The Journal of Neuroscience, June 1993, 13(6): 2273–2286

Feature Article

Peptides and the Primary Afferent Nociceptor

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Animal tissues are innervated by sensory neurons that respond with increasing discharge frequency to stimuli that have the potential to cause tissue damage. In vertebrates these neurons are called primary afferent nociceptors (PANs); they have their cell bodies [dorsal root ganglion (DRG) neurons] in sensory ganglia and a central process that connects synaptically to sensory transmission neurons in the CNS. It is now clear that the PAN does more than simply relay information about intense thermal, mechanical, and chemical stimuli; rather, the PAN is subject to modulation at its central and peripheral terminals, has a peripheral neuroeffector function, and undergoes activity-dependent long-term changes. This expanded view of the function of the PAN has resulted, in part, from a more comprehensive description of the peptides contained in PANs and of their functions. This article focuses on the role of neuropeptides in PAN function.

Activation and sensitization of primary afferent nociceptors

Activation

Activation of the PAN at its peripheral terminal requires intense mechanical, thermal, or chemical stimulation. Although the concentration of many endogenous chemicals that can activate PANs (e.g., 5-HT, histamine, and hydrogen and potassium ions) increases in an area of tissue damage, among the peptides that do so, only bradykinin (BK) has been extensively studied (Kumazawa et al., 1991). Bradykinin is a nonapeptide (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) cleaved by enzymes (kallikreins) from certain proteins that circulate in the plasma. Kallikreins are rapidly activated at sites of tissue injury, leading to the local production of large amounts of BK (Garrison, 1990). There are two known types of BK receptor (B_1 and B_2). Activation of PANs by BK is mimicked by B_2- but not B_1-type agonists and is selectively antagonized by B_2 antagonists (Dray and Perkins, 1988; Haley et al., 1989; Kumazawa et al., 1991). Similarly, BK-induced pain in humans appears to be mediated by the B_2-type receptor (Whalley et al., 1989). BK-induced activation of PANs involves the generation of diacyl glycerol and activation of protein kinase C, leading to an increase in a sodium conductance (Dray et al., 1988; Burgess et al., 1989; but see Dunn and Rang, 1990). Although tachyphylaxis develops rapidly when BK is repeatedly applied (Kumazawa et al., 1991), other inflammatory mediators can prolong BK activation of PANs (King et al., 1976; Kirchhoff et al., 1990; Lang et al., 1990).

Direct sensitization

In contrast to the constant stimulus–response function for many sensory receptors, repeated noxious stimulation or tissue damage produces prolonged increases in PAN excitability. This phenomenon, termed sensitization, is manifested by an increase in spontaneous activity, a lowered threshold for activation, and increased and prolonged firing to a suprathreshold stimulus (Meyer and Campbell, 1981; LaMotte et al., 1982, 1983). Sensitization of PANs contributes to hyperalgesia, which is defined as a lowered threshold for evoking behavioral indicators of pain in animals and the tenderness that is associated with inflammation in humans.

Several endogenous peptides generated at a site of injury or inflammation can sensitize PANs directly. These include interleukin-1 (IL-1), neutrophil-chemotactic peptides, and NGF-octapeptide (NGF-OP). Because of the clinical importance of hyperalgesia, the identity and mechanisms of action of hyperalgesic mediators is a major area of interest. The technique currently used to study direct sensitization involves cultured DRG neurons. These can be identified as probable nociceptors if they are depolarized by capsaicin (a nonpeptide pain-producing ingredient in chili peppers), which *in vivo* predominantly activates nociceptive small-diameter primary afferents (Helme et al., 1986; Jonsson et al., 1986; Saria et al., 1988; Lynn, 1990). Nonpeptide agents that directly sensitize PANs include prostaglandin E_2, prostaglandin I_2, the lipoxygenase product of arachidonic acid, 8*R*,15*S*-diHETE (Taiwo et al., 1987), adenosine [acting at an A_2-type receptor (Taiwo and Levine, 1990)], and 5-HT [acting at a 5-HT_{1a} receptor (Taiwo and Levine, 1992)]. Prostaglandin E_2 has recently been shown to increase the calcium conductance and to stimulate the release of peptide transmitter from cultured DRG cells (Nicol et al., 1992).

Indirect sensitization

Both neural and non-neural cellular elements are required for a variety of peptide mediators to act upon PANs. Thus, in contrast to hyperalgesic prostaglandins (prostaglandin E_2 and

Key words: spinal cord, inflammation, substance P, CGRP, pain, hyperalgesia, opioid, analgesia

This work was supported by NIH Grants DA-01949, NS-21445, NS-21647, DE-08973, and AM-32634, Grants DE/NIDA-08973 and NS-14627, and a grant from Bristol-Meyers Squibb.

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I_2), which appear to sensitize PANs directly (Pitchford and Levine, 1991), most hyperalgesic peptides act on cells other than the PANs; these cells (e.g., sympathetic postganglionic neurons and white blood cells), in turn, release a hyperalgesic agent that acts directly on the PAN.

Bradykinin. Although BK activates PANs directly, BK-induced *sensitization* of PANs to mechanical stimuli is largely indirect and depends on prostaglandin synthesis (Lembeck et al., 1976; Gonzales et al., 1989; Kumazawa et al., 1991). BK hyperalgesia is blocked by the prostaglandin E_2 receptor antagonist SC19220 (Taiwo and Levine, 1988). Furthermore, there is evidence that BK-induced hyperalgesia to mechanical stimuli depends on sympathetic postglanglionic neuron (SPGN) terminals; it is markedly attenuated by their chemical destruction with 6-hydroxydopamine (Levine et al., 1986c). BK-induced hyperalgesia is also blocked by mepacrine (a phospholipase A_2 antagonist), suggesting that BK-induced production of the directly acting hyperalgesic agent prostaglandin E_2 is a phospholipase A_2–dependent activity (Taiwo et al., 1990).

BK sensitizes PANs to thermal as well as mechanical stimuli in animals, and in humans produces thermal hyperalgesia (Raja et al., 1990). The duration of the sensitization to thermal stimuli is relatively brief (Koltzenburg et al., 1991; Kumazawa et al., 1991) and is blocked by prostaglandin synthesis inhibitors (Kumazawa et al., 1991). In contrast to BK-induced mechanical hyperalgesia, Koltzenburg et al. (1991) reported that BK-induced sensitization to thermal stimuli is not attenuated by surgical sympathectomy. Although this suggests a direct BK effect on PANs, surgical sympathectomy is less complete than chemical sympathectomy produced by 6-hydroxydopamine (Fischer et al., 1964; Thoenen, 1972).

