has been identified and is very similar to that determined for another tetrodotoxin-resistant VGSC, SCN5a (cardiac).[156,157] The gene is located on chromosome 9, contains 27 exons, and is approximately 90 kilobases in length. Comparison of the genomic structure of SNS to that of other VGSCs reveals that exon and exon–intron junction structure is well conserved, but length of the introns varies considerably (up to 200-fold).[156]

Depolarization causes an extracellular shift of normally intracellular residues in S4, which, in turn, causes opening of the channel.[158] Tetrodotoxin-resistant VGSCs show slow sodium conductance with slowly activating and deactivating currents.[130,131] This is unlike tetrodotoxin-sensitive VGSCs, which demonstrate fast activation–inactivation kinetics. Like tetrodotoxin-resistant sodium channels found on small sensory neurons, functional SNS sodium channels reveal a depolarized activation potential with slow inactivation kinetics.[131,138] Compared with cardiac and skeletal muscle sodium channels, SNS has a more depolarized (positive) activation potential with a depolarization midpoint of approximately 20 mV.[138,155]

In general, physiologic modulation of VGSC function may result from posttranslational phosphorylation by inflammatory mediators.[132] Phosphorylation of VGSC by protein kinases A, C, and G occurs at several sites and results in reduction in sodium current amplitude.[146] Posttranslational phosphorylation also occurs in tetrodotoxin-resistant VGSCs of sensory neurons.[132] Hyperalgesic agents, such as prostaglandin E_2, modulate tetrodotoxin-resistant currents through protein kinase A and PKC.[44,159]

Transcriptional regulation of tetrodotoxin-resistant VGSC may occur through nerve growth factor (NGF) or possibly neuron-restrictive silencer element.[160,161] NGF appears to participate in regulation of VGSC gene expression *in vivo* as there is upregulation of α-SNS mRNA levels and increase in tetrodotoxin-resistant current density in NGF-treated axotomized DRG neurons.[161] Upregulation of sodium channel α- and β1-subunit mRNA occurs in cultured embryonic DRG neurons treated with NGF.[162] NGF regulates expression of a unique SNS mRNA by a novel transsplicing mechanism; however, the functional significance of this exon duplication is unknown.[163] Neuron-restrictive silencer elements are regulatory proteins that repress transcription of several neuronal genes, including a type II sodium channel.[160] It is not known if neuron-restrictive silencer elements may repress other types of sodium channels such as SNS. Thus, genetic data are available for a gene-based approach to regulate SNS expression.

Antagonist, Antisense Knockdown, and Knockout Data. At this time there are no specific agonists or antagonists for tetrodotoxin-resistant VGSCs. Khasar *et al.*[136] reported the use of antisense ODN against SNS sodium channels. Intrathecal antisense, sense, or mismatch ODNs was given once daily for 3 days through an indwelling catheter. Twenty-four hours after the last dose of ODN, the investigators administered intradermal prostaglandin E_2 into the hind paw. Administration of antisense ODN, but not of sense or mismatch ODNs, resulted in a significant reduction of prostaglandin E_2–induced hyperalgesia and tetrodotoxin-resistant current density in sensory neurons.[136] There was partial recovery of prostaglandin E_2–induced hyperalgesia 4 days after the last ODN injection, with full recovery in 7 days. The investigators concluded that tetrodotoxin-resistant sodium channels contributed to mechanical nociception and inflammatory hyperalgesia *in vivo*.[136] The specific role of SNS3 in mediating tactile allodynia and thermal hyperalgesia after spinal nerve ligation is supported by another recent antisense ODN study.[154] In contrast, antisense ODN targeting SNS2, another tetrodotoxin-resistant sodium channel, had no effect on painful behaviors. Consistent with the anatomic and antisense ODN data, SNS3 knockout mice demonstrated analgesia to noxious mechanical stimuli and delayed development of inflammatory hyperalgesia.[164] Preliminary data indicate that gene therapy targeting SNS may provide antinociception with no significant side effects. Restriction of SNS to sensory neurons, its presence after nerve injury, and its role in neuropathic pain suggests that a gene-based approach to SNS expression may be promising.

Cannabinoid Receptors

Evidence of Role in Nociception. Cannabinoids, which include marijuana and its active compound Δ-9-tetrahydrocannabinol (THC), have been shown to possess antinociceptive properties.[165] Presently, there are

two known cannabinoid receptors. The cannabinoid1 receptors are found centrally (basal ganglia, cerebellum, cortex, hippocampus, periaqueductal grey, and spinal cord) and peripherally in certain tissues (uterus, heart). CB-2 receptors are only found in peripheral tissues, including spleen and macrophages.[166–169] A truncated isoform of the CB-1 receptor (CB-1a) with similar pharmacology and distribution to the CB-1 receptor has also been described.[170,171] Anatomically, CB-1 receptors are located in close proximity to areas involved in nociception.[172,173] Many of the CB-1–like immunoreactive neurons also are located in areas involved in GABA-mediated transmission, thus leading to the possibility that cannabinoid receptors may have a role in modulating GABA-mediated neurons.[173] Although CB-2 receptors are generally considered to be involved in mediating lymphocyte function, recent data suggest that the CB-2 receptor also may produce an antinociceptive effect against inflammatory pain.[167,174]

