Presynaptic control of nociceptor signalling: Differential influence of mu opioid and GABAergic systems

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The relative contribution of pre- and postsynaptic controls to the flow of nociceptive information at the level of the spinal cord has been one of Ron Melzack's longstanding interests and a key issue in the formulation of the gate control theory. The authors review their own studies, in which they monitored internalization of the neurokinin-1 receptor to examine specifically the action of two classically inhibitory systems – mu opioid and gamma-amino butyric acid (GABA) – on noxious stimulus-evoked tachykinin signalling in the rat spinal cord. Evidence that opioids and GABAergic controls operate differently on the central consequences of any noxious stimulus-induced substance P release is provided. Whereas at least 80% of the tachykinin signalling remained intact after even the highest concentration of spinal morphine or D-Ala2, NMe-phe4, Glyol5-enkephalin administration, spinal administration of the $GABA_B$ receptor agonist baclofen had a dramatic inhibitory effect. These findings are discussed in light of the disappointing clinical utility of baclofen and neurokinin-1 receptor antagonists to combat pain.

Key Words: *Gamma-amino butyric acid; Mu opioid; Neurokinin 1 receptors; Receptor internalization; Spinal cord; Tachykinins*

Commande présynaptique de la transmission des signaux par les nocicepteurs : mécanisme d'action des opioïdes de type mu et du GABA

Le rôle relatif des commandes pré- et postsynaptiques dans la transmission des signaux nociceptifs dans la moelle épinière, en plus de susciter l'intérêt soutenu de Ron Melzack, a constitué un pilier de la théorie du ‹‹ portillon ››. Les auteurs ont passé en revue leurs propres études dans lesquelles ils se sont penchés sur l'endocytose des récepteurs de la neurokinine 1 afin d'étudier le mécanisme d'action de deux inhibiteurs classiques, les opioïdes de type mu et l'acide gamma-aminobutyrique (GABA), sur la transmission de la tachykinine déclenchée par un stimulus nocif dans la moelle épinière du rat. Ils ont recueilli des preuves selon lesquelles les opioïdes et le GABA agissent différemment sur les effets centraux de la libération de la substance P, provoquée par un stimulus nocif. Tandis qu'au moins 80 % de la transmission des signaux par la tachykinine étaient restés intacts même après les concentrations les plus fortes de morphine dans la moelle épinière ou après l'administration d'enképhaline D-Ala2-Nme-ph4-Glyol5, l'administration d'un agoniste des récepteurs du $GABA_B$ dans la moelle épinière, le baclofen, a eu un effet inhibiteur marqué. Suit une discussion à la lumière des résultats cliniques décevants du baclofen et des inhibiteurs des récepteurs de la neurokinine 1 dans la lutte contre la douleur.

It is difficult to pick a topic to discuss that adequately honours Ron Melzack's many years of pain research. This is particularly problematic if one is cognizant of the tremendous emphasis that Ron puts on the distinction between pain and nociception. Indeed, the thread that binds so many of Ron's studies is that the complexity of the pain experience will not be understood if one exclusively concentrates on the processing of nociceptive messages at the level of the periphery or spinal cord. Ron always emphasized the importance of affective and cognitive components of the pain experience. These elements of the pain experience, so beautifully and memorably discussed in Melzack and Casey's (1) theoretical review, must always be included when this topic is addressed. Dr Allan Basbaum was a student of Ron's in an undergradu-

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ate class at McGill University, Montreal, Quebec. The topic was motivation. It is still easy to recall the enthusiasm of the lecturer and of the students. The complexity of our perceptions and the influence of that experience on perceptions were common themes of the course. This was true whether the discussion was about the famous hawk-goose experiments of the German ethologists or the maladaptive behaviours of the beagles that Ron raised in restrictive environments (2). These animals would approach a hot flame repeatedly, apparently unable to adapt to these stimuli or appreciate that they were the source of the pain that was generated. Habituation was altered to such an extent that previously experienced stimuli (even 'painful' stimuli) always appeared novel. Apparently, noxious stimuli were not perceived as painful, but not because the stimulus was not transmitted faithfully (ie, nociceptive processing was intact). Clearly an understanding of spinal circuitry and neurochemistry could not, by itself, explain the behaviour of these dogs.

Of course, Ron did not ignore the obvious contribution of nociceptive processing to the experience of pain. Despite his emphasis on the contribution of the brain to the experience of pain, his most famous legacy is the 'gate control theory of pain' (presented in an article co-authored by Pat Wall), which concentrates on dorsal horn circuitry (3). Students today can probably describe gate control theory's dorsal horn circuitry, through which large diameter afferents were hypothesized to regulate the output of the 'T' cell – probably a lamina V neuron in present day terminology. Indeed, nociceptive processing and segmental regulatory mechanisms generated in the dorsal horn are currently the topic of much research and, in fact, are the focus of the present article. But the spinal cord 'gate' is also influenced by supraspinal controls (the 'central control trigger'). This 'trigger' regulated nociceptive processing via activation of cortical and subcortical mechanisms that are put into play through experience. It is not important whether the specific pathways that were hypothesized have proven to be true. What was important was the emphasis on the cortical and limbic contributions to the experience of pain. Basbaum and Fields' (4) studies on the descending control of nociceptive processing were strongly influenced by the pioneering principles that the 'central control trigger' concept articulated.

