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Review Article

Substantia gelatinosa of the spinal cord. Morphofunctional organization

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Abstract

The superficial layers of the dorsal horn of the spinal cord and the caudal subnucleus of the trigeminal nerve, especially the substantia gelatinosa (or lamina II of Rexed), are regions that have traditionally been associated with the modulation of nociceptive information since Ranson's classic clinical studies. Considerable attention to this area was generated in the 1960s and 1970s by the publication of physiologically based theories of pain, which postulated the existence of synaptic circuits involving interneurons and afferent fibers carrying various input signals. These theories introduced the basic concept that nociceptive transmission can be altered by simultaneous activation of other fiber systems. In recent years, significant progress has been made in understanding the anatomical and neurochemical characteristics of the relevant cells and systems. However, our knowledge in this area is still far from complete, and, unfortunately, in textbooks and even in reviews one can find too many simplified schemes/

Keywords: morphofunctional organization; substantia gelatinosa; spinal cord; rat; brain

Introduction

The superficial layers of the dorsal horn of the spinal cord and the caudal subnucleus of the trigeminal nerve, especially the substantia gelatinosa (or lamina II of Rexed), are regions that have traditionally been associated with the modulation of nociceptive information since Ranson's classic clinical studies. Considerable attention to this area was generated in the 1960s and 1970s by the publication of physiologically based theories of pain, which postulated the existence of synaptic circuits involving interneurons and afferent fibers carrying various input signals. These theories introduced the basic concept that nociceptive transmission can be altered by simultaneous activation of other fiber systems. In recent years, significant progress has been made in understanding the anatomical and neurochemical characteristics of the relevant cells and systems. However, our knowledge in this area is still far from complete, and, unfortunately, in textbooks and even in reviews one can find too many simplified schemes [1]. This article provides an overview of the rat spinal cord substantia gelatinosa, focusing on its anatomical, ultrastructural, and immunocytochemical aspects. While most of the information will be on lamina II, or the substantia gelatinous proper, lamina I (the marginal layer) and lamina III (the superficial part of the nucleus propria) will also be briefly described because of their close relationship and physiological significance.

Definition

The substantia gelatinosa of the spinal cord received its name from Rolando in 1824 because of the translucent and gelatinous appearance it exhibits when fresh tissue is examined. In the cat, Rexed used 100-µm-thick frozen microtome sections stained for Nissl substance to divide the spinal cord into several horizontal lamellae based on cell density and size, and Nissl body morphology. Lamina II had a particularly high cellular density due to the presence of many small neurons. Rexed made a second lamina of the corresponding gelatinous substance, which he divided into dorsal (more cellular) and ventral (less cellular and thicker) parts. In more recent studies, lamina II is usually divided into outer lamina II (or lamina IIo) and inner lamina II (or lamina IIi). Rexed's cytoarchitectonic classification has been adapted to rats and other species such as monkeys [1]. The laminar pattern can also be recognized in samples examined in the dark field (eg, cryostat sections processed for receptor binding studies), fiber-stained (eg, Mahon's method), or osmicated and embedded in epon. Using epon-embedded semi-thin sections of 1 or 2 µm thickness, lamina I can be separated from outer lamina II by the abundant small myelinated fibers in the former; they are less numerous in the outer lamina II and are almost completely absent in the inner lamina II. The lamina II–III boundary is easily identified in semithin sections because of the numerous small myelinated fibers found in lamina III. Thus, at the light microscopic level, the subdivisions of the superficial plates in the rat are very similar to those observed in other mammals such as cats and