Cannabinoid receptor activation at central (spinal and supraspinal) and peripheral sites may produce antinociception.[165,174] Intravenous administration of WIN 55,212-2 (a selective cannabinoid agonist) to rats results in inhibition of noxious stimulus–evoked activity of wide-dynamic-range neurons and prevention of development of hyperalgesia.[175,176] WIN 55,212-2 does not change evoked activity in nonnociceptive neurons, and administration of an inactive enantiomer, WIN 55,212-3, does not alter the noxious stimulus–evoked activity of wide-dynamic-range neurons. Administration of anandamide, an endogenous agonist, completely prevents carrageenan-induced thermal hyperalgesia.[177]

Central mechanisms of cannabinoid-induced antinociception most likely involve both spinal and supraspinal components.[178] In support of a supraspinal mechanism, intraventricular administration of WIN 55,212-2 and CP-55,940, two potent synthetic and selective cannabinoids, reduces rat responses to noxious thermal stimuli.[179] At supraspinal levels, cannabinoids appear to produce antinociception through modulation of rostral ventromedial medulla activity in a fashion similar to that of morphine.[180] Microinjection of THC and its analogs into various supraspinal locations indicate that the posterior ventrolateral periaqueductal grey may also be important in mediating cannabinoid antinociception.[181] Both the periaqueductal grey and rostral ventromedial medulla are important in antinociception.[182] Endogenous cannabinoids also appear to tonically regulate antinociception at supraspinal sites as exclusive administration of selective CB-1 receptor antagonists results in hyperalgesia.[180,183]

Spinal mediation of antinociception by cannabinoid receptors occurs as CB-1 receptors have been localized to the dorsal horn of the spinal cord. Intrathecal injection of THC produced antinociception in intact mice and those that had their spinal cord transected at the T12 level.[88] Brain concentrations of THC in spinally transected mice that received intrathecal THC resulting in antinociception were lower than those measured in mice that received intravenous THC, which provided ineffective antinociception, suggesting the presence of a spinal mechanism of THC-mediated antinociception. Endogenous cannabinoids also appear to tonically modulate basal thermal nociceptive thresholds at the spinal level and carrageenan-induced hyperalgesia.[177,184] The antinociceptive action of cannabinoid agonists extend to persistent pain states as well. Intrathecal administration of WIN 55,212-2 reduced mechanical allodynia and prevented wind-up of spinal nociceptive.[185,186] Although the exact mechanism by which cannabinoids act at the spinal level is unclear, cannabinoids may activate descending noradrenergic neurons and provide antinociception *via* spinal α_2 receptors or prevent neurosecretion from primary afferent fibers.[178] Other mechanisms of cannabinoid-induced spinal antinociception may include indirect interactions with κ opioids and spinal release of dynorphin.[187,188] The relative contribution of spinal and supraspinal mechanisms to cannabinoid-induced antinociception is not clear at this time.

Peripheral mechanisms of antinociception by cannabinoid receptors has been described previously.[174,189] Existence of CB-2 receptors on peripheral nerves and antinociceptive properties of CB-2 receptors have been demonstrated.[189,190] In rat models of visceral and somatic inflammatory pain, antinociceptive effects were noted after administration of both anandamide and palmitylethanolamide, an analog of anandamide and CB-2 agonist devoid of central CB-1 receptor activity.[174] Analgesia produced by palmitylethanolamide was reversed by a CB-2 antagonist (SR 144528) but not by naloxone or a CB-1 antagonist (SR141716A).[189] Antinociceptive effects of palmitylethanolamide were more pronounced when given locally than systemically. Although traditionally thought of as a central receptor, CB-1–like receptors have been found outside the CNS and may act with CB-2 receptors to produce synergistic inhibition of peripheral nociception.[189] Furthermore, recognition that a natural metabolite of arachidonic acid, anandamide, serves as an endogenous cannabinoid ligand enhances the physiologic relevance of cannabinoid receptor–mediated antinociceptive mechanisms.[191]

Thus, both CB-1 and CB-2 demonstrate antinociceptive properties. Recent data have modified the traditional view of CB-1 and CB-2 receptors as confined to the CNS and periphery, respectively. CB-1 antinociceptive effects appear to have a supraspinal and spinal component, and there may be synergistic interactions between cannabinoid subtypes to produce antinociception.

Molecular Biology and Receptor Function.

All cannabinoid receptors belong to the superfamily of G protein–coupled membrane receptors, and critical

regions, such as intracellular loops required for G-protein interaction, are conserved.[192,193] Characteristics shared by all G protein–coupled receptors are described elsewhere, and some of these have been previously mentioned (see Tachykinin NK-1 Receptors).[194] Many G protein–coupled receptors contain conserved cysteine residues in extracellular regions between TM2–TM3 and TM4–TM5 that appear to stabilize the tertiary structure; however, cannabinoid receptors lack cysteine residues between TM2 and TM3 but contain two or more cysteine residues between TM4 and TM5.[195] Cysteine residues are critical for cell surface expression of CB-1 and ligand binding for CB-2 receptors.[196] For the CB-1 receptor, the lysine-192 residue appears to be critical for receptor recognition by several cannabinoid agonists.[197]