Interleukin-1. IL-1 refers to two polypeptides (IL-1α and IL-1β), classified as cytokines, that stimulate proliferation of and protein synthesis in a variety of cells (Dinarello, 1989). Cytokines are produced by leukocytes and other cells in response to infection, exposure to bacterial toxins, and inflammatory mediators. IL-1β is a potent hyperalgesic agent with a probable peripheral site of action (Ferreira et al., 1988). Interestingly, a tripeptide analog of IL-1β acts as a peripherally acting *analgesic.* This tripeptide blocks hyperalgesia induced by both IL-1β and carrageenan, a proinflammatory plant polysaccharide. Since IL-1 induces E-type prostaglandin production in non-neuronal cells (Dayer et al., 1986), it is not surprising that the prostaglandin synthesis inhibitor indomethacin inhibits IL-1β hyperalgesia.

Chemotactic peptides. There are two major pathways of arachidonic acid metabolism: the cyclooxygenase pathway, which leads to the production of prostaglandins, and the lipoxygenase pathway, which produces leukotrienes (Lewis, 1989). Metabolites of both pathways sensitize nociceptors. Although these compounds are not peptides, studies of their actions have led to the discovery of a novel class of hyperalgesic peptides, namely, those that attract and activate white blood cells.

Leukotriene B_4 (LTB_4) sensitizes PANs and produces hyperalgesia in animals (Rackham and Ford-Hutchinson, 1983; Levine et al., 1984a, 1985b, 1986b; Martin et al., 1988; Madison et al., 1992), and produces a tender and indurated lesion after intradermal injection in humans (Soter et al., 1983; Lewis et al., 1984). LTB_4-induced hyperalgesia is distinguished from that induced by prostaglandin E_2 and BK by its dependence on white blood cells and its independence of the SPGN and of the cyclooxygenation of arachidonic acid (Levine et al., 1984a, 1985b). The hyperalgesic factor released by white blood cells in response to LTB_4 has been identified as 8*R*,15*S*-diHETE, another lipoxygenase product (Levine et al., 1986b). The hyperalgesic peptides that activate white blood cells include formyl Met-Leu-Phe, a tripeptide generated during the degradation of bacterial cell wall proteins, and C_{5a}, a fragment of the fifth component of the complement cascade.

Nerve growth factor–derived octapeptide. Cleavage of the amino-terminal end of NGF produces an octapeptide (NGF-OP: Ser-Ser-Thr-His-Pro-Val-Phe-His; Burton et al., 1978). Since enzyme inhibition studies indicated structural relatedness of NGF-OP and BK, we examined NGF-OP in nociceptive tests. NGF-OP produces a behavioral hyperalgesia that is dose dependent (Taiwo et al., 1991). Like BK-induced hyperalgesia, sympathectomy or indomethacin pretreatment attenuates NGF-OP hyperalgesia. NGF-OP action, however, is distinct from that of BK. NGF-OP induces hyperalgesia only in the setting of tissue injury. Since NGF production is markedly increased after nerve injury (Heumann et al., 1987a), it is possible that NGF-OP contributes to the pain and hyperalgesia associated with nerve injury. NGF gene expression (Heumann et al., 1987b; Lindholm et al., 1987) can also be enhanced by IL-1, the hyperalgesic cytokine mentioned above.

In summary, tissue damage or inflammation generate mediators, including peptides, that produce a prolonged lowering of the PAN threshold. Importantly, the lowering of PAN threshold depends on the presence of cells other than the PAN such as white blood cells and SPGNs. Thus, these cells must play a crucial role in the sensory transduction process. Analgesic and anti-inflammatory drugs such as aspirin and indomethacin block the generation of these mediators. The development of receptor blockers for these peptide mediators represents an important avenue for development of new analgesic agents.

Modulation of the peripheral terminals of primary afferent nociceptors by opioids

In addition to their well-characterized antinociceptive actions in the CNS (see below), opioids act in the periphery to modulate PAN function (Basbaum and Levine, 1991). Opioid binding sites, synthesized in the DRG, are transported into the peripheral, as well as the central, terminals of sensory neurons (Young et al., 1980; Laduron, 1984). Indeed, local injection of opioids into inflamed tissue reduces activity in PANs (Russell et al., 1987). Behavioral studies demonstrated a naloxone-antagonizable analgesic effect of opioids directly injected into tissue that is injured or inflamed (i.e., hyperalgesic) (Ferreira and Nakamura, 1979; Abbott, 1988; Levine and Taiwo, 1989; Stein et al., 1989) but not after injection into normal tissue (Hargreaves et al., 1987; Russell et al., 1987; Smith et al., 1988; Stein et al., 1988, 1989). In a recent clinical trial using injection of opioids directly into the knee joint, Stein et al. (1991) provided evidence that a peripheral action of opioids may relieve pain in patients following knee surgery.

Of the three major classes of opioid receptor ligands (μ, δ, κ), μ-ligands appear to be the most potent of the peripherally acting agonists (Joris et al., 1987; Levine and Taiwo, 1989; Stein et al., 1989). μ-Receptor–specific agonists appear to act on the terminals of the PAN (Taiwo and Levine, 1991a). Thus, intradermal injection of DAMGO, a μ-opioid receptor–selective agonist, but neither the δ-selective ligand DPDPE nor the κ-selective ligand U50,488H, inhibits the hyperalgesia induced by prostaglandin E_2 (Levine and Taiwo, 1989). The analgesic effect of the peripherally administered μ-ligand is prevented by per-