monkeys. Unfortunately, ultrastructural observation of the rat dorsal horn poses unexpected problems. In fact, unlike the dorsal horn of the monkey and cat, lamina II externus in the rat is virtually devoid of synaptic endings. Inner lamina II has a narrow dorsal stripe rich in type I synaptic terminals (with electron-dense central varicosities) and very few type II terminals (which have light and large central varicosities). [1,2]. The more extensive ventral-most portion of lamina II is rich in type II synaptic terminals and contains very few type I terminals. On the contrary, in cats and monkeys, dense type endings predominate in lamina IIo. This difference in the distribution of synaptic terminals most likely results from interspecies differences in the distribution of primary sensory fibers. Therefore, although the cytoarchitectonically defined outer lamina II appears similar in rats, cats, and monkeys, there are likely differences in primary afferent input between species. It is as if the rat outer lamina II had certain features of lamina I. As a result of these interspecies differences in the distribution of synaptic terminals, it is preferable to use an alternative nomenclature when working with the rat dorsal horn: lamina IIA (instead of lamina IIo) and lamina IIB (instead of lamina IIi) . Next, lamina IIB is divided into two subplates: subplate IIBd (corresponding to the most dorsal part of the inner lamina II) and subplate IIBv (corresponding to most of the inner lamina II) [2]. In transverse sections of the cervical dorsal horn (level C4–C5) of young adult rats (weight 200–250 g), the thickness of lamina I is about 20 µm, lamina IIA and subplate IIBd are 20 µm each, subplate IIBv is 40 µm with a thickness of up to 60 µm. At the lumbar level, which is often used for studies in animal models of chronic pain, the main difference is that the thickness of the most superficial laminae in the lateral portion is thinner than in the intermediate and medial portions. However, at the midlumbar level, Todd et al suggest lamina I in the middle is significantly thicker than in the lateral and medial portions of the dorsal horn. This view is based on the distribution of projection neurons and the immunostaining pattern of substance P receptors and does not correspond to standard cytoarchitectonic criteria. Todd et al compared this area of possibly thicker lamina I to the "dorsal cap" described by Snyder in the cat [1,2,3]. When defining the boundaries of the main lamellae, it is important to adhere to the parameters determined in studies using classical cytoarchitectonic methods, as outlined in the chapter by Grant and Kerber. Unfortunately, this is not often followed [2,3]. As a result of the use of poorly defined criteria in delimiting the laminae of the dorsal horn, many published micrographs and diagrams show that lamina I is too thick and includes part of lamina II. One approach to identifying lamellae on sections processed for immunocytochemistry is to stain an adjacent section using the Nissl method. Rexed plates can be easily noted on micrographs of Nissl-stained sections and on a transparent plate superimposed on images of immunostained sections [3].

Characteristics Of Neurons In The Superficial Laminae Of The Spinal Cord

Lamina I

Lima and Coimbra described four morphological types of neurons in rats using the Golgi method, a classification that is still followed by most researchers. Fusiform neurons are elongated rostrocaudally and are more numerous in the lateral part of the plate. Multipolar neurons have characteristic radial dendritic trees and predominate in the medial part of the plate. Pyramidal neurons have triangular shaped cell bodies and are found throughout the mediolateral extension of lamina I, always at the edge of the white matter. Flattened cells have dendritic trees that extend in the mediolateral and rostrocaudal axes. Cells of each of the four types are sometimes (6% of the total) two to three times their normal size. Larger variants of pyramidal and flattened cells probably represent classic Waldeyer cells. Plate I is considered an important area of projection to higher structures [4]. The main sites of projection of lamina I are the thalamus and some areas of the brainstem, especially the lateral reticular nucleus, parabrachial nucleus and periaqueductal gray matter. Although some experimental data have suggested that the morphological types of lamina I neurons differ in the content of neurotransmitters/modulators and the nature of supraspinal projections, this issue remains controversial. In fact, evidence is accumulating in favor of a correlation between the morphological and physiological properties of lamina I neurons.