We hope that Ron is not disappointed with the following discussion that we have written in his honour. The review focuses almost exclusively on the spinal cord networks through which nociceptive inputs are regulated. In some respects, it is a modern day look at how to close the 'gate' that is located in the superficial dorsal horn. Interestingly, it focuses on the balance of pre- and postsynaptic controls of nociceptive messages by dorsal horn neurons – a topic that burned intensely in the debate that arose soon after the publication of the gate control theory paper. In keeping with the ultimate conclusion that both pre- and postsynaptic controls are important, we provide evidence that opioids and gamma-amino butyric acid (GABA)ergic controls operate differently on the central consequences of noxious stimulus-induced substance P (SP) release.

PHARMACOLOGICAL CONTROLS OF PRIMARY AFFERENT NEUROTRANSMITTER RELEASE

In the quest for new and improved analgesics with minimal side effects, considerable attention has been directed at which systems can regulate neurotransmitter release from the central terminals of primary afferent nociceptors (5). Methods used have ranged from studying how candidate agents affect neurotransmitter release in vivo and in vitro to electrophysiological studies monitoring dorsal root ganglion (DRG) responsiveness and postsynaptic neuronal firing. Although glutamate is almost certainly the primary mediator of nociceptive transmission at this first synapse, other neurochemicals, notably the tachykinin peptides SP and neurokinin (NK) A, are major contributors (6,7). These peptides are present in a proportion of the small primary afferent neurons that terminate in the outer laminae of the dorsal horn, lamina I and lamina II (8,9); are released by high intensity stimulation (10-16); enhance the excitability of spinal neurons to noxious inputs (17-19); and contribute to a 'window' of noxious stimulus-evoked behaviours (20). SP and glutamate colocalize in the terminals of small diameter primary afferent neurons in the dorsal horn (21,22).

Investigations into which systems influence tachykinin release (the majority assaying SP release) have often produced conflicting findings. Among the many molecules that purportedly regulate SP release are noradrenaline, serotonin and neuropeptide Y (5). This article focuses only on the action of two classically inhibitory systems, mu opioid and GABA. We highlight recent studies from our laboratory that used a novel neuroanatomical approach to evaluate the contribution of SP to nociceptive processing. Most importantly, rather than assessing the release of SP, we monitored a marker of the functional consequences of the interaction of SP with postsynaptic dorsal horn neurons – NK-1 receptor internalization.

INTERNALIZATION OF NK-1 RECEPTOR: A RELIABLE MEASURE OF THE FUNCTIONAL CONSEQUENCES OF SP RELEASE

A range of methodologies have been used to study the release of SP from sensory afferents in vitro or in vivo. Commonly, these have involved measuring the levels of SP in perfusate collected from slices of spinal cord (23), from intact spinal cord (10), or from within the spinal cord tissue itself using push-pull cannulae or microdialysis fibres (24,25). To improve the spatial resolution within which the sites of SP release can be detected, another method was developed that uses immobilized antibodies to SP on microelectrodes. The antibodies bind any locally released peptide and, thus, provide a spatial marker of the locus of peptide release. Importantly, this technique is associated with relatively minor tissue damage, which can induce the release of primary afferent neurotransmitters (26). However, even these 'antibody microprobes' can only measure extrasynaptic levels of peptide. As a result, although an inhibition of SP release may be mirrored by a reduction in peptide levels detected in the perfusate or by reduced binding to microprobes, none of these