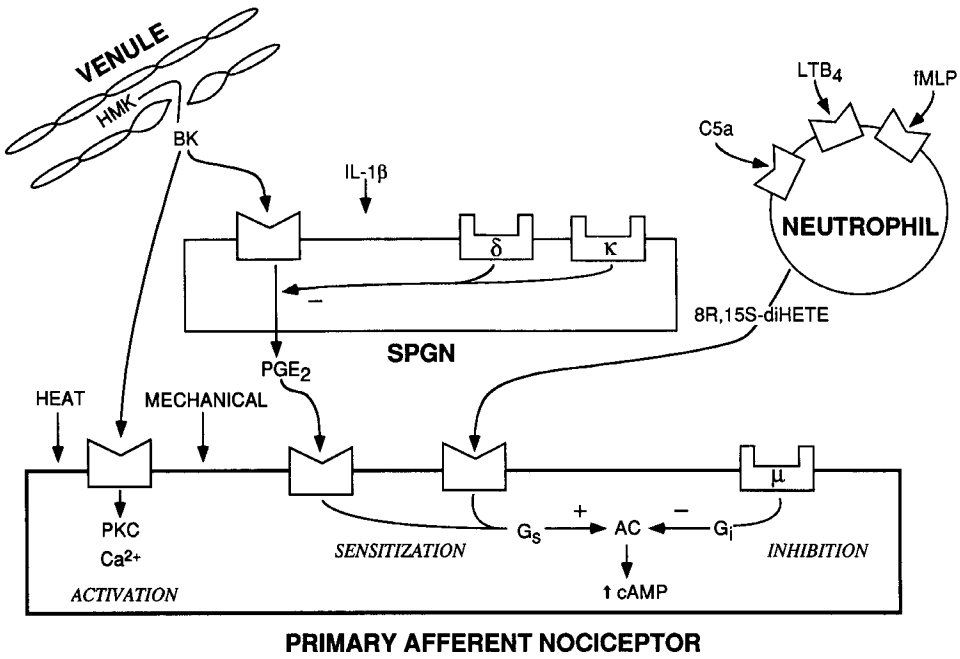

Figure 1. Sites of peptide action in peripheral pathways of pain and hyperalgesia. The inflammatory peptide bradykinin (*BK*), cleaved from high-molecular-weight kininogen (*HMK*) circulating in the venules, can activate the primary afferent nociceptor (PAN) in a protein kinase C (*PKC*)- and Ca^{2+}-dependent mechanism or sensitize the PAN through the production of PGE_2 in sympathetic postganglionic neurons (*SPGN*). Interleukin-1β (*IL-1β*) also can sensitize the primary afferent through a similar mechanism. The chemotactic peptides C_{5a} and *fMLP* activate the neutrophil, as does leukotriene B_4 (LTB_4), and causes the release of the lipoxygenase product *8R,15S-dHETE*, which sensitizes the primary afferent directly. Primary afferent sensitization probably involves a stimulatory G-protein (G_s) and the *cAMP* second messenger system. Opioid ligands of the δ- and κ-classes can inhibit this sensitization at the level of the SPGN, and the μ-class opioid ligand can do so at the level of the PAN via an inhibitory G-protein (G_i).

tussis toxin, suggesting that the effect is mediated by G-protein–coupled inhibition of cAMP. κ- and δ-ligands, which do not block prostaglandin E_2-induced hyperalgesia, block BK-induced hyperalgesia (Taiwo and Levine, 1991b). Since κ- and δ-opioid receptors are located on SPGN terminals (Illes et al., 1980a,b, 1985; Hughes, 1981; Wuster et al., 1981; Berzetei et al., 1987, 1988), and since BK hyperalgesia depends on SPGN terminals, it is likely that δ- and κ-ligands reduce the hyperalgesia through an action on the sympathetic terminals (see Fig. 1).

Receptors on the peripheral terminals of nerves may, of course, respond to endogenous as well as exogenous opioids, including those arising in the pituitary, adrenal cortex, and/or local inflammatory cells, specifically lymphocytes, which synthesize (Zurawski et al., 1986; Rosen et al., 1989) and release (Smith et al., 1986; Kavelaars et al., 1989) opioid peptides (Stein et al., 1990).

Figure 1 schematically illustrates our current understanding of the peripheral sites and mechanisms of action of peptides in pain and hyperalgesia.

Peptides in primary afferent nociceptors

Distribution of peptides in dorsal root ganglion neurons

The list of peptides found in primary afferents is large and growing. The most extensively studied primary afferent neuropeptide is substance P (SP), an undecapeptide that is present in about 20% of DRG neurons. SP is a member of a family of peptides, the tachykinins or neurokinins, which have a common C-terminal amino acid sequence. Despite intensive study, it has not been shown that the release of SP, or for that matter of any individual peptide, is correlated with the activity of a single physiologically defined class of primary afferents. Thus, although SP is present in some PANs (Leah et al., 1985; Cameron et al., 1988; Plenderleith et al., 1990), it is not restricted to nociceptors. In fact, the identification of a specific peptide with a specific physiological class of sensory receptor is unlikely in view of the coexistence, in various combinations, of up to four peptides in single DRG neurons (Cameron et al., 1988). For example, up to 80% of SP-containing DRG neurons in the cat cocontain calcitonin gene–related peptide (CGRP; Garry et al., 1989); the fraction may be even higher in the rat (Wiesenfeld-Hallin et al., 1984). Almost 26% of the SP-containing neurons also contain somatostatin (SOM); 34% of the SOM neurons contain SP and 22% of the SOM-containing neurons also contain CGRP. Furthermore, the pattern of peptide content is developmentally regulated (Hammond and Ruda, 1991), and the coexistence pattern differs considerably among species. Thus, SOM and SP appear not to coexist in the rat (Tuchscherer and Seybold, 1985). Furthermore, a significant fraction of small-diameter DRG neurons contain neither SP nor SOM.

Although the complement of neuropeptides in primary afferent neurons does not correlate well with cutaneous sensory modality (Wall and Fitzgerald, 1982; Leah et al., 1985; see below), it may be related to the type of tissue innervated (Green and Dockray, 1987; McMahon and Gibson, 1987; Molander et al., 1987; Ositelu et al., 1987; O'Brien et al., 1989). In general, DRG neurons that innervate visceral targets are enriched in SP and CGRP compared to those innervating the skin. For example, of the population of DRG neurons that innervates the rat stomach antrum, up to 85% contain CGRP and up to 60% contain SP (Green and Dockray, 1988); this compares to 15% and 10%, respectively, for DRG neurons that innervate the skin. When cutaneous nerves are cut and forced to reinnervate stomach antrum, the levels of SP and CGRP in the reinnervating DRG neurons increase significantly (Horgan and Van der Kooy, 1992).

Plasticity in primary afferent nociceptors

The concentrations of peptides in DRG neurons change after tissue injury or nerve damage. For example, hindlimb injection of formalin, which evokes a characteristic but short-lived pain syndrome, is associated with a significant increase in the number of DRG neurons expressing preprotachykinin (PPT) mRNA (Noguchi et al., 1988). In adjuvant-induced arthritis, the concentrations of SP and CGRP, but not SOM, are markedly increased in the DRG neurons that innervate the affected joints (Smith et al., 1992). On the other hand, Weihe et al. (1988) found little change in dorsal horn neurokinin immunoreactivity

in response to inflammation (despite dramatic increases in the levels of dynorphin in second-order neurons of the dorsal horn). These changes in the DRG neurons outlast the pain behavior evoked by the same formalin stimulus.