Lamina II

Despite several studies, our understanding of lamina II neurons in rats is less than that in cats. In the latter species, lamina II cells have been extensively studied using anatomical and physiological approaches. The cells of lamina II, since the work of Ramón y Cajal, have been divided into two main morphological types: the central cell, which is widely distributed throughout the lamina, and the limiting cell, which occurs in the outer band near the border of lamina I–II. These types were identified by Gobel in cats and named islet cells and stalked cells, respectively. In rats, Todd and Lewis used the Golgi method to confirm the presence of both stalked and islet cells in lamina II. Stem cells corresponded to half of the stained cells in the outer portion of lamina II, whereas islet cells were found throughout the lamina and corresponded to approximately one-third of the entire stained neuronal population. However, Todd and Lewis also reported that about half of the cells in lamina IIBv could not be classified as either stalked or islet cells, although they could be subdivided into groups based on their dendritic arborization. The axons of these cells either moved into lamina III or remained in lamina II [1,4]. In the cat, both islet and stem cells were electrophysiologically characterized, filled with horseradish peroxidase (HRP), and studied at the light and electron microscopic levels by Bennett, Goebel and co-workers. At least some stalked cells with axonal arborizations in lamina I appear to transmit excitatory impulses to lamina I cells and therefore represent feedforward excitatory interneurons. The major electrophysiological findings were that the physiological properties of islet cells differed depending on their location: cells located in deep lamina II did not respond to noxious stimuli, whereas cells in outer lamina II responded specifically to these stimuli. This is consistent with previous studies in cats by Light and co-authors, who found that cells in the outer half of lamina II responded to noxious skin stimuli, while cells in the inner half of lamina II responded only to innocuous stimuli. Also in cats, when studying lamina II cells, the type of response evoked seemed to have little correlation with their morphology, but depended more on the localization of dendritic branches. Because dendrites receive most of their information from incoming fibers, fibers terminating deep in the second lamina do not appear to convey nociceptive information in the cat. [5]. However, this is unlikely in rats due to differences in the pattern of sensory fiber terminations. In fact, as explained below, the non-peptidergic subpopulation of small-diameter sensory fibers in rodents terminates primarily in the outermost part of the internal lamina II (sublamina IIBd), and available evidence indicates that these fibers are nociceptive. Consistent with this, C-fiber studies combining intracellular recording with intracellular marker injection revealed significant termination of unmyelinated multimodal nociceptive fibers in a specific region II of the ventral lamina of the guinea pig. Evidence suggests that in animals such as the guinea pig and rat, unmyelinated fibers terminate deeper in lamina II than in cats and monkeys [1,5]. Initially, lamina II was considered a closed system, receiving afferents but not projecting to any region of the brain. However, there is now evidence that a small number of lamina II neurons project to the brain (thalamus, lateral cervical nucleus, or

pontomedullary junction) [2]. One study suggests that a significant number of islet cells project to the reticular formation of the medulla.

Lamina III

In cats, lamina III neurons have been described as a heterogeneous population of non-nociceptive cells based on intracellular injections of physiologically characterized neurons. However, the idea that all cells are nonnociceptive requires revision, at least in rats, based on the discovery in lamina III and IV of neurons that express the substance P receptor and possess dorsally oriented dendrites that branch in lamina I and II [6]. Most of these neurons project to supraspinal levels. Little is known about other populations of lamina III neurons in rats.

Ultrastructure Of the Dorsal Horn

Signs that allow each lamina and sublamina to be characterized under an electron microscope are the density of small myelin fibers and the distribution of synaptic endings. In this part we will focus on synaptic terminals.

Synaptic endings

In the rat, the ultrastructure of the dorsal and medullary dorsal horn has been studied in detail. The most striking ultrastructural feature of the dorsal horn is the presence of synaptic terminals, which are complex synaptic structures in which a "central" (core) axonal bouton is surrounded by several dendrites and axonal boutons (surrounding boutons). The axonal bouton nucleus (C) is of primary sensory origin, as shown by studies showing its degeneration after multiple dorsal root transections or labeling following tracer injections. Bouton C interacts with the dendrites of spinal cord interneurons or projection neurons. Some of these dendrites contain synaptic vesicles (presynaptic dendrites) and are presynaptic to the C bouton and/or to other dendrites. Synaptic terminals are thought to play an important role in sensory mechanisms as they constitute a significant portion of the synaptic population of the superficial dorsal horn and exhibit complex synaptic mechanisms [7].