The CNR1 locus that codes for the human CB-1 receptor is located on chromosome 6; the CNR2 locus that codes for the CB-2 receptor has not yet been identified.[195] Although the complete extron–intron structure of the human CB-1 or CB-2 receptor has not been published, it appears that the coding sequence for CB-1 is located within a single exon.[195] Other exons code for the 5' sequence in rat cDNA (exon A) and leader sequence in human cDNA (exon B).[195,198] A sequence corresponding to a 5' intron has most likely been identified.[171,195] The peptide sequences for both CB-1 and CB-2 have been documented.[169,195] The CB-2 receptor has a 44% overall homology to the CB-1 receptor with a 68% homology in the helical regions.[199]

Although CB-1 and CB-2 receptors differ in their coupling to signal transduction pathways, both receptors will inhibit adenylate cyclase through a pertussis toxin-sensitive G protein.[200] Unlike CB-2 receptors, CB-1 receptors will inhibit N- and Q-type calcium channels, activate inwardly rectifying potassium channels, and may actually stimulate cAMP during certain conditions.[200–203] CB-2 receptor activation results only in inhibition of adenylate cyclase but not ion channel modulation.[192,204] Thus, cannabinoid receptor activation may effect antinociception by altering neurotransmitter release (*e.g.*, acetylcholine, noradrenaline, and glutamate).[200,205,206] Cannabinoids may also mediate receptor-independent effects such as inhibition of arachidonic acid acylation and release of arachidonic acid and intracellular calcium.[207,208]

Cannabinoid 1 receptor expression may be affected by several factors. Glucocorticoid and dopamergic activity reduces CB-1 receptor mRNA levels.[209,210] Decreases in glutamergic neurotransmission occur in association with decreases in CB-1 receptor mRNA levels.[211] It is not clear whether the observed changes are the result of transcriptional or posttranscriptional effects. Thus, partial data on the genomic structure and regulation of the cannabinoid receptors are available, and the creation of a recently described CB-1 knockout mouse will facilitate further development of cannabinoid receptor–based strategies for antinociception.

Knockout and Antisense Knockdown Data. Although endogenous cannabinoids and cannabinoid receptor agonists exist, there are many disadvantages of using THC as an agent for antinociception as a result of its biologic profile. Spinal administration of THC in mice produces not only potent antinociception but also hypoactivity, hypothermia, and catalepsy.[88] In rhesus monkeys, cannabinoids produce decreases in respiratory minute ventilation and tidal volume.[212] Cardiovascular effects include tachycardia, bradycardia, and hypotension.[213] Side effects in humans include drowsiness and dysphoria.[165]

Although there are no published data for a CB-2 receptor knockout, a recent report described lack of effect of cannabinoids in CB-1 receptor knockout mice.[214] Spontaneous nociceptive thresholds between mutant and normal mice are similar; however, antinociceptive properties of THC are not observed in mutant mice, unlike those observed in normal mice. The investigators concluded that the CB-1 receptor is important in mediating physiologic (hypothermia, hypolocomotion, hypotension) and behavioral (addiction, motivation) properties of cannabinoids.[214] In addition, the CB-1 receptors may manifest as highly relevant for pain control as this receptor appears to mediate reinforcing and withdrawal properties of morphine.[215]

Edsall *et al.*[166] describe use of antisense ODN against both CB-1 and CB-2 receptors. Intracerebroventricular injections of both antisense ODN to the CB-1 receptor (complimentary to the 5' end) and mismatched ODN to mice for a period of 3 days results in complete antinociception after administration of the cannabinoid agonist CP-55,940 in control mice or those treated with mismatched ODNs. Mice treated with antisense ODN to the CB-1 receptor demonstrate inhibition of antinociception after administration of CP-55,940, thus providing further evidence that the CB-1 receptor is responsible for antinociception.[166] No adverse symptoms or side effects occur after antisense ODN administration. An *in vitro* study of antisense ODN to the CB-1 and CB-2 receptors suggests that cannabinoid receptor activation may mobilize arachidonic acid, resulting in increased eicosanoid (*e.g.*, anandamide) synthesis.[216] Administration of antisense ODN against the CB-1 and CB-2 receptors and a CB-1 antagonist (SR 141716A) results in a significant decrease in THC-initiated release of arachidonate and anandamide; however, random, mismatched ODN were ineffective in reducing THC-induced release of arachidonic acid.[216] Thus, initial data for cannabinoid receptor gene-based therapy appear promising, with the limitation being that a peptide agonist of the cannabinoid receptor amenable to overexpression by viral vectors has not been described. In theory, an alternative strategy of enhancing synthesis of anandamide, an endogenous

cannabinoid- receptor agonist, through overexpression of the synthetic enzymes could achieve the same end point of enhanced antinociception.

Muscarinic and Nicotinic Acetylcholine Receptor

Acetylcholine can stimulate both muscarinic acetylcholine receptor (mAChR) and nicotinic acetylcholine receptor (nAChR) and may produce supraspinal and spinal-mediated analgesia. mAChRs and nAChRs have different structure, function, agonists–antagonists, and receptor activation pathways. Evidence for analgesia of and differences between the two receptors are highlighted in the following sections.

Evidence of Role in Nociception.