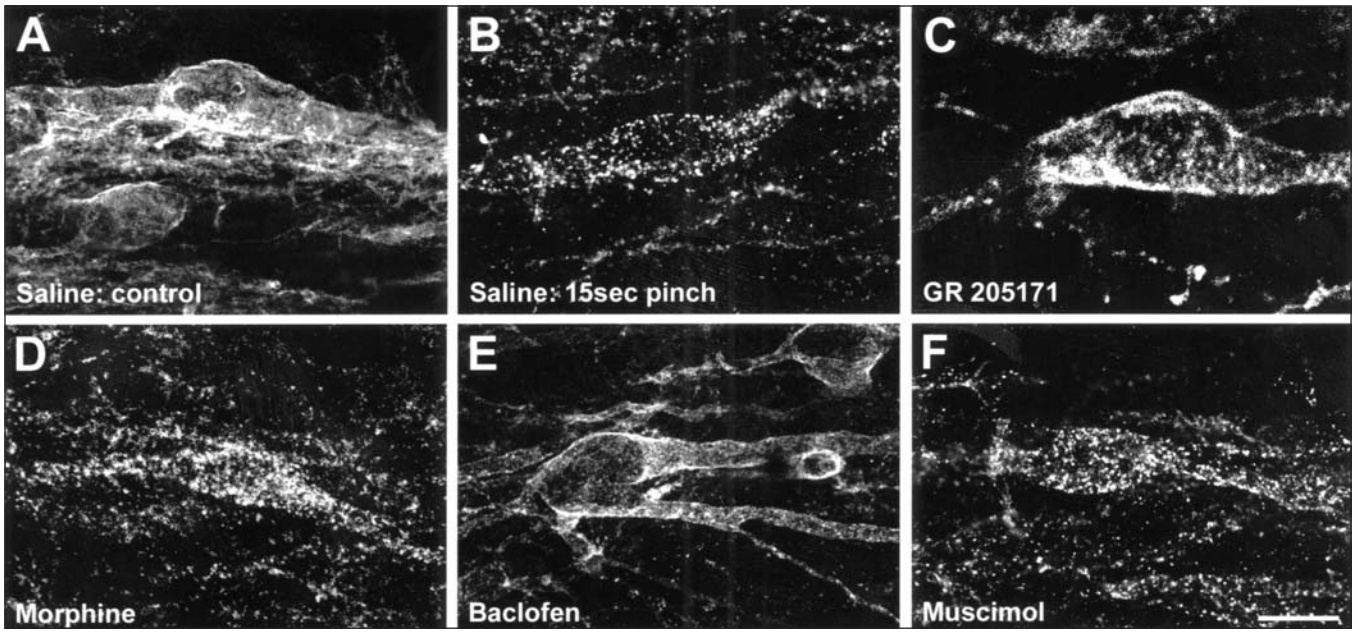

Figure 1) These confocal images (A-F) illustrate the effect of mu opioid and gamma-amino butyric acid (GABA) receptor agonists on noxious stimulus-evoked internalization of the neurokinin (NK) -1 receptor in neurons of lamina I of the spinal cord, viewed here in saggital section. In the absence of, or contralateral to, the side of a noxious stimulation, NK-1 receptor-like immunoreactivity is localized to the plasma membrane (A). However, after noxious stimulation of the periphery, the immunoreactivity is present in endosomal-like structures throughout the cell body, and little or no plasma membrane labelling is evident (B). Receptor internalization is reduced by the systemic administration of the NK-1 receptor antagonist GR205171 (10 mg/kg, subcutaneously) (C). Neither systemic nor spinal morphine had a significant effect on the incidence of NK-1 receptor internalization (30 μg intrathecally) (D). In contrast, spinal administration of the $GABA_B$ receptor agonist baclofen (10 μg intrathecally) (E) but not the $GABA_A$ receptor agonist muscimol (10 μg intrathecally) (F) dramatically reduced the magnitude of NK-1 receptor internalization. All animals were subjected to a noxious mechanical compression of the distal hindpaw for 15 s and perfused 5 min later. Drugs were injected either directly into the intrathecal space (20 μL) or subcutaneously in 1.0 mL saline, approximately 20 to 25 min before stimulation. Scale bar (shown in F) = 20 μm

techniques can predict the functional consequences of this reduced SP release. Only by monitoring the neurons that respond to the released peptide can this be determined.

The majority of the primary afferent-released SP is thought to act via NK-1 receptors located in the spinal cord dorsal horn. NK-1 receptors are part of a larger family of NK receptors, including the NK-2 and NK-3 receptors that, respectively, show high affinity for NKA and the nonprimary afferent-derived tachykinin NKB (27). As is characteristic of many other G-protein coupled receptors, NK-1 receptors rapidly internalize upon agonist binding (28-30). After internalization, the receptor dissociates from the ligand. The receptor recycles to the plasma membrane (within approximately 1 h of internalization); the ligand is typically shuttled to lysosomes, where it is degraded. Our group and others have used internalization of the NK-1 receptor as a quantifiable indicator of when neurons that express these receptors are activated in vivo (31-33). In the absence of internalization, NK-1 receptor-like immunoreactivity (NK1R-LI) is uniformly distributed on the surface of cell bodies and dendrites in lamina I (Figure 1A). This was confirmed in electron microscopic studies that also showed that there is a significant mismatch between the presence of SP terminals and the NK-1 receptor; the latter had a much more extensive distribution (34), suggestive of extrasynaptic transmission (35,36, but see 37). In fact, despite the presence of dense SP immunoreactivity in lamina II (the substantia gelatinosa), the only element that expresses the NK-1 receptor in this region corresponds to the dorsally directed dendrites of large NK-1 receptor-expressing neurons in lamina III (38,39).

After noxious stimulation of the rat hindpaw, the distribution of the receptor is dramatically altered. The majority of the immunoreactivity is then present as bright immunofluorescent 'endosome-like' structures in the cytoplasm (Figure 1B). Because the dendrites of these lamina I neurons arborize in the rostrocaudal direction, the incidence of internalization is usually quantified on sections cut in the saggital plane, each neuron being scored for the presence or absence of internalized receptor. In agreement with the consensus of previous studies of SP release (see above), all modalities (mechanical, thermal or chemical) of noxious, but not innocuous, peripheral stimulation were found to induce internalization of the NK-1 receptor (31,32,40). The internalization is dependent on ligand binding; coadministration of the selective NK-1 receptor antagonist GR 205171 significantly reduced noxious stimulus-induced internalization of the NK-1 receptor in spinal cord neurons (Figure 1C) (32).