There is no consistent definition in the literature of what should be considered a synaptic terminal. However, correct identification is important because the endings can be excellent markers for identifying sensory fiber endings at the ultrastructural level. Fiber terminals derived from the brainstem or neurons native to the spinal cord (defined by antigenic markers such as serotonin and GABA) are sometimes the main element of synaptic mechanisms that are simpler than synaptic terminals. In an isolated electron micrograph, a complex synaptic device can be classified as a synaptic terminal if it meets all of the following criteria: (a) it must have a C bouton containing agranular round synaptic vesicles, (b) the C bouton must be in apposition to at least four " surrounding" dendritic profiles (one or more may be replaced by axonal boutons and presynaptic dendrites) and (c) two or more synaptic specializations should be found between C and the surrounding profiles [3, 7]. Types of surrounding profiles: (1) dendrites lacking synaptic vesicles (“simple” or “general” dendrites – D), (2) vesicle-containing or presynaptic dendrites (V1), and (3) surrounding axonal boutons (V2). In the rat (but not in the cat or monkey) lamina I has very few synaptic endings. The endings become abundant only in lamina IIB, especially in sublamina IIBd. Endings are quite common in lamina III.

Types of synaptic terminals

Two main types of synaptic terminals have been described in rats. Type I terminals have a relatively small Bouton C scalloped outline with tightly packed synaptic vesicles and very few mitochondria. Two varieties can be described. Type Ia (or type I "non-peptidergic") endings have a particularly electron-dense C-bout, with vesicles having a very wide range of diameters and having on average one V1 terminal and one V2 terminal per ending. Type Ib (or "peptidergic" type I) terminals contain more than three dense-core vesicles in the C bouton, are immunoreactive for sensory peptides, and have a simplified synaptic architecture (virtually all surrounding profiles are dendrites postsynaptic to the central bouton) [3,8]. Type II endings have a larger C bouton, a less jagged outline, that is lighter and richer in mitochondria than their Type I counterparts. Moreover, Type II endings are richer in surrounding axonal boutons (V2) than Type I endings. Two varieties of type I endings can be distinguished II: type IIa (without neurofilament bundles in bouton C) and type IIb (with neurofilament bundles in bouton C). Type IIb endings are especially rich in V2 boutons.

Functional role of synaptic terminals

The functional role of the endings is far from known. Most varicose lesions of primary sensory fibers are not associated with endings. However, the available evidence strongly suggests that the endings are “multiplier systems”, i.e. devices through which primary sensory information is transmitted to several dorsal horn neurons via a single axonal bouton. In turn, synaptic terminals are important integrators, often being postsynaptic to other neuronal profiles. Thus, synaptic terminals are very important elements of sensory transmission. Most likely, type I (CI) C terminal boutons represent unmyelinated nociceptive fibers because they correspond to the terminals of capsaicin-sensitive fibers [7,9]. However, capsaicin also damages smaller Aδ fibers; therefore, some CI boutons may represent termination of Aδ fibers. It is tempting to argue that all type I C boutons represent nociceptive sensory fiber endings. If this is the case, then type I endings are of primary importance for the transmission of pain-related information. Most small-diameter peptidergic primary afferents are not located in synaptic terminals. However, about 20% of type I endings are of the peptidergic type. These peptidergic (or type Ib) terminals most likely represent only multiplier systems, since their peptide-containing core boutons share an important characteristic with the terminals of the same population of fibers: the fact that they are almost never postsynaptic to other neuronal profiles. This is in complete contrast to the arrangement of non-peptidergic type I (type Ia) terminals, in which both presynaptic dendrites and peripheral axons are presynaptic to the main bouton and are therefore likely also very important integrating devices [1,10]. These non-peptidergic terminals correspond to fibers that express the P2X3 receptor and bind the IB4 lectin. Regarding type II endings, we can extrapolate from ultrastructural studies of physiologically characterized fibers in cats that show ending morphology very similar to the C-bouton morphology of type II (CII) endings. Therefore, the C boutons of type IIa endings likely represent the endings of Aδ D hair fibers, and the type IIb boutons (with neurofilaments) are the endings of thicker fibers. In rats, both varieties of type II terminals display a fairly complex synaptic arrangement, including both presynaptic dendrites (V1) and surrounding axonal boutons (V2 in type IIa or V2 in type IIb).

Electron microscopic properties of lamina II neurons

Stem and islet cells were studied at the ultrastructural level by Todd using the Golgi method. As previously shown in cats, stem cells do not give rise to presynaptic dendrites, unlike islet cells. Both cell types participated in synaptic terminals through their dendritic processes [7].