In contrast to inflammation, sciatic nerve section is associated with a *decrease* in the levels of PPT, CGRP, and SOM mRNA in DRG neurons (Noguchi et al., 1989, 1990); there is a corresponding decrease in the level of peptide immunoreactivity in terminals in the dorsal horn (Barbut et al., 1981). On the other hand, the levels of the peptides galanin and vasoactive intestinal peptide *increase* in DRG neurons after peripheral nerve section (Hökfelt et al., 1987; Noguchi et al., 1989). In part, this occurs in DRG neurons that previously expressed CGRP (Doughty et al., 1991). Neuropeptide Y mRNA and peptide, which are undetectable in DRG neurons of normal rats, rise to very high levels in rats with partial nerve section (Wakisaka et al., 1991). Taken together, these results indicate that nerve injury can alter the phenotype of the PAN.

Peripheral neuroeffector function of primary afferent nociceptor peptides

Peptides released from the peripheral terminals of PANs have potent biological activity. SP, the peptide studied most extensively in this regard, is released from the peripheral terminals of PANs when they are activated by noxious stimuli or by antidromic activation of the peripheral nerve (Bill et al., 1979; Brodin et al., 1981; Moskowitz et al., 1984; White and Helme, 1985). Direct application of SP to peripheral tissues produces vasodilatation and increases vascular permeability (Lembeck and Holzer, 1979; Saria, 1984), attracts white blood cells (Helme and Andrews, 1985; Saito et al., 1986), activates the phagocytic function of neutrophils (Payan et al., 1984) and macrophages (Bar-Shavit et al., 1980; Hartung et al., 1986), increases production and release of inflammatory mediators (e.g., lysosomal enzymes and eicosanoids) from these cells (Hartung et al., 1986), and degranulates mast cells resulting in local release of histamine (Johnson and Erdos, 1973). SP also stimulates release of prostaglandin E_2 and collagenase from cells in joints of patients with rheumatoid arthritis (Lotz et al., 1987), and induces the release of IL-1, tumor necrosis factor α, and IL-6 from white blood cells (Lotz et al., 1988). This suggests a possible mechanism for the regulation of host defense responses by the nervous system.

That SP released by PANs actively contributes to joint inflammation is suggested by the observation that the destruction of PANs with capsaicin reduces the severity of experimental arthritis (Levine et al., 1986a). Furthermore, SP antagonists inhibit the neurogenic inflammation produced by PAN stimulation (Couture and Cuello, 1984), and the leakage of plasma induced by SP or by antidromic electrical stimulation of sensory nerves is completely inhibited by a selective antagonist of the NK_1 receptor (Garret et al., 1991; Lembeck et al., 1992), one of three neurokinin receptors (NK_1, NK_2, and NK_3) that have been cloned and sequenced (Masu et al., 1987; Yokota et al., 1989; Shigemoto et al., 1990). These studies raised the possibility that neuropeptides released by primary afferents contribute to the inflammatory response in a variety of diseases, including arthritis (Levine et al., 1984b, 1985a), asthma (Barnes et al., 1991a,b), inflammatory bowel disease (Mantyh et al., 1988, 1989, 1991), and migraine headache (Moskowitz et al., 1984), and that neurokinin antagonists may be of clinical value for their amelioration.

Central terminals of primary afferent nociceptors: peptides as central neurotransmitters and neuromodulators

Neurokinins as primary afferent nociceptor neurotransmitters. PAN-derived SP is concentrated in laminae I and II [the substantia gelatinosa (SG)] of the spinal cord dorsal horn. These laminae contain nociresponsive neurons (Hökfelt et al., 1975) and, although iontophoresis of SP onto these neurons excites them (Henry, 1976), these results were not generally accepted as showing that SP is a "neurotransmitter" of PANs. To the contrary, the demonstration that dorsal horn nociresponsive neurons can be activated even when the SP content of the dorsal horn is reduced (e.g., after sciatic nerve section) led to the suggestion that peptides released by primary afferents serve a poorly defined "neuromodulatory," rather than neurotransmitter, function (Wall et al., 1981). Even the recent demonstration that SP-immunoreactive terminals of undoubted primary afferent origin (Carlton et al., 1989; see below) make synaptic contact with physiologically defined nociresponsive neurons did not reveal how SP contributes to the transmission of nociceptive input.

A variety of new approaches, including the development of better methods to monitor the release of peptides in the spinal cord, the design of more selective peptide antagonists, and the refinement of ultrastructural double labeling techniques (which allow several amino acids and peptides to be examined simultaneously in the same synaptic profile), have provided powerful evidence in favor of the hypothesis that SP is indeed a central neurotransmitter of PANs. To implicate peptides in the transmission of nociceptive messages from PANs to second-order neurons requires the demonstration that they are released by pain-producing stimulation. In fact, the release of SP into the cerebrospinal fluid (CSF) of the cat can be evoked by peripheral nerve stimulation, but only at intensities that elicit pain-related responses, such as increased blood pressure and dilation of the pupils. Increased SP release is also produced by application of capsaicin to the skin, which preferentially activates PANs (Go and Yaksh, 1987).

Although CSF levels of SP are presumed to result from overflow secondary to release from the central terminals of PANs, recent evidence suggests an alternative source. Using antibody-coated glass microelectrodes, Duggan and Hendry (1986) evaluated the spatial distribution of released peptide in spinal cord. They found that electrical stimulation of cutaneous nerves at intensities high enough to activate PANs evokes the release of SP in the SG *and* at the surface of the spinal cord. They suggested that the peptide detected in the CSF derives from the peripheral terminals of branches of SP-containing primary afferents that innervate the pia mater of the spinal cord, rather than from central terminals of PANs that synapse upon spinal cord neurons.

The distance within the spinal cord over which neurokinins may act has also been studied by means of the antibody microprobe technique. Noxious stimuli evoke the release of SP and neurokinin A (NKA; a neurokinin that co-occurs with SP and that is presumably coreleased from primary afferent terminals; see below). However, NKA persists in spinal tissue long after SP can no longer be detected (Duggan et al., 1990; Hope et al., 1990a,b). Furthermore, NKA can be detected at a considerable distance from its presumed site of release in the SG. Since SP, but not NKA, is rapidly degraded by neutral endopeptidase 24.11 (enkephalinase), it was proposed that the persistence and

diffusion of NKA after release is due to its slower degradation. These results raise the possibility that some peptides released from PANs can exert effects at a considerable distance from their site of release, an observation consistent with the presence of mismatches between the distribution of peptides and their respective binding sites (Herkenham, 1987; Kruger et al., 1988a,b).