In the absence of tissue damage, only NK-1 receptors expressed by lamina I neurons show any evidence of internalization after noxious stimulation; however, in the setting of tissue inflammation or nerve injury, NK-1 receptors located more ventrally, in laminae III to VI, also begin to internalize

in response to noxious stimulation (32,41,42). In some cases, innocuous stimuli applied to inflamed tissue may evoke NK-1 receptor internalization, but exclusively in lamina I. More recently, Schwei et al (43) found that palpation of the limb of a rat with a cancerous femur was an effective stimulus to drive the internalization process. The source of the SP that produced NK-1 receptor internalization in response to innocuous stimuli was unclear in these studies. Several possibilities are proposed. First, there may be peripheral sensitization of the SP-containing primary afferent nociceptor. Second, because there is evidence of de novo synthesis of tachykinins by large diameter, myelinated afferents in the setting of persistent inflammation (44), it is possible that the condition of bone cancer alters the neurochemical phenotype of mechanoreceptors. Finally, the possibility that interneurons and/or descending fibres (45-47) are the source of the SP that induces internalization in novel populations cannot be ruled out. These neurons may be activated by innocuous stimuli in the setting of injury.

Our assumption that receptor internalization provides a robust indicator of the extent of NK-1 receptor activation is based on studies in vitro linking NK-1 receptor internalization to intracellular signalling (48-51). However, all these studies used transfected non-neuronal cell lines, and comparable investigations had not been performed using neurons. To ensure that our endpoint of internalization correlated with NK-1 receptor-mediated signalling, we performed a series of in vitro studies using primary cultures of spinal cord neurons. In these studies, we compared the dose-response curves for the magnitude of SP-mediated increases in intracellular calcium ion and NK-1 receptor internalization. Importantly, we showed that SP-induced calcium ion mobilization was highly correlated with the magnitude of SP-induced NK-1 receptor internalization in dorsal horn neurons. In addition, we showed that the NK-1 receptor antagonist GR 205171 reduced the incidence of noxious stimulus-induced internalization of the NK-1 receptor in a dose-dependent manner. Thus, in contrast to traditional release methods that monitor the extrasynaptic levels of SP (ie, the spillover from that necessary for receptor activation), NK-1 receptor internalization provides a measure of the functional consequences of the SP interaction with the NK-1 receptor.

OPIOID CONTROL OF NOCICEPTOR SIGNALLING

Radioligand binding studies (52-57), and more recently immunocytochemical (58-65) and in situ hybridization studies (66-69), have located opioid receptors on the cell bodies and terminals of primary afferent neurons, including some small diameter capsaicin-sensitive afferents (54,55,64) that synthesize SP (58,59,69). Minami and co-workers (69) investigated by double in situ hybridization the extent to which each of the mu, delta and kappa subtypes of opioid receptor colocalizes with preprotachykinin A (PPTA), the precursor protein of SP. They found that, whereas 90% and 30% of PPTA-positive neurons expressed mu and kappa receptor mRNAs at high levels, respectively, only about 3% of PPTA mRNA-positive neurons expressed delta receptor mRNA at high levels. In addition, electrophysiological studies on cultured or dissociated DRG neurons have shown that opioids active at mu, delta and kappa receptors can suppress voltage-dependent calcium ion channel conductances (70-72), which would be expected to contribute to a reduction of neurotransmitter release at the presynaptic terminal. Further, by recording the responses of trigeminal ganglion neurons retrogradely labelled from the tooth pulp, which represents a nearly pure nociceptive population, mu opioid receptor (MOR) regulation of calcium ion channels has been proposed to be limited to the slow-conducting, unmyelinated (C fibre) nociceptors (73)

That opioids reduce a calcium ion conductance is consistent with the demonstration that mu opioids, such as morphine, can inhibit the potassium ion-evoked release of SP from brain slices of the trigeminal nucleus caudalis (74). This key observation led to the hypothesis that inhibition of SP release at the presynaptic terminal is a major mechanism by which opiates produce analgesia. The hypothesis gained support from a number of other investigations that measured the levels of evoked tachykinin (typically SP) in perfusate collected from the spinal cord in vivo (10,13,75,76). However, as Duggan (5) noted, in all these studies, morphine was added to the superfusate from which the levels of SP were subsequently estimated. No group has reported a reduction of stimulus-evoked release of SP when morphine was administered intravenously in analgesic doses (25,77,78). Additionally, other studies in slices failed to find a uniform inhibition of SP release by mu opioids (23,79,80). By contrast, a multiphasic modulation of SP release by morphine has been proposed (80).

OPIOIDS ONLY MINIMALLY AFFECT NOXIOUS STIMULUS-EVOKED NK-1 RECEPTOR INTERNALIZATION

In light of the questions raised by Duggan (5) and because there is now a measure of SP-mediated signalling, we re-examined the question of the regulation of SP effects by opioids. If there is a functionally relevant presynaptic inhibitory control of SP release, this should be reflected in a reduction of noxious stimulus-evoked internalization of the NK-1 receptor. In these studies, we used acute noxious mechanical or thermal stimulation to evoke SP release, both in normal rats and in rats with a persistent hindpaw inflammation. In contrast to the majority of SP release studies cited above, we found that neither spinal nor systemic morphine had a significant effect on the incidence of NK-1 receptor internalization in lamina I neurons of the lumbar spinal cord (Figure 1D) (33). Only intrathecal administration of high doses of morphine (30 μg) resulted in a small, albeit significant, reduction in the percentage of lamina I neurons that contain internalized NK-1 receptors after mechanical stimulation of the hindpaw in normal rats. Importantly, the same result was obtained whether the occurrence of internalization was estimated by our usual 'all or none' criterion, namely scoring neurons as internalized if they contained greater than a threshold number