Muscarinic. Although mAChRs are involved in supraspinal analgesia, mAChRs seem especially important in mediating analgesia at the spinal cord level.[217–220,221] Autoradiographic studies demonstrate localization of muscarinic binding to laminae II–III of the dorsal horn with a two to three times higher number of mAChRs than nAChRs.[222,223] Immunocytochemistry demonstrates the presence of a cholinergic interneuron within the dorsal horn.[224] Presynaptic muscarinic receptors are also located on primary afferent nerve terminals in the dorsal horn as there is rapid disappearance of cholinergic receptors after dorsal rhizotomy.[225,226] Electrophysiologic data reveal depolarization and hyperpolarization after muscarinic agonist administration, suggesting that muscarinic receptor–induced spinal antinociception may result from hyperpolarization of dorsal horn neurons and depolarization of inhibitory spinal interneurons.[227,228]

There is also pharmacologic evidence for muscarinic receptor–induced analgesia. Administration of selective muscarinic agonists intrathecally produces antinociception to tail-flick and thermal stimuli.[229–231] Intrathecal administration of indirect muscarinic agonists produces analgesia that is antagonized by muscarinic antagonists.[232,233] Analgesia to thermal nociception after spinal cholinesterase inhibition is antagonized by muscarinic but not nicotinic antagonists.[227] However, in females, a significant nicotinic component appears to be present.[234] Spinal muscarinic receptors may also mediate the anti-allodynic effects of clonidine in neuropathic pain.[220] Mechanisms of muscarinic receptor–induced analgesia may involve stimulation of nitric oxide formation.[218,221,235] Thus, based on anatomic, genetic, and pharmacologic data, there appears to be evidence for muscarinic receptor–induced analgesia at the spinal level.

There are at least five muscarinic receptor subtypes (M1–M5), and it is unclear which subtype(s) mediate mAChR-induced spinal analgesia. Previous data suggested the possibility that M1 and/or M2 subtypes mediate muscarinic antinociception at the spinal cord level.[236,237] The M3 subtype may also mediate spinal muscarinic receptor–induced antinociception.[230] Unlike previous studies suggesting the presence of the M1 subtype in spinal cord, more recent data reveal the absence of M1 from and presence of M2, M3, and M4 in rat spinal cord.[238] Reverse-transcriptase polymerase chain reaction reveals an abundance of M1 receptor mRNA in cortex but a barely detectable level in spinal cord.[239] Some limitations to neuroanatomic localization of specific muscarinic subtypes are discussed elsewhere.[240] Pharmacologic data with selective agonists also suggest that central muscarinic analgesia is mediated by subtypes other than M1.[241] Other data using selective muscarinic agonists and antagonists suggest that neither M2 nor M3 mediate mAChR spinal analgesia; however, intrathecal administration of epibatidine analogs with M4 activity demonstrate antinociception.[229,242–244] In addition, a mouse line lacking functional M2 demonstrates antinociception in both the tail-flick and hot-plate tests.[219] Further neuroanatomic and pharmacologic evidence is needed to elucidate definitively muscarinic subtypes mediating spinal antinociception. Antisense ODN–mediated knockdown of specific mAChR isoforms and examination of the resultant pain phenotype is one possible approach to a deeper understanding of the functional importance of receptor heterogeneity.

Nicotinic. Although nAChR will mediate both supraspinal and spinal analgesia, nAChR-mediated supraspinal antinociception may be more prominent and spinal antinociception may not be as important as that mediated by muscarinic receptors.[230,245,246] Although many subunits of nicotinic acetylcholine receptors are known, virtually all of the neuronal nAChRs consist of α_4 and β_2 subunits and the homooligomer α_7.[247] Use of cRNA probes reveals distribution of $\alpha_{4\beta2}$ throughout the CNS with a strong hybridization in thalamus and reticular core, to a lesser extent, with weak hybridization found throughout the spinal cord.[248] An immunochemical study also locates nAChR to thalamus, reticular core, and substantia gelatinosa.[249] Electrophysiologic studies demonstrate direct excitation of isolated septal and peduncular neurons cells with exogenously applied nicotinic agonists.[250,251] *In vitro* ion flux studies with a DRG cell line demonstrate that nAChR agonists induced release of SP in a concentration-dependent fashion is inhibited by nAChR antagonists, suggesting that nAChRs may play a role in modulating nociceptive transmission.[252] In addition, a mutant mouse lacking an α_4 subunit demonstrates reduction of antinociception, suggesting that the α_4 nAChR subunit is crucial for nicotinic-elicited antinociception.[245]

Pharmacologic data support the role of nAChR in antinociception at both supraspinal and spinal sites. Supraspinally, nAChRs are located presynaptically and may act to modulate release of transmitters from presynaptic terminals.[253] Microinjection of nicotine and carbachol into the mesencephalic periaqueductal grey, pedunculopontine tegmental nucleus, and nucleus raphe magnus

produces antinociception independent of endogenous opioid mechanisms.[254,255] ABT-594, a potent neuronal nicotinic agonist that produces analgesia in animal models of acute thermal, persistent chemical, and neuropathic pain, also produces antinociception to thermal stimuli when directly injected centrally into the nucleus raphe magnus.[246,256,257] Activation of supraspinal nAChR may produce antinociception through activation of descending inhibitory systems, cholinergic and possibly enhancement of β-endorphin–induced antinociception.[237,248,257–260]