As described above, the effects of neurokinins result from an action on at least three different receptors, NK_1, NK_2, and NK_3. The NK_1 and NK_2 receptors are thought to be targeted by SP and NKA, respectively. Consistent with their putative contribution to nociceptive processing, NK_1 binding sites are densely concentrated in dorsal horn laminae I and II and along the medial half of the laminae III–X, as well as in the intermediolateral cell column. NK_2 sites overlap considerably with the NK_1 sites; NK_3 sites are more restricted in their distribution, being densely concentrated in laminae I and II.

The antibody-coated glass microelectrode was also used to address the nature of the stimuli that evoke peptide release. Kuraishi et al. (1988) had reported that SP was released by noxious mechanical, but not thermal, stimulation. Duggan and colleagues confirmed that, at the temperature used by Kuraishi et al. (<48.5°C), there is no SP release; however, noxious thermal stimuli did evoke the release of SP at temperatures above 52°C (Duggan et al., 1988). Although the release of SP at the higher temperature may simply reflect an increased number of impulses invading the central terminal of thermally sensitive nociceptors, it is possible that the higher temperatures induce tissue damage with associated inflammation and that chemicals released as a consequence may secondarily activate a separate population of chemosensitive, SP-containing PANs that are not directly sensitive to thermal stimuli. Consistent with the idea that inflammatory mediators in the periphery also act as stimuli for central release of SP, Schaible et al. (1990) reported that inflammation of the knee joint evokes the release of SP in the cat spinal cord.

Behavioral effects of substance P. In addition to the anatomical studies of SP distribution in the dorsal horn and the demonstration that it is released by noxious stimuli, the involvement of SP in the transmission of nociceptive messages by PANs is supported by the finding that nociceptive neurons of the dorsal horn are excited by local iontophoresis of SP (see below) and that lumbar intrathecal injection of SP, in both rats and mice, evokes a syndrome characterized by biting and scratching of the hindlimbs (Hylden and Wilcox, 1981; Piercey et al., 1981; Seybold et al., 1982). Based on the limited rostral spread of the peptide, Piercey et al. (1981) concluded that these behavioral effects indicate pain and result from a spinal action. These effects are mimicked by the NK_1-selective agonist septide, but not by the NK_3-selective agonist senktide (Papir-Kricheli et al., 1987).

Although some investigators have argued that these behaviors do not indicate pain (Bossut et al., 1988a,b; Frenk et al., 1988), other studies more directly link SP with pain behavior. For example, intraspinal injection of SP lowers the threshold for eliciting the thermal-evoked tail-flick reflex (Cridland and Henry, 1988c), and antagonists of SP block the facilitation of the tail-flick response produced by a concurrent noxious stimulus (Cridland and Henry, 1988b). The selective nonpeptide NK_1 antagonist CP-96345 reduces the pain behavior produced by subcutaneous formalin (Yamamoto and Yaksh, 1991). Taken together, these results provide evidence that SP is a neurotransmitter of nociceptive PANs and that the intensity of the stimulus, its modality (Wiesenfeld-Hallin, 1986a,b; Ohno et al., 1988), and/or the degree to which the stimulus provokes inflammation are critical factors that determine the extent to which SP contributes to the transmission of nociceptive signals.

Substance P actions on second-order neurons in spinal dorsal horn. Stimulation of dorsal roots in rat spinal cord *in vitro* produces an early, fast and a late, slow depolarization in dorsal horn neurons. The late, slow depolarization is blocked by an SP antagonist and cannot be demonstrated in spinal cords taken from rats in which C-fibers are destroyed by pretreatment with capsaicin (Urban and Randic, 1984). When SP is bath applied, dorsal horn neurons show a depolarization that is similar in time course to the late, slow depolarization evoked by dorsal root stimulation. Voltage-clamp studies show that this depolarization is due largely to the activation of a time- and voltage-dependent inward Ca^{2+} current (Murase and Randic, 1984). The analysis of SP effects on ionic conductances is somewhat complicated, however, because SP also activates a Ca^{2+}-activated voltage-sensitive K^+ current (which would oppose the SP-activated inward Ca^{2+} current) and a nonspecific cationic conductance that would act synergistically with the Ca^{2+} current (Murase et al., 1989). *In vivo* electrophysiologicial studies have shown that locally iontophoresed SP can produce an excitatory effect that is slow in onset, of long duration, and selective for dorsal horn neurons that respond to noxious stimulation (Henry, 1976; Salter and Henry, 1991). A selective NK_1 antagonist, CP 96345, blocks responses of cat dorsal horn neurons to both noxious heat and iontophoretically applied SP (Radhakrishnan and Henry, 1991). Excitation of identified spinothalamic tract neurons by iontophoretically applied SP has also been demonstrated (Willcockson et al., 1984). Finally, using receptor-selective drugs, Fleetwood-Walker et al. (1990) found that both SP, acting via the NK_1 receptor, and NKA, acting via the NK_2 receptor, excite nociceptive dorsal horn neurons.

Although SP has a predominantly excitatory effect at the synapse between PANs and dorsal horn nociceptive neurons, other *in vivo* experiments indicate that a subpopulation of dorsal horn neurons is *inhibited* by SP, acting via the NK_1 receptor (Fleetwood-Walker et al., 1990). In fact, bath-applied SP evokes a hyperpolarizing response in about one-third of dorsal horn neurons (Murase and Randic, 1984). This hyperpolarization is abolished by either TTX or high-Mg^{2+}, low-Ca^{2+} solutions, suggesting that it is an indirect effect produced through an action of SP on an interneuron that synapses upon the recorded cell. A corollary of this observation is that some of the cells activated by SP inhibit other dorsal horn neurons. Since SP produces both direct excitation and indirect inhibition of dorsal horn nociceptive neurons, it is of interest that CNS injection of SP exerts both nociceptive and naloxone-reversible antinociceptive effects (Fredrickson et al., 1978). The antinociceptive effect may result from an SP-induced release of opioid peptides from dorsal horn interneurons. Indeed, such release has been detected (Del Rio et al., 1983). More recently, however, Krumins et al. (1989) raised the possibility that the N-terminal fragment of SP might interact with opioid receptors and, in fact, demonstrated that SP1-9 and SP1-4, but not SP5-11 (i.e., the C-terminus of SP), reduce the affinity and increase the binding capacity of the μ-opioid ligand DAMGO in mouse brain and spinal cord. They also provided evidence that the naloxone-antagonizable "behavioral desensitization" produced by repeated injections of SP is a consequence of the breakdown of SP into (1) a pronociceptive C-terminal fragment and (2) a desensitizing N-terminal fragment that

exerts its effects through the μ_1 opioid binding site (Larson, 1988; Igwe et al., 1990a,b).