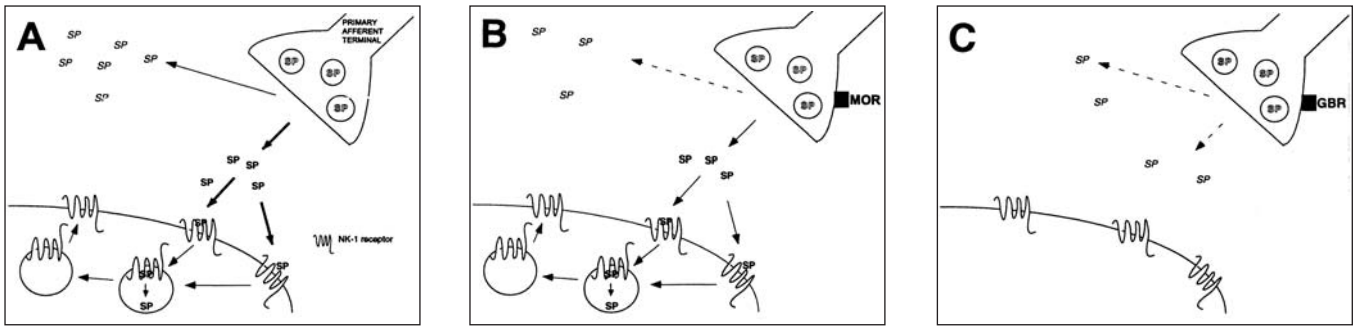

Figure 2) This schematic diagram illustrates the hypothesized mechanisms through which regulation of substance P (SP) release from primary afferents by presynaptic mu opioid receptor (MOR) or gamma-amino butyric acid (GABA)$_B$ receptors (GBR) differentially affects neurokinin (NK)-1 receptor internalization. In the absence of exogenous drugs (A), noxious stimulation of the periphery results in the release of SP (SP) from the terminals of small diameter, unmyelinated primary afferents. Some of this SP (**SP**) diffuses to target cells, where it interacts and activates NK-1 receptors. This SP is internalized along with the NK-1 receptor into endosomes. Acidification of these endosomes dissociates the SP from the NK-1 receptor. The SP is degraded, and the NK-1 receptor is recycled to the membrane. Unbound SP (*SP*) diffuses into the extracellular space and eventually into the cerebrospinal fluid, where it may be broken down by endopeptidases. In the presence of the MOR agonist morphine (B), the amount of SP released by noxious stimulation is reduced but not to a sufficient extent to limit the internalization of the postsynaptic NK-1 receptors. In other words, the functional pool of SP is sufficient for near maximal activation and internalization of NK-1 receptors. In contrast, after the spinal administration of the GABA$_B$ agonist baclofen, very little SP is released; therefore, little or no SP is available to activate the NK-1 receptors

of NK-1 receptor-positive endosomal-like structures, or when we counted the total number of NK-1-containing endosomes per neuron. In fact, no difference in the average number of NK-1 receptor-positive endosomes per neuron between groups was found.

In subsequent studies, we found that only high concentrations of the selective mu receptor agonist D-Ala2, NMephe4, Glyol5-enkephalin (DAMGO) (1.0 µg), but neither the selective delta agonist [D-Pen(2), D-Pen(5)]-enkephalin (DPDPE) (30 µg) nor the selective kappa receptor agonist U-50488H (100 µg) decreased the number of neurons with internalized receptor. This suggests that the minimal effect of morphine was mediated by the MOR. Despite reports of enhanced morphine potency following inflammatory injury (81), morphine was less effective in the setting of injury (see below). All these doses of morphine and selective opioid receptor agonists have been found to produce analgesia in animals (82). Because at least 80% of the tachykinin signalling was estimated to remain intact after even the highest concentration of spinal morphine administration, it was concluded that NK-1 receptor signalling is only slightly reduced under conditions of profound opioid analgesia.

We have argued that it is the magnitude of the inhibition of release that is critical. We do not disagree with the claim that morphine reduces the release of SP from primary afferent nociceptors. However, even when a reduction of the level of SP in cerebrospinal fluid can be detected, the residual SP (presumably at the synaptic cleft) is of sufficient magnitude to exert maximal signalling (and NK-1 receptor internalization) in postsynaptic neurons (Figure 2B). To test this hypothesis specifically, morphine (10 mg/kg) and an ineffective dose of the NK-1 receptor antagonist GR 205171 (1.0 mg/kg) were administered simultaneously. The concurrent administration of these drugs significantly reduced the percentage of lamina I cells showing internalization following mechanical stimulation of the hindpaw compared with that in the saline-treated group (45% in the L4-L5 segment) (33). Thus, an ineffective dose of NK-1 receptor antagonist 'uncovered' the relatively large decreases in total SP released. Consistent with this proposal was the finding that this combination of morphine and GR 205171 in rats with persistent hindpaw inflammation was no longer able to reduce significantly the incidence of NK-1 receptor internalization; the highest dose of NK-1 receptor antagonist (10 mg/kg) administered alone produced less of a reduction. Conceivably, this latter result occurred because of the concurrent upregulation of SP in DRG (83-85) and NK-1 receptor in dorsal horn cells (86-89) in the setting of inflammation. This increased SP release would be expected to reduce the effectiveness of the competitive NK-1 receptor antagonist.