Neurochemistry Of the Dorsal Horn

Neurokinins

The three major mammalian neurokinins are substance P, neurokinin A, and neurokinin B. All are found in the superficial laminae of the dorsal horn.

Substance P

Currently, there is undoubted evidence of the participation of neurokinin substance P in the processing of sensory information in the region of the first sensory synapse [7,11]. Immunocytochemically, substance P has been shown to occur in either unmyelinated or thinly myelinated sensory fibers, which terminate mainly in laminae I and II. Substance P immunoreactivity is especially pronounced in lamina I and outer lamina II, but is significantly reduced in inner lamina II. In lamina III, SP immunoreactivity decreases even more and represents predominantly crossing fibers toward the deeper lamina. Laminae IV–V contain clusters of substance P-immunoreactive (IR) fibers and boutons separated by areas of sparse immunoreactivity. It should be clearly stated that, contrary to popular belief, not all substance P immunoreactivity in the superficial dorsal horn is of primary sensory origin, since multiple dorsal rhizotomies and capsaicin treatment were not able to completely deplete substance P immunoreactivity. In addition, cell bodies containing substance P were identified in spinal laminae I and II by both immunocytochemistry and in situ hybridization. Additionally, although most substance P-containing systems descending from the brainstem terminate in the ventral horn, some may terminate in the superficial laminae of the dorsal horn. It is interesting to note that most, if not all, substance P-IR cell bodies in the dorsal horn of the spinal cord colocalize enkephalin immunoreactivity.

At the ultrastructural level, substance P immunoreactivity in the central boutons of synaptic terminals is particularly significant, since such profiles have a known sensory origin [12,13]. Substance P immunoreactivity has also been detected in C terminal boutons in several animal species, including rats. In the rat, substance P immunoreactivity was detected in 10% of the C boutons of the synaptic endings of lamina II. All of these synaptic terminal boutons had large vesicles with a dense core (characteristic of type Ib terminals).

Other neurokinins

Virtually all substance P neurons in rats express precursors that produce both substance P and neurokinin A, which means their distribution is essentially the same. However, neurokinin B is derived from a different precursor and, unlike substance P, is not found in primary sensory neurons [1,7]. A recent light and electron microscopic immunocytochemical study of neurokinin B in the dorsal horn of the spinal cord showed that in the superficial lamina its signal is found in the axon terminals of lamina I–II, with a peak in lamina IIB, and in cell bodies and dendrites predominantly in lamina IIB. Plate III showed much less immunolabeling. Thus, in contrast to substance P, immunoreactivity for neurokinin B increased from lamina I to lamina IIB. Interestingly, neurokinin B immunoreactivity was observed in dendrites of type I terminals, suggesting its involvement in the modulation of nociception.

Neurokinin receptors

Initial descriptions of substance P receptor (neurokinin-1 receptor) immunoreactivity in the dorsal horn reported that it was present in neurons with cell bodies located in lamina I and in deeper layers (laminas III–IV). According to these reports, the substantia gelatinous itself did not have cell bodies immunoreactive for the neurokinin-1 receptor (NK-1r) and was much less immunoreactive than lamina I because it was limited to cell processes mainly from neurons in more deep plates [1,7]. However, more recent studies have reported NK-1r immunoreactivity in cell bodies in both lamina I and outer lamina II (LIIA). It should be noted that most of these NK-1r-IR neurons project to higher levels: the thalamus, parabrachial nucleus, lateral reticular nucleus, dorsal part of the caudal medulla and, to a minor extent, the periaqueductal substance. Unlike NK-1r, the neurokinin A receptor (neurokinin receptor-2) is scarcely found in the CNS, indicating that neurokinin A acts either through another receptor in the CNS or acts primarily in the periphery [14,15]. In the superficial lamina of the dorsal horn, some neurokinin-2 receptor immunoreactivity was detected in a narrow band in the lateral portion of lamina I but appeared to be localized to glial cells. As for the preferred neurokinin B receptor, neurokinin receptor-3, it is found in cell bodies located in lamina I and mainly in lamina II of the spinal cord.