Multiple primary afferent nociceptor neurotransmitters and their interactions

Among the many peptides that are located in DRG neurons, CGRP provides one of the best examples of a neuromodulator, in the sense of a compound that exerts only limited effects by itself, and yet dramatically potentiates the effects of other compounds (particularly SP). Unlike SP (Helke et al., 1982), CGRP-containing terminals in the dorsal horn appear to derive exclusively from DRG cells. Dorsal rhizotomy (Chung et al., 1988; Traub et al., 1989) virtually eliminates CGRP staining in the cord. Since neonatal capsaicin treatment destroys a large proportion of PANs, it is not surprising that it also significantly reduces the level of CGRP in the spinal cord (Franco-Cereceda et al., 1987; Hammond and Ruda, 1989). The CGRP-containing axons are largely unmyelinated or small-diameter myelinated and constitute almost 30% of the primary afferent axons of Lissauer's tract, the major afferent input to the superficial laminae of the dorsal horn (McNeill et al., 1988). Unlike SP, which is distributed extensively in both dorsal and ventral horn, CGRP terminals are concentrated in dorsal horn laminae I and II and in the reticulated region of lamina V (Carlton et al., 1988). Electron microscopic studies have established a direct connection between CGRP-containing primary afferents and second-order nociceptive spinothalamic tract neurons (Carlton et al., 1990). Since SP and CGRP coexist in primary afferent dorsal horn terminals (Merighi et al., 1988; Fried et al., 1989; Plenderleith et al., 1990), the fact that CGRP at spinal levels is found only in primary afferents means that there are some direct SP-containing PAN connections to dorsal horn neurons (Carlton et al., 1989).

In the primate many CGRP-immunoreactive terminals contact one another. This indicates that primary afferent terminals make axoaxonic contact with each other. This arrangement may provide the anatomical substrate for a regulation by CGRP of amino acid release from PANs. In fact, there is evidence that both SP and CGRP can regulate the release of amino acid neurotransmitters from primary afferent fibers (Kangrga and Randic, 1990; Smullin et al., 1990). Paradoxically, neither CGRP nor neurokinin binding sites have been demonstrated on primary afferent fibers [indeed, dorsal rhizotomy results in an increase in the numbers of such binding sites in the dorsal horn (Charlton and Helke, 1985)]. There is, in fact, an apparent mismatch in the distribution of CGRP-containing terminals and CGRP binding sites in the dorsal horn. Specifically, the binding sites are notably low in abundance in the SG (Kruger et al., 1988a).

Iontophoretically applied CGRP produces a slow-onset, long-lasting excitation of nociceptive dorsal horn neurons *in vivo* (Miletic and Tan, 1988). *In vitro* studies have demonstrated that CGRP produces a slow depolarization by a direct action on nociceptive dorsal horn cells (Ryu et al., 1988). Importantly, Biella et al. (1991) demonstrated that concentrations of CGRP that alone have little or no consistent effect, markedly potentiate the excitatory effect of either SP or noxious stimulation on rat dorsal horn neurons *in vivo*. Furthermore, CGRP increases Ca^{2+} conductance in DRG cells (Ryu et al., 1988) and enhances the release of SP from spinal cord slices (Oku et al., 1987).

Consistent with CGRP contributing to nociceptive processing in the dorsal horn, Morton and Hutchison (1990), using the antibody microprobe technique, found that noxious thermal, mechanical, or electrical stimulation evokes the release of CGRP in the superficial dorsal horn. Furthermore, direct application to the spinal cord of antisera to CGRP has an antinociceptive action (Kuraishi et al., 1988), and CGRP enhances the effects produced by SP (Wiesenfeld-Hallin et al., 1984). For example, coadministration of SP and CGRP significantly increases the excitability of a nociceptive flexion reflex in the rat (Woolf and Wiesenfeld-Hallin, 1986). Although the mechanism through which the interaction between SP and CGRP is produced is not clear, there is evidence that CGRP may retard the enzymatic degradation of SP (Le Greves et al., 1985).

In addition to the interactions between peptides, there are also interactions between peptide and excitatory amino acid transmitters on dorsal horn neurons. Noxious stimulation evokes the release of glutamate and other amino acids that co-occur with the peptides in primary afferent terminals (Battaglia and Rustioni, 1988; De Biasi and Rustioni, 1988; Skilling et al., 1988; Smullin et al., 1990). *In vivo,* iontophoretically applied SP potentiates NMDA-induced responses in identified spinothalamic tract neurons (Dougherty and Willis, 1991). In whole-cell voltage-clamp studies of dissociated dorsal horn neurons, SP markedly potentiates an inward glutamate-gated current (Randic et al., 1990). This modulatory effect of SP on the actions of excitatory amino acid transmitters contributes to the wind-up phenomenon, the property of nociceptive dorsal horn neurons whereby repeated activation of PANs results in a progressive increase in their discharge to each stimulus. This enhancement of discharge is blocked by either an SP (Kellstein et al., 1990) or an NMDA (Dickenson and Sullivan, 1990) antagonist.

In summary (Fig. 2), the available evidence is consistent with a model in which SP and CGRP produce slow-onset, long-duration depolarizations that act synergistically to excite second-order nociceptive dorsal horn neurons, including identified spinothalamic tract neurons. In addition, there is a slow-onset, long-lasting potentiation of a glutamate-induced fast excitation. Since multiple neuromediators are released when PANs are active, a burst of activity in PANs should be sufficient to induce long-lasting activation of dorsal horn neurons.

Other excitatory primary afferent nociceptor neuropeptides: cholecystokinin

In some species cholecystokinin (CCK) is colocalized with SP and/or CGRP (Tuchscherer et al., 1987). Unfortunately, there is immunological cross-reactivity between CCK and CGRP, so the presence of CCK in primary afferents has been called into question (Duggan and Weihe, 1991). CCK immunoreactivity is also present in dorsal horn neurons and in the terminals of axons descending from supraspinal regions. Iontophoresis of CCK excites dorsal horn neurons in the rat (Jeftinija et al., 1981; Kellstein et al., 1991); however, this effect is weak, inconsistent, and neither dose related nor blocked by CCK antagonists. A more consistent and provocative effect is for CCK to antagonize and CCK antagonists to potentiate opioid suppression of C-fiber activation of dorsal horn neurons (Kellstein et al., 1991). These electrophysiological data are consistent with behavioral studies (e.g., Watkins et al., 1984) and with some clinical reports of an analgesic effect of CCK antagonists (see Baber et al., 1989, for review). The antianalgesic action of CCK was recently put into a behavioral context by Wiertelak et al. (1992). Whereas fear and stress (e.g., produced by painful shocks) activate an endog-

enous opioid-mediated analgesic effect, cues that signal safety from painful shocks reverse this antinociception as well as the antinociceptive effect of morphine. CCK antagonists block the reversal of both morphine and stress antinociception produced by such safety cues.