The results of these studies led to the conclusion that opioids produce their analgesic effect predominantly via postsynaptic inhibitory controls that are exerted upon dorsal horn neurons. Of course, presynaptic inhibitory controls of primary afferents that do not release SP cannot be ruled out. Opioid regulation of the release of glutamate, which coexists with SP in some nociceptors, may also contribute. However, overall there is limited electrophysiological evidence to support a direct inhibitory action of opioids on the central terminals of small diameter myelinated and unmyelinated primary afferents (90,91). Rather, opioids, at least MOR-preferring ligands, have mainly been reported to have hyperpolarizing actions on dorsal horn neurons by increasing outward potassium conductance (92-97).

GABAERGIC SYSTEMS IN THE SUPERFICIAL DORSAL HORN

Given that opioids were found to have little effect on SP signalling, it was of interest to compare their effects with those of other neurochemicals also proposed to exert inhibitory controls on the flow of nociceptive information in the spinal cord. There is a significant body of literature to support the

possibility that GABA, a major inhibitory neurotransmitter in the central nervous system (CNS), like the MORs, exerts presynaptic control of SP-containing nociceptors (reviewed in 98). GABA operates chiefly through one of two receptor subtypes. The $GABA_A$ receptor is a ligand-gated ion channel receptor that elicits fast inhibitory postsynaptic currents by increasing the membrane conductance to chloride ion; the $GABA_B$ receptor is a G-protein-coupled receptor that generates inhibitory potentials of slower onset and more prolonged duration. These may involve the opening of adjacent, inwardly rectifying potassium ion channels, the closure of voltage-dependent calcium ion channels and/or the inhibition of adenylate cyclase (99). Both $GABA_A$ and $GABA_B$ receptors have been reported to exert presynaptic actions on nerve terminals, for example, in the CA1 region of the hippocampus, in the molecular layer of the cerebellum, and in the dorsal horn of the spinal cord and trigeminal caudalis (99,100).

Early binding studies using titrated muscimol and baclofen (selective agonists for the $GABA_A$ and $GABA_B$ receptors, respectively) provided some evidence for a presynaptic location for both receptors on capsaicin-sensitive (ie, small diameter) primary afferent terminals (101,102). This binding accounted for a high percentage of $GABA_B$ sites in the dorsal horn (approximately 40% to 50% [102]), but less for $GABA_A$ (approximately 20% to 30% [101]). Dorsal rhizotomy produced a reduction in $GABA_B$-binding sites in the superficial laminae comparable with that produced by neonatal capsaicin treatment, suggesting that these presynaptic $GABA_B$ receptors are preferentially localized to capsaicin-sensitive, small diameter myelinated and unmyelinated, typically nociceptive, afferents (103). Even though the $GABA_B$ receptor has recently been cloned (104), the precise location of the receptor in DRG neurons has yet to be investigated fully by in situ hybridization or immunohistochemistry. By contrast, $GABA_A$ receptors appear to be more ubiquitously expressed by sensory afferents, as shown by quantification of receptor subunit mRNAs in DRG (105). At an electrophysiological level, studies in vitro (106-108) as well as in vivo (109-113) support both $GABA_A$ and $GABA_B$ receptor-mediated effects on nociceptor function. Other groups have, however, described a preferential presynaptic action of the selective $GABA_B$ receptor agonist, baclofen, on monosynaptic (typically low threshold, large diameter afferent-mediated, proprioceptive) pathways in the spinal cord (114,115).

Studies of the effect of GABA receptor agonists on SP release have produced inconsistent findings. In part, the different conclusions may have resulted from differences in the route of drug administration, as well as in the types of release methodologies used in these studies. For example, whereas studies in vivo failed to demonstrate an effect of systemically administered baclofen on SP release (13,116), those involving the application of baclofen onto spinal cord slices reported dramatic reductions in superfusate levels of SP (117, but see 118,119). In a similar way, direct spinal (100,120) but not systemic (116) injection of baclofen has been reported to reduce the release of calcitonin gene-related peptide (CGRP), a more generalized marker of small diameter primary afferents (121). Likewise, previous reports on the effect of $GABA_A$ agonists (muscimol and isoguvacine) on neurotransmitter release have been inconsistent, and overall have reported no effect (117,122, but see 13,118). In part, this may reflect the more complex action of $GABA_A$ receptor agonists on primary afferents. While the role of presynaptic $GABA_B$ receptors appears to be exclusively inhibitory, reducing neurotransmitter release via reduction of a calcium ion conductance (106,123,124), the consequence of activating presynaptic $GABA_A$ receptors is more controversial. Because the $GABA_A$ receptor antagonist bicuculline inhibits the primary afferent depolarization (PAD) produced in the dorsal grey matter of the spinal cord after an intense afferent volley (125), PAD is likely mediated by $GABA_A$ receptor, via some shunting of the membrane conductance to chloride ion (126). However, the functional significance of PAD is not clear; it has been implicated in both excitatory (113) and inhibitory (112,115) events.