Spinal administration of nicotine and nicotinic agonists produces antinociception.[261,262] Antinociception produced by epibatidine is blocked by a nicotinic channel blocker but not inhibited by naloxone or atropine.[261] Activation of spinal nAChR may produce antinociception through several mechanisms, including activation of inhibitory interneurons or spinal–supraspinal modulatory circuits.[261] Potent nAChR agonists will cause not only antinociception but also adverse side effects such as hypertension, neuromuscular paralysis, and seizures.[246] Although anatomic and functional evidence indicates that nAChRs at both supraspinal and spinal levels mediate antinociception, taking advantage of this knowledge for therapeutic purposes will require a method that allows for pharmacologic and anatomic modulation of the receptor function.

Molecular Biology and Receptor Function.

Muscarinic. Mucarinic receptors belong to the G protein–coupled receptor superfamily and share structural similarities to other G protein–coupled receptors.[263] The muscarinic receptors consist of a number of subtypes, and cloned receptor subtypes (m1–m5) correspond to M1 through M5 muscarinic receptor subtypes, respectively. The deduced amino acid sequences of muscarinic subtypes are described elsewhere.[264] The genomic structure of several subtypes has been described.[265,266] In general, the coding exon lacks introns and is preceded by two noncoding exons and an intron.[265] Transcription initiation occurs at different sites, and the promoter regions for the different subtypes have been identified.[267,268] In the M1 and M4 mAChR, initiation of transcription occurs at two sites upstream from the 5' end of the intron, whereas the M2 mAChR contains five transcription sites. Promoters for mAChR contain consensus sites for recognized transcription factors.[265] There appears to be a neuron-restrictive silencer element for the M4 and M4 promoter region but not the M1 subtype.[266,269] Detailed knowledge of the intron–extron structure of the muscarinic receptor genes will allow identification of targets particularly susceptible to antisense knockdown. Alternatively, dominant-negative mutants can be expressed using endogenous promoters for a selective elimination of the receptor function only in cells normally expressing a particular receptor function.

Second messenger signaling differs among the subtypes. "Odd" subtypes (M1, M3, M5) mediate their effects through increases in intracellular calcium, whereas "even" subtypes (M2, M4) mediate their effects through decreases in cAMP production.[263] Odd muscarinic receptors couple to the α subunit of Gq and G11 and activate phospholipase C, resulting in mobilization of intracellular calcium and activation of diacylglycerol and, subsequently, PKC. There is also activation of phospholipase D, which may be involved with signaling of deeper cellular compartments associated with gene expression, mitogenesis, and vesicular trafficking.[270] Activation of odd muscarinic subtypes will inhibit voltage-dependent, noninactivating K^+ current ("M current") and Ca^{2+}-activated K^+ channels ("SK").[270,271] Even muscarinic subtypes couple to the α subunit of G_i and G_o and inhibit adenylyl cyclase activity and cAMP formation. This results in a reduction in protein kinase A–dependent phosphorylation of calcium channels and proteins involved in regulating gene expression.[270] Activation of even subtypes results in opening of inward rectifier (Kir) channels and inhibition of neuronal (N-type) Ca^{2+} currents.[271] Knowledge of downstream mediators of the muscarinic receptor–induced analgesia opens up the possibility of neuron-specific expression of these ion channels as a means of providing analgesia by bypassing the muscarinic receptor. Thus, the genomic structure and receptor function of mAChR are known and provide the basis for a gene-based approach for analgesia.

Nicotinic. Unlike muscarinic receptors, nicotinic receptors are ligand-gated ion channels. At least 11 nAChR subunits (α_2–α_9 and β_2–β_4) are recognized. The genomic structure of human nAChR α_4, β_2, and α_7 subunits are known.[272–274] Both the α_4 and β_2 nAChR subunits contain six conserved exons and five introns, whereas the α_7 nAChR subunit contains 10 exons.[272–274] In addition, the promoter regions of these nAChR subunits are known and contain several regulatory elements.[275–279] The amino acid sequences of nAChR subunits are described elsewhere.[280–282] Posttranslational mechanisms, including phosphorylation, may increase the number of functional $\alpha_4\beta_2$ nAChR on cell surfaces.[283]

With the exception of nAChR subunits α_7–α_9, neuronal nAChR exists as a pentameric arrangement containing both α and β subunits with some neuronal nAChR consisting of two α and three β subunits.[283] The pentameric arrangement creates a transmembrane channel allowing Na^+ and Ca^{2+} to pass during activation. Although each nAChR subunit consists of four hydrophobic transmembrane domains (M1–M4) with extracellular NH_2 and COOH terminals, the lengths of each subunit vary. The loops between transmembrane domains are intracellular and also vary in length and amino acid composition between nAChR subunits.[283]

Unlike mAChRs, which are G protein–coupled receptors, nAChRs are ligand-gated channels where binding of an agonist causes conformational change of the channel

to allow influx of Na^+ and Ca^{2+}. Neuronal nAChRs show functional diversity, and functional properties of the ion channels are determined by the composition of the nAChR subunits. A detailed discussion of functional properties of nAChR is beyond the scope of this review and is discussed elsewhere.[284] Activation of neuronal nAChR produces significant increases in intracellular Ca^{2+} and may play a role in cellular signaling.[285] When compared with muscle nAChRs, neuronal nAChRs are more permeable to Ca^{2+}, reinforcing the possibility that neuronal nAChRs may mediate long-term potentiation and synaptic transmission.[284] Thus, the genetic data are well established for neuronal nAChR α_4, β_2, and α_7 subunits.