Inhibitory neuropeptides in primary afferent nociceptors: somatostatin and galanin

The classic view that primary afferents release only excitatory neurotransmitters is complicated not only by the finding that SP has both proalgesic and analgesic actions, but also by the evidence that SOM and galanin (GAL) (Randic et al., 1987) *inhibit* the firing of nociceptive-specific spinal cord neurons (Randic and Miletic, 1978; Miletic and Randic, 1982; Sandkuhler et al., 1990). *In vitro* studies indicate that the SOM-induced inhibition of dorsal horn cells is associated with a hyperpolarization of these neurons (Murase et al., 1982), which may be due to a G-protein–mediated reduction of Ca^{2+} conductance (Sah, 1990). Unfortunately, the lack of a specific antagonist makes it difficult to prove that the observed inhibition occurs under physiological conditions associated with SOM release.

GAL, which colocalizes with SP and CGRP in large numbers of primary afferent, capsaicin-sensitive C-fibers (Skofitsch and Jacobowitz, 1985; Klein et al., 1990), may also have an inhibitory effect on nociceptive transmission. In some studies of thermal nociception, intrathecal GAL is reported to have an antinociceptive effect (Cridland and Henry, 1988a; Post et al., 1988), although it lowered the threshold for vocalization in mechanical tests at higher doses. Other studies reported that low doses of intrathecal GAL *increase* the excitability of the flexion reflex, while higher doses of GAL produce a prolonged depression of thermal nociceptive reflexes (Wiesenfeld-Hallin et al., 1988, 1989, 1990). The higher doses also blocked the facilitatory effect of SP, CGRP, or conditioning stimulation of C-fibers (Xu et al., 1989, 1991). Thus GAL, like SP, appears to exert both pro- and antinociceptive effects.

Modulation of primary afferent neurons at their central terminals

Opioids applied directly to the spinal cord block behavioral responses to noxious stimulation in animals and produce profound antinociception in humans (see Yaksh et al., 1988, for review). Primary afferent terminals, local interneurons, and the dendrites of projection neurons whose somata lie in deeper laminae are present in the SG and are potential targets of opioids. Opioids both reduce transmitter release from primary afferents and directly inhibit dorsal horn neurons. Although either of these actions could contribute to spinal mechanisms of antinociception, this article will focus on control of transmitter release from primary afferents.

The central terminals of small-diameter primary afferents contain μ, δ, and κ opioid binding sites (LaMotte et al., 1976; Fields et al., 1980; Zajac et al., 1989; Gouardères et al., 1991). Studies of spinal cord slices clearly demonstrate that met-enkephalin, acting presynaptically, can inhibit glutaminergic input to neurons in lamina I (Hori et al., 1992). Electrophysiological studies of cultured DRG neurons provide evidence for a direct opioid action on primary afferents. Opioids shorten the plateau of the action potential, which is mainly due to a Ca^{2+} current (Mudge et al., 1979). In mouse DRG neurons, κ-opioid ligands block this voltage-dependent Ca^{2+} current. In contrast, μ- and

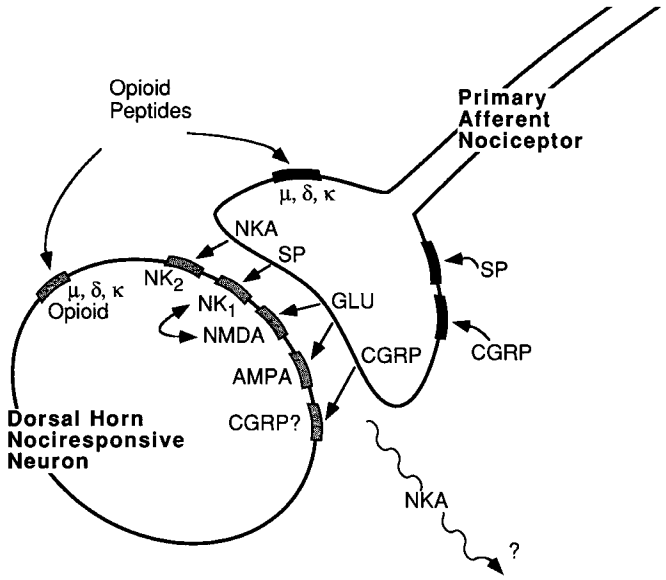

Figure 2. Primary afferents and peptide actions in the CNS. The PAN releases a variety of co-occurring neuropeptides (*NKA, SP, CGRP*) and excitatory amino acids [e.g., glutamate (*GLU*)]. These act at several postsynaptic receptors: the NK_1 and NK_2 tachykinin receptors, the *CGRP* receptor, and the *NMDA* and *AMPA* excitatory amino receptors. *NKA* may diffuse to act at a distance from its site of release. There is evidence that *SP* and *CGRP* also act at autoreceptors at neuropeptide-containing primary afferent terminals. In addition, opioid peptides act upon both pre- and postsynaptic μ-, δ-, and κ-opioid receptors to modulate transmitter release and the firing of second-order nociresponsive neurons.

δ-ligands increase K^+ conductance, which secondarily shortens the Ca^{2+} current (Werz and MacDonald, 1983; MacDonald and Werz, 1986). If this reduction in inward Ca^{2+} current also occurs at the central terminals of adult DRG neurons, then transmitter release would be reduced. On the other hand, although Shen and Crain (1989) confirmed that micromolar concentrations of opioids shorten the action potential of cultured DRG neurons, they found that nanomolar concentrations of μ, δ, or κ ligands prolong the action potential. Since this action potential is, in large part, due to an inward Ca^{2+} current, its prolongation should increase transmitter release. We should point out, however, that in these *in vitro* studies, the DRG populations did not respond uniformly to opioids and probably included several different physiological classes of primary afferent neurons.

It is generally accepted that opioids inhibit the release of SP from primary afferents *in vitro* (Jessell and Iversen, 1977; Mudge et al., 1979; Chang et al., 1989) and *in vivo* (Yaksh et al., 1980; Yonehara et al., 1986; Go and Yaksh, 1987; Aimone and Yaksh, 1989). Although K^+-evoked SP release could derive from intrinsic dorsal horn neurons as well as primary afferents, the fact that the opioid-induced reduction of K^+-evoked SP release is not observed in rats whose unmyelinated primary afferents are destroyed by neonatal treatment with capsaicin indicates that opioids do in fact inhibit SP release from PANs (Pohl et al., 1989a). Furthermore, opioids also reduce K^+- and capsaicin-evoked release of CGRP, which at spinal levels is derived almost completely from primary afferents (Pohl et al., 1989b). On the other hand, recent experiments have raised questions about the interpretation of studies of the modulation of neuropeptide release that are based on sampling PAN neuropeptides in CSF.