$GABA_B$ BUT NOT $GABA_A$ RECEPTOR ACTIVATION LIMITS NOXIOUS STIMULUS-EVOKED NK-1 RECEPTOR INTERNALIZATION

To address the contribution of $GABA_A$ and $GABA_B$ receptor activation to SP-mediated signalling in the spinal cord dorsal horn, we again monitored the internalization of the NK-1 receptor after acute noxious mechanical or thermal stimulation of the rat hindpaw. In agreement with the majority of SP release studies cited above, there was evidence for a $GABA_B$ but not $GABA_A$ receptor-mediated inhibition of tachykinin release and/or signalling in the superficial dorsal horn. Intrathecal administration of R(+) baclofen (10 μg) dramatically reduced the percentage of NK1R-LI lamina I neurons that contained internalized receptors after an acute noxious mechanical stimulation of the rat hindpaw (Figure 1E). By contrast, administration of even high doses of the $GABA_A$ receptor agonists muscimol (10 μg) or isoguvacine (50 μg) did not produce an effect (Figure 1F). The inhibitory effect by baclofen was prevented by the coadministration of a selective $GABA_B$ receptor antagonist, CGP55845 (10 μg), confirming the selectivity of the effect to $GABA_B$ receptors (127). These effects are consistent with those seen by Marvizon et al (128), who examined the effect of GABA agonists on NK-1 receptor internalization induced by electrical stimulation of dorsal roots in a spinal cord slice preparation. The robustness of the inhibitory effect of baclofen on tachykinin signalling is illustrated not only by how ineffective opioids are on NK-1 receptor internalization under similar noxious conditions (as presented above), but also by how comparable the maximal reduction of NK-1 receptor internalization produced by intrathecal baclofen (approximately 73% in L4-L5) is with that produced by the NK-1 receptor antagonist GR205171 (approximately 78% in L4 [32]).

Although baclofen is predominantly used as a muscle relaxant for the treatment of spasticity in humans (100), it exerts potent analgesic actions in animal models of acute (129-131) and persistent pain (132-135), especially when given by

the spinal route (131). In our studies, although motor side-effects were found to occur at the higher doses of intrathecal baclofen tested, lower doses, which produce minimal or no motor weakness, and have also been shown to be analgesic (129,130), still significantly reduced the incidence of receptor internalization after noxious stimulation. In keeping with the findings of previous groups (13,116,119), we found that systemic administration of baclofen was only minimally effective in limiting the internalization of the NK-1 receptor, although motor effects were still evident. We conclude that baclofen can exert a powerful inhibitory influence on nociceptor activity and tachykinin release via presynaptic actions at $GABA_B$ receptors located on the small diameter nociceptive afferents that terminate in the superficial dorsal horn (Figure 2C).

IS THERE TONIC CONTROL OF PRIMARY AFFERENT NEUROTRANSMITTER RELEASE?

While release studies that monitor the effect of exogenously administered compounds are informative from a pharmacological perspective, they do not address the physiological relevance of these inhibitory controls, ie, whether such modulation occurs in vivo (5). To this end, there is a notable lack in the superficial dorsal horn of axo-axonic synapses in which the presynaptic terminal contains enkephalin (an endogenous ligand of mu and delta opioid receptors) (136-139). Instead, in the cat spinal cord, enkephalin-LI axonal boutons have been found presynaptic to physiologically identified nociresponsive neurons in the superficial dorsal horn (139). These are primarily thought to be of intrinsic origin (140-142). More recently, ultrastructural analyses in the rat trigeminal dorsal horn have demonstrated that MORs are more often found postsynaptic to, but infrequently located on, SP-containing terminals in laminae I and II, and that a third of these MOR-positive dendrites colocalize the NK-1 receptor (35). This synaptic arrangement may provide the basis for the MOR inhibition of nociceptor signalling proposed in this review. Of course, the possibility of diffusion of neuronal-released enkephalin to more distant targets must be considered, and is certainly likely given the nonsynaptic interactions that characterize other peptide neurotransmitter systems, including the tachykinins (143,144). On the other hand, because we found minimal control of tachykinin release by exogenously administered opioids, our results suggest that the anatomy may accurately reflect the functional endogenous circuitry, ie, that mu opioids primarily modulate the postsynaptic responses to primary afferent-released SP and glutamate, rather than their actual release. Conceivably, circulating opioids from the pituitary and adrenal medulla may be the source of the endogenous activation of MORs on primary afferents (139,145).

In sharp contrast and in keeping with the dramatic effects of $GABA_B$ receptor agonists on tachykinin signalling reported here, there is strong evidence that GABAergic neurons are positioned to presynaptically regulate SP-containing primary afferent terminals. Notably, GABAergic terminals make axo-axonic synapses with small diameter primary afferent terminals in the substantia gelatinosa (146-150), some of which may be unmyelinated (148,150) and contain SP (150, but see 151-154). The proposed sources of the GABA are the terminals of 'islet cell' interneurons of laminae II and III in the rat spinal cord (155), although a contribution from descending GABAergic projections (156) cannot be ruled out. There is also morphological evidence to suggest that small diameter primary afferents activate GABAergic interneurons of the superficial dorsal horn (149,150,152,157,158).