Calcitonin gene-related peptide (CGRP)

CGRP immunoreactivity has been shown to arise in dorsal root ganglia and primary sensory fibers that project primarily to the superficial laminae of the spinal cord. In the dorsal horn, CGRP-IR boutons occur primarily in laminas I, IIA, and IIBd, as well as in portions of lamina V. One interesting feature of CGRP immunoreactivity in sensory systems is its colocalization with substance P. In fact, substance P immunoreactivity almost always colocalizes with CGRP in dorsal root ganglion cells, although CGRP immunoreactivity occurs in a significantly higher percentage of these cells than substance P [3,16]. Another interesting feature of CGRP immunoreactivity in the dorsal horn is its almost complete disappearance after dorsal rhizotomy. This finding suggests that all CGRP immunoreactivity in the dorsal horn is derived from primary sensory fibers, a finding supported by in situ hybridization studies that do not identify any dorsal horn neurons synthesizing the peptide [17]. Therefore, it seems legitimate to use the colocalization of CGRP and substance P in the same terminal as a marker of primary sensory origin. At the ultrastructural level, CGRP occurs primarily in nonglomerular varicosities of the dorsal horn of the rat spinal cord, although some varicosities are of the glomerular type. Most CGRP immunoreactivity in the dorsal horn colocalizes with either substance P or somatostatin immunoreactivity.

The distribution of CGRP receptors in the spinal cord has been studied using ligand binding approaches. They occur at high densities in lamina I and in the deeper laminae, but occur at low densities in lamina II. However, after peripheral denervation, significant CGRP binding was detected in lamina II, indicating that neurons have the ability to produce the receptor.

Somatostatin

Somatostatin-like immunoreactivity occurs both in primary sensory fibers and in neurons of the spinal cord. In the superficial lamina, somatostatin-IR neurons are found predominantly in lamina II [2]. Somatostatin receptors form a family of five receptors (sst1 to sst5), all of which belong to the G protein-coupled receptor superfamily. Immunoreactivity for receptor subtypes was detected in cell bodies and processes of the superficial laminae of the dorsal horn.

Opioid peptides

Enkephalin

Since their discovery, endogenous opioid peptides have been considered important candidates for presynaptic interactions in the dorsal horn of the spinal cord. The opioid peptides met- and leu-enkephalin occur in high concentrations in laminae I and II of the spinal cord and have been found in nerve cell bodies in laminae I–III. Double labeling studies combining radioimmunocytochemistry and DAB-based immunocytochemistry have

shown that sometimes enkephalin and substance P-IR varices form separate synapses on a common dendrite and that glomerular boutons of substance P-IR C are presynaptic to enkephalin-IR dendrites in rats [1,7]. Enkephalin-IR boutons were never presynaptic to substance P-IR boutons. These results indicate (together with the data indicated below) that substance P containing glomerular C boutons excites the dendrites of enkephalinergic interneurons of the substantia gelatinosa and that the axons of such neurons inhibit the dendrites of neurons excited by substance P. Discovery of the colocalization of substance P and enkephalin in a significant number of neurons and axonal varicosities in both rats and cats added a new dimension to the problem. In fact, almost all substance P-IR neurons in the rat dorsal horn colocalize enkephalin, and approximately 50% of enkephalin-IR cells colocalize substance P. It seems likely that the majority of enkephalin immunoreactivity comes from neurons intrinsic to the dorsal horn. Enkephalin is localized in serotonergic neurons of the raphe nuclei extending into the spinal cord, but most of these fibers terminate in the anterior horn. In addition, some enkephalin immunoreactivity may originate from primary sensory fibers [3]. However, enkephalin has never been detected in a significant number of dorsal root ganglion neurons. Based on the above, it is clear that colocalization with enkephalin can be used as a marker of substance P immunoreactivity in nerve endings of the dorsal horn of origin. In cats, enkephalin-IR boutons have been shown to form synapses on spinothalamic neurons and on dorsal column postsynaptic pathway neurons.

Dynorphins

Dynorphin immunoreactivity was detected in neurons of lamina I and II.