Antisense Knockdown and Knockout Data.

Muscarinic. Mice deficient in M1 and M2 subtypes have been generated.[219,286] Although no tests for antinociception were performed on the M1 mAChR knockout mice, the M2 mAChR-deficient mice demonstrated significantly reduced antinociceptive responses to tail-flick and hot-plate tests after muscarinic agonist administration.[219] The M2 subtype was found to play a critical role in mediating muscarinic receptor–dependent movement and temperature control.[219]

Use of antisense ODN against M1 mAChR has been reported.[287] Intraventricular administration of antisense ODN for 3 days reduced M1, but not M2, receptor mRNA by 64%. [^{3}H]Pirenzepine binding to M1 receptors was decreased by 43%. In another study, antisense ODN against mRNAs encoding receptors with seven-transmembrane spanning regions (G protein–coupled receptor superfamily) was administered for a period of 4–6 days *in vitro*.[288] Although not specifically targeting muscarinic receptors, the antisense but not mismatch ODNs decreased total muscarinic binding sites by 40% and completely eliminated M2 mAChR. Thus, preliminary studies demonstrate the possibility of antisense ODNs in reducing expression of mAChR mRNA; however, other gene-based approaches to regulate expression of mAChR subtypes may be more successful in producing antinociception.

Nicotinic. Mice deficient in α_4 and β_2 subunits have been generated.[245,289,290] Marubio *et al.*[245] demonstrated reduction of antinociception to nicotine in mice lacking neuronal nAChR α_4 or β_2 subunits. There was no detectable α_4 or β_2 mRNA by *in situ* hybridization or compensatory upregulation of other nAChR subunits.[245,289] In addition, there was loss of nicotine-elicited currents in patch clamp recordings of neurons in the raphe magnus and thalamus in mutant mice, suggesting the importance of nAChR α_4 and β_2 subunits in mediating supraspinal nicotine antinociception. β_2 subunits may also be important in mediating the reinforcing properties of nicotine.[290]

Antisense ODNs targeting genes encoding nAChR subunits in sympathetic neurons have been described previously.[291] Selective deletion of individual subunits was performed; however, this was an *in vitro* study, and antinociceptive properties of the nAChR subunits were not examined. Another study attempted a different antisense approach to inhibit nAChR function. Antisense RNAs inhibited the appearance of functional nAChR on surface membranes *in vitro*.[292] The antisense RNA hybridized with its counterpart mRNA to inhibit mRNA translation of nAChR.

Although viral overexpression of nicotinic or muscarinic AChRs is feasible since both mediate antinociception, increasing the expression of these receptors may only indirectly affect nociception. Both muscarinic M2 and nicotinic α_4 knockout animals demonstrate no intrinsic hyperalgesic effects but clearly influence nociception by decreasing the analgesic effects of exogenous pharmacologic agonists for the respective receptors. Complicating this picture are the differences demonstrated between men and women in their response to nicotinic analgesia and the likely interaction between cholinergic, adrenergic, and nitric oxide systems.[221,234] Direct enhancement of AChR expression may or may not be useful in producing antinociception, and alternate gene-based approaches to enhancing the receptor function (*e.g.*, upregulation of acetylcholine synthetic enzymes, local downregulation of cholinesterase, *etc.*) may be useful in producing antinociception when using the nicotinic or muscarinic systems.

N-*methyl-D-aspartate Receptor*

Evidence of Role in Nociception. Glutamate receptors of the α-amino-3-hydroxy-5-methyl-4-isoazolepropionate (AMPA)–kainate type preferentially mediate spinal monosynaptic reflexes[293–296] and acute nociceptive responses.[297] Conversely, NMDA receptors are implicated in polysynaptic spinal pathways[298,299] and chronic nociceptive responses. The presence of NMDA receptors and subunits (NR1–NR3) in spinal cord and their involvement in pain processing is supported by receptor autoradiography[300,301] and *in situ* hybridization.[302] Recently, immunohistochemical studies using a specific antibody revealed that the NMDA NR1 subunit is located in both presynaptic and postsynaptic components in the dorsal horn of rat spinal cord in regions that are important to nociceptive processing.[303] NR1 immunoreactivity has also been observed in small-diameter DRG cells.[304] Moreover, it has been observed that NR1 mRNA predominates in ascending spinal pathways when compared with interneurons.[305]

The NR2 subunits have also been evaluated in the spinal cord. mRNA for NR2A or NR2B was not detectable in rat spinal cord, whereas NR2C and NR2D showed low levels of expression in the superficial laminae and in regions surrounding the central canal in rat.[306] In contrast, NR2A, NR2C, and NR2D were observed in human cord.[307] Taken together, these data demonstrate an en-

dogenous NMDA receptor system located in regions of the spinal cord known to be involved in nociceptive processing.