Specifically, using the antibody microprobe technique in cat dorsal horn, Morton et al. (1990) failed to demonstrate inhibition by morphine of the noxious stimulation–evoked release of either SP (Morton et al., 1990), NKA (Lang et al., 1991), or CGRP (Morton and Hutchison, 1990).

To some extent these discrepant results may be due to simultaneous action of opioids at different receptor sites. This issue has been addressed by the use of opioid receptor type–selective ligands. For example, in rat dorsal cord slices, selective δ-ligands reduce capsaicin-evoked release of SP, but μ-selective ligands significantly increase SP release (Pohl et al., 1989b). In these studies and in other *in vivo* work (Hirota et al., 1985), κ-ligands had no effect on SP release. Moreover, in slices of trigeminal dorsal horn, Suarez-Roca et al. (1992) found a complex dose–response curve for morphine: inhibition of K^+-evoked SP release at low nanomolar and at low micromolar concentrations, and enhancement of release at high nanomolar and at high micromolar doses. Using receptor-selective antagonists, they showed that the low-nanomolar inhibition and the high-nanomolar facilitation of SP release are μ-effects; the low-micromolar inhibition is primarily δ-mediated (see also Collin et al., 1991), and the high-nanomolar morphine facilitation is a κ-effect (Suarez-Roca and Maixner, 1992; H. Suarez-Roca and W. Maixner, unpublished observations).

Finally, it is possible that the studies of the action of opioids at their peripheral terminals discussed above may be relevant to the actions of these drugs at the central terminals of PANs. Thus, opioids not only block transmitter release from the peripheral terminals of primary afferents (Yaksh, 1988), but also increase nociceptive threshold through inhibition of the cAMP second messenger in peripheral tissues (Levine and Taiwo, 1989; Taiwo and Levine, 1991a). To what extent a comparable action contributes at the central terminals of PANs must be examined.

In summary, each of the three major classes of opioid receptor may contribute to the opioid-induced modulation of transmission of nociceptive information in the dorsal horn. There is reasonably good evidence that both μ- and δ-ligands act presynaptically to modulate peptide release from primary afferents, and postsynaptically to inhibit dorsal horn nociresponsive neurons directly, including identified projection cells. μ-Ligands produce consistent and selective antinociceptive actions when applied to the SG, where the bulk of unmyelinated PANs terminate (Hope et al., 1990c). δ-Ligands are active when applied to the surface of the cord and in lamina I, where myelinated PANs terminate. The contribution of κ-ligands is unresolved since both antinociceptive and pronociceptive actions have been seen when it is applied to the cord in different ways (Fleetwood-Walker et al., 1988).

Although it is clear that opioids can modulate the release of neuropeptides from PANs, the contribution of this effect to the modulation of nociceptive transmission in the dorsal horn is uncertain. μ- and δ-opioid agonists selectively inhibit the response of dorsal horn neurons to noxious stimulation when applied in the region of termination of PANs in superficial dorsal horn (Duggan and North, 1984; Fleetwood-Walker et al., 1988). While this effect could result from a reduction in transmitter release from PANs, postsynaptic actions on intrinsic dorsal horn neurons have also been demonstrated (Yoshimura and North, 1983; Jeftinija, 1988; Hope et al., 1990c).

If one accepts the likelihood that opioid modulation of the release of transmitter from the spinal terminals of PANs contributes to antinociception, it is natural to ask whether endogenous opioid peptides could be involved in this control. In fact, there is a dense concentration of preproenkephalin-derived opioid peptides in terminals in the superficial dorsal horn. Although this distribution overlaps precisely the terminal fields of PANs, axoaxonic connections with enkephalin immunoreactivity onto PAN terminals have been looked for but not found (Glazer and Basbaum, 1984).

Support for the concept that enkephalins are indeed involved in the control of transmitter release from PANs derives from studies of enzymes that inactivate enkephalins. A prime candidate for inactivation of enkephalins is a neutral endopeptidase (NEP) that cleaves both met- and leu-enkephalin at the amide bond between glycine and phenylalanine. Immunocytochemical studies show a dense concentration of NEP immunoreactivity in the superficial dorsal horn, with its greatest density in the SG largely overlapping that of enkephalin and SP (Waksman et al., 1986; Back and Gorenstein, 1989; Pollard et al., 1989). Importantly, application of an NEP inhibitor to the spinal cord produces antinociception in animals (Oshita et al., 1990) and inhibits nociceptive dorsal horn neurons (Dickenson et al., 1987), although it is not clear that these effects are due to an action on PAN terminals.

Summary

An expanding knowledge of neuropeptides and their function has led to a profound change in our view of how the PAN contributes to pain. In addition to their expected direct action on postsynaptic cells in the dorsal horn, neuropeptides can modify transmitter release from nearby terminals of other PANs and/or diffuse to act on dorsal horn neurons at a considerable distance from their site of release (Fig. 2). Contrary to early expectations and despite the evidence that several neuropeptides excite central nociceptive neurons, there is no clear correspondence between neuropeptide content and physiologically defined classes of small-diameter primary afferents. There is, however, a tendency for populations of afferents innervating different organs to differ consistently in their peptide content. In fact, the peptide content of primary afferents is, in part, determined by specific factors in the tissues that they innervate. Furthermore, peptide content can change dramatically in response to certain prolonged stimuli or nerve damage. The lack of correspondence of peptide content and physiological response pattern, the plasticity of peptide content, its tissue specificity, and the possibility for action at a distance from the site of their release from central PAN terminals strongly suggest that PAN peptides have functions that are fundamentally different from those of the short-range actions of amino acid neurotransmitters that are also found in the PAN. Finally, nowhere is the plasticity of function of the PAN more evident than at its peripheral terminals. Long-term changes are produced in these terminals by a host of peptides that derive from a variety of cell types. The complexity of this transduction process is augmented by the activity-induced release of peripherally active neuropeptides from the PAN itself.

In addition to the variety of fundamental neurobiological issues that recent studies of PANs have raised, they have also generated a great deal of clinical interest, in view of the role of the PAN in inflammation and its accessibility for study and for therapeutic intervention.

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