Indeed, based on this evidence, it has been proposed that this circuit underlies a negative feedback mechanism that limits the further release of excitatory amino acids and/or peptides from the primary afferent nociceptor (100). Because the majority of NK-1 receptor-positive neurons in the spinal cord, including lamina I, are not GABA-immunoreactive (159), this pathway is likely to be indirect. Increases in the superfusate levels of SP (132) and glutamate (122) from spinal cord slices have been reported following $GABA_B$ receptor antagonist administration. However, this potentiation of SP release was only evident three weeks after intraplantar injections of Freund's complete adjuvant (132), and no such potentiation of CGRP release by $GABA_B$ receptor antagonists was observed (98). More recently, Marvizon et al (128) monitored the internalization of the NK-1 receptor in superfused dorsal horn slices and found that 2-hydroxysaclofen (a selective $GABA_B$ receptor antagonist) altered the stimulus-response function for inducing NK-1 receptor internalization. Normally ineffective, low frequency stimulation (1.0 or 10 Hz) of dorsal roots at C-fibre intensities in the presence of 2-hydroxysaclofen induces NK-1 receptor internalization of comparable, if not greater, magnitude than that produced by high frequency stimulation (100 Hz) in the absence of the antagonist.

We addressed the question of whether there is tonic control of primary afferent neurotransmitter release in our in vivo studies using different modalities (mechanical compared with thermal) and intensities (45°C compared with 50°C) of noxious stimulation in the presence of CGP55845. We were unable to detect even a trend toward an increase in the number of neurons with internalized NK1R-LI. Although a recent study reported an increase in the expression of the immediate early gene protein, Fos, induced by peripheral noxious stimulation (160), because the $GABA_B$ receptor antagonist CGP35348 was administered systemically in that study, the locus of the antagonism could not be determined. It may involve $GABA_B$ receptors at the brainstem as well as the spinal level (131). Thus, we believe that it is still to be proved whether $GABA_B$ receptor-mediated tonic regulation of acute nociceptive processing occurs in vivo.

CLINICAL IMPLICATIONS

Unlike morphine and other mu receptor opioids, $GABA_B$ receptor agonists such as baclofen have proved to be disappointing in the treatment of human clinical pain (100). Interestingly, this dichotomy in preclinical compared with clinical effectiveness is also true for the use of NK-1 receptor

antagonists as analgesics (161). These findings, taken together with the results using NK-1 receptor internalization, suggest that disruption of tachykinin signalling is not essential or sufficient for effective analgesia. The possibility remains, however, that blockade of NK-1 signalling can potentiate the actions of morphine, as supported by a number of preclinical (20,162,163) and clinical (164) studies. This may be partly due to differences in the neuronal pathways targeted by these agents. Indeed, using Fos as marker of neuronal activation following noxious peripheral stimulation, we found that, whereas lamina I neurons that express the NK-1 receptor are sensitive to baclofen treatment, they are relatively refractory to opioid treatment (33), particularly the ones that project to the parabrachial nucleus (165). In addition, in recent studies using mice carrying a disruption of the preprotachykinin A gene, we found that tachykinins only come into play at relatively high stimulus intensities (20).

Thus, although it remains to be proved that $GABA_B$ receptor-mediated tonic regulation of nociceptive processing can occur in vivo, the profound inhibitory effects of exogenous baclofen that we observed must not be underestimated. We believe that the search for new approaches in the treatment of persistent pain will be enhanced by further studies that address this form of GABAergic control in primates and humans. Indeed, an understanding of why baclofen is not particularly useful in the treatment of pain (rather than spasticity) in patients may provide important insights into the specific contribution of tachykinin systems to clinical pain conditions.

CONCLUSIONS

Gate control theory stimulated the research that led to our new appreciation of the complexity of nociceptive processing. This theory emphasized the basic principle of gating that is a hallmark of the transmission of nociceptive messages in the dorsal horn. Our understanding of the complexity of gating, of course, has changed considerably. The 'gate' of gate control theory was part of a hardwired circuit that controlled the flow of nociceptive information in the dorsal horn. The key to opening and closing the gate corresponded to a neuron in the substantia gelatinosa. Thirty-five years of research since publication of the theory has taught us that the dorsal horn nociceptive circuits are remarkably plastic – not hardwired. The circuits change subtly through use and dramatically in the setting of injury because of molecular and cellular alterations of the component neurons. More important, perhaps, is that we have learned that there are multiple keys to opening and closing the gate and that the locks are changeable, but the focus of the modern day dorsal horn locksmiths remains the substantia gelatinosa. Understanding gating mechanisms in the dorsal horn, we believe, is still critical to understanding nociceptive processing and to the development of novel therapeutic agents for the treatment of pain.

There is no question that pain is one of the most interesting areas of neuroscience in which to work, and there is also no question that Ron Melzack continues to influence how we think about this fascinating problem. Our own studies are built upon the insights into the relationship between nociception and pain that Ron Melzack has so clearly articulated. Of course, how the output of these dorsal horn circuits is translated into altered perceptions of pain and where in the brain such processing occurs are fascinating questions that should not be ignored and indeed are the focus of Ron Melzack's research to this day. To some extent, our concentration on spinal cord circuitry is one of expedience. Studying the neurochemistry and circuitry in the thalamus and cortex in the context of the 'pain' percept is extremely difficult, particularly in animals. We are aware that nociception and pain are not equivalent and that changes in the expression of a particular gene or of the internalization of a particular receptor do not equate with pain. On the other hand, we believe that a practical approach to developing new treatment regimens will be greatly benefited by studies that characterize the circuitry through which nociceptive messages are processed.

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