Data investigating NMDA receptor activation support the role of NMDA receptors in mediating nociception. First, spinal administration of NMDA evokes a dose-dependent, selective, and reversible hyperalgesia in mice and rats.[308–310] In addition, increased spinal release of glutamate has been observed after various forms of peripheral nociceptive stimulation.[311–313] Moreover, spinal NMDA receptor activation has been shown to participate in neuronal plasticity such as "wind-up," which contributes to the phenomenon of central sensitization.[297–314]

The role of NMDA receptor activation in animal models of chronic pain also has been established. Behavioral studies demonstrate that the hyperalgesia, which occurs in the carrageenan,[315,316] formalin,[317] and mustard oil[318] models of inflammation, is inhibited by spinal administration of selective NMDA antagonists. NMDA receptor activation also has been implicated in mediating hyperalgesia in neuropathic pain models.[319] There have been some studies that suggest that NMDA may mediate development of chronic pain through activation of peripheral NMDA receptors.[320–322] This concept is supported by findings in human trials where local injection of NMDA antagonists resulted in decreased hyperalgesia during burn injury.[323]

Molecular Biology and Receptor Function. Excitatory amino acids are a major class of neurotransmitters that bind to ionotropic (ligand-gated cation channels) and metabotropic receptors (G protein–coupled receptors). Ionotropic receptors can be subdivided into three major distinct types based on their preferred agonists, referred to as NMDA, AMPA, and kainate. The NMDA receptor, which plays a major role in glutamate transmission in the CNS, is a Ca^{2+}-permeable, ligand-gated ion channel in a heterooligomeric assembly. The NMDA receptor possesses a Mg^{2+} binding site that confers a voltage-dependent block on the channel.[324,325] Therefore, NMDA receptor activation is dependent on both the presence of transmitter and membrane potential of the cell. At resting potential, the synaptic current consists mainly of a fast AMPA receptor–mediated component. When the cell is depolarized, the Mg^{2+} block is removed and the NMDA-mediated component revealed.[326] This allows the receptor to function as an activity sensor, which permits an influx of Ca^{2+} and Na^{+} through NMDA channels only in cells with high-frequency synaptic inputs. This type of high frequency synaptic input occurs during wind-up and central sensitization, which are fundamental spinal processes known to facilitate chronic pain states.

The NMDA receptor is pharmacologically complex, with at least nine distinct binding sties[327–331] by which receptor activity can be regulated. NMDA receptor activity, through these regulatory sites, can be modulated by occupation of these sites to include facilitation by glycine, activation by polyamines, and inhibition by Zn^{2+} and intracellular Na^{+} concentration.[332] Gycine acts as a co-agonist of NMDA by binding to the GlycineB site. Once occupied, the GlycineB site acts to decrease NMDA desensitization, thereby prolonging the NMDA effect. The NMDA receptor quickly repolarizes, as slow kinetics limit NMDA activation in the absence of glycine, and is blocked by extracellular Mg^{2+} in a voltage-dependent manner.

Several genes encoding NMDA receptor subunits have been identified.[333–335] These genes fall into three categories: the NMDA-R1 (NR1), NMDA-R2A-D (NR2A-D), and NMDA-R3 (NR3) subunits. There are at least eight splice variants of the NR1 protein; however, whether these differ functionally is unclear. The NR1 protein has a large N-terminal region, a core region including four transmembrane domains, and a C-terminal extension. NR2 subunits display little sequence homology to NR1 (15–20%) and are considerably larger than NR1. Although NR1 subunits are able to form functional homooligomeric receptors in *Xenopus* oocytes, the NR1 subunits require NR2 subunits for expression of functional receptors in eukaryotic cells.[333–336] Differing expression of NR2 isoforms shifts the affinity of NMDA channel blockers, whereas altering NR1 splice variants has no effect.[337] Expression of NR1 is ubiquitously expressed, although mRNA levels vary regionally. For example, the highest NR1 mRNA levels observed in the adult CNS are in the olfactory bulb, whereas the lowest are in the spinal cord.[338] An increase in NR1 levels is generally observed in adults when compared with postnates.

NR2 subunits are regionally localized and differentially expressed during development.[339] In general, NR2A (highest mRNA levels in hippocampus and cerebral cortex and lowest in pons-medulla[340]) and NR2B[341] predominate early in development, whereas expression of NR2C and NR2D increases in the adult.[342–344] Localization of the NR2A subunit is generally ubiquitous throughout the brain, whereas expression of NR2B is more restricted. Interestingly, NR2B proteins are virtually absent in the cerebellum,[340,345] whereas expression of the NR2C is predominant. It has been suggested that the development shifts that occur in expression of the NR2 subunits may be activity dependent.[346–348] Thus, receptor diversity may be conferred through expression of NR2 variants. The NR2 subunits can be functionally divided into two groups (NR2A and NR2B *vs.* NR2C and NR2D),[349] primarily by sensitivity to Mg^{2+} blockade. In addition, a recently characterized regulatory subunit, NR3A, is primarily expressed during brain development and most highly expressed in spinal cord, brain stem, and thalamus.[350]

Of particular interest, with regard to nociception, is the fact that PKC has differential effects on calcium permeability that is dependent on the NR2 subunit type

that is expressed. It has been demonstrated that PKC activation of recombinant NMDA-mediated calcium increase is enhanced by expression of NR1–NR2A or NR2B, whereas calcium increases are suppressed by coexpression of NR1–NR2C or NR2D.[351] These distinguishing features may account for the differential effects of PKC in different tissues.