Endormorphins

Of the two endomorphins, endomorphin-2 is most abundant in the superficial laminae of the dorsal horn, where it occurs in laminae I and IIA with a distribution similar to that of substance P, with which it colocalizes in sensory fibers. In contrast, endomorphin-1 is inherent in the central nervous system and is found in fibers of lamina I and II.

Opioid receptors

Because dorsal rhizotomy results in decreased binding of both μ- and δ-opioid receptors, it has been proposed that such receptors must be, at least in part, localized to primary sensory fibers. In situ hybridization cytochemistry confirmed the presence of opioid receptors in dorsal root ganglion neurons and dorsal horn neurons. Combined light and electron microscopy studies using antibodies against the δ-opioid receptor have shown conflicting results [1,3]. One group argues that receptors occur both on the cell bodies and dendrites of dorsal horn neurons and in axon terminals. Surprisingly, another group found that the δ-opioid receptor associates primarily with dense core vesicles on sensory fibers rather than with the plasma membrane as expected for a G protein-coupled receptor. The receptor has been shown to colocalize in terminals with enkephalin and in sensory fibers with substance P. In contrast, the κ receptor is localized predominantly postsynaptically, whereas μ receptor immunoreactivity occurs predominantly in lamina II, in axon terminals, in dendritic profiles, and in cell bodies dorsal horn neuron cells. Interestingly, the vast majority of μ-opioid receptor-immunoreactive cell bodies, which were primarily located in lamina II, contained neither GABA nor glycine immunoreactivity, suggesting that μ-receptor-expressing neurons may be in mainly excitatory interneurons.

Glutamate

Immunocytochemical methods have shown that glutamate is localized in almost all sensory fibers and that at the ultrastructural level it is found in almost all central glomerular varicosities, which confirms the hypothesis that glutamate is a rapid mediator of excitation of primary sensory fibers. Glutamate and substance P have been shown to colocalize in a significant number of dorsal root ganglion cells and dorsal horn terminals. Aspartate colocalizes with glutamate in some of these sensory fibers, especially in the small diameter fibers. Glutamate receptors have been studied in the dorsal horn using receptor binding and in situ hybridization, as well as immunocytochemistry. Recent studies have shown that AMPA receptors are widely distributed in dorsal root ganglion cells and the dorsal horn [1]. One study showed that Ca2+-permeable AMPA receptors are localized predominantly in GABAergic inhibitory interneurons and NK-1r-IR neurons. Interestingly, light and electron microscopy have shown that NMDA receptors are also found in sensory fibers, and this localization has also been described for metabotropic glutamate receptors.

Inhibitory amino acids

GABA and glycine

Some neurons have been shown to be immunoreactive for GABA or GAD or to take up [3H]GABA and are considered GABAergic, while others specifically incorporate [3H]glycine or are immunoreactive to anti-glycine antibodies and are considered glycinergic. When immunocytochemistry was combined with Wallerian degeneration in rats or intracellular filling of identified sensory fibers in the cat, GABAergic neurons were shown to be presynaptic to primary sensory boutons [18]. Evidence indicates that GABA, like enkephalin, is present primarily in local circuit neurons, and colocalization of both neurochemicals has been demonstrated at the light microscopic level in the superficial dorsal horn. [18,19]. We confirmed this finding at the ultrastructural level. Moreover, it has been suggested that virtually all glycinergic neurons in lamina I–III are also GABAergic. However, only about half of GABAergic cells colocalize glycine. Recent evidence also indicates storage of GABA and glycine in the same vesicles at superficial dorsal horn synapses. Glycinergic varicosities, like GABAergic ones, can be presynaptic to the primary sensory fibers of the terminals.

GABA and glycine receptors

$GABA_A$ receptors have been characterized by in situ hybridization techniques in both dorsal root ganglion and spinal cord cells, supporting morphological evidence that GABA-IR fibers are often presynaptic to sensory fibers. Using immunocytochemistry, it was shown that $GABA_A$ receptor subunits are found in laminae I–III. An ultrastructural study with an antibody raised against the β2–β3 subunits of the $GABA_A$ receptor