Antagonists, Antisense Knockdown, and Knockout Data. Several clinical trials have been performed using selective NMDA antagonists in the treatment of a variety of disorders. Unfortunately, compounds such as MK-801 have limited clinical usefulness because of issues of toxicity.[352–354] Less selective NMDA antagonists have gained recent attention as these compounds (*e.g.*, ketamine and dextrophan) were previously approved for human use as anesthetics and cough–cold therapy. When these compounds are administered intrathecally or systemically, their antinociceptive effects are accompanied by side effects such as nausea, fatigue, dizziness, visual impairment, and loss of memory.[355–358] However, it has been suggested that peripheral administration of these compounds may avoid untoward side effects while still providing adequate pain control.[359]

Because of multiple functions of the NMDA receptor and ubiquitous expression of the NR1 receptor subunit, it is not surprising that targeted disruption of the gene encoding the NR1 subunit would be lethal.[360] Knockout of genes encoding NR2A, C, and D subunits are viable but render no distinct phenotypes. Of interest are mice that express a truncated form of the NR2A that exhibit impaired synaptic plasticity,[361] since such processes are likely to resemble central sensitization. Knockout mice of the NR2B gene die perinatally.[361,362] Overexpression of the NR2B subunit in forebrains of transgenic mice yield mice that display enhanced learning and memory.[363] Should these higher cognitive functions correlate with neuroplasticity at the spinal cord level responsible for central sensitization, selective knockdown of the NR2B subunit may be a useful strategy for limiting neuropathic pain.

Many studies have successfully demonstrated knockdown of the NMDA R1 subunit using antisense strategies in the CNS. For example, antisense strategies directed against the NR1 subunit of the NMDA receptor have been shown to block production of the NR1 subunit, protect cortical neurons from neurotoxicity, and reduce and/or prevent ischemic infarctions.[364,365] Moreover, NR1 knockdown has been shown to be anxiolytic and increased the latency of NMDA and sound-induced seizures.[366,367] In addition, the relative selectivity of this strategy has been demonstrated since antisense directed against the NR1 subunit has been shown to depress NMDA receptor–mediated synaptic transmission in hippocampal slices, while AMPA-mediated responses and glycine binding are kept intact.[366,368] Finally, in mice lacking the NR3A subunit, increases in NMDA current and dendritic spine density in early postnatal cerebrocortical neurons have been observed.[350]

In a recent study, antisense ODNs targeted against the NR1 subunit were administered into the ventral posterior lateral nucleus of the thalamus, a supraspinal site for nociceptive processing.[369] It was observed that knockdown of the NR1 subunit resulted in blockade of thermal and reduction of mechanical hyperalgesia in response to peripheral inflammation. In addition, we demonstrated that antisense directed against the NR1 subunit reduces NMDA- and formalin-induced behaviors when administered spinally.[370] In our study, we observed greater antinociception when ODNs had phosphothiolate modifications. Both of these studies demonstrate the feasibility of the use of an antisense strategy in reduction of pain behaviors. Although toxicity was not specifically tested, both studies report absence of motor deficit, weight loss, or overt signs of distress as a result of the antisense regimen. However, since the NR1 subunit is ubiquitously expressed (though it is enriched in the dorsal horn), it is feasible that more selective blockade would come from targeting NR2 subunits. In particular, NR2C and NR2D would be logical targets because of their localization to the spinal cord. What is currently unknown is the state of subunit expression in the spinal cord during development of chronic pain. Such information would be critical in the selection of specific NMDA receptor targets for development of novel compounds that could lack deleterious side effects.

Future Directions

Our enhanced understanding of the neurobiology of pain and great progress in molecular biologic technology, particularly in gene therapy, render a gene therapeutic approach to pain management a realistic possibility. The process of nociception involves intricate interactions between a large number of cellular and molecular targets and includes many classes of potential targets for gene therapy. We have presented only a selected subset of targets that may be amenable to a gene therapeutic approach for attenuation of nociception; however, the strategies and methods of gene therapy that we have provided may be applied to other current or future nociceptive targets.

Admittedly, many gaps in our understanding of the roles of receptors and other potential targets for gene therapy for pain exist. However, as outlined in this review, much is known about the physiology, pharmacology, and molecular biology of potential therapeutic targets. Subsequent elucidation of the neurobiology of pain and progress in the tools available for gene therapy will advance the possibility and reality of gene therapy for the management of pain. Strategies and methods currently available for implementing gene therapy for the management of pain are not ideal, as reviewed in part I,[3A] but sufficiently elucidated for an initial clinical

trial. Anesthesiologists, by the virtue of our understanding and intimate familiarity with the clinical problems of pain management, are situated particularly well to become the leaders in translating the dramatic developments in the neurobiology of pain to clinical gene therapy for pain management.

The authors thank Roger A. Johns, M.D. (Professor and Chair, Johns Hopkins University, Baltimore, Maryland), for reviewing the manuscript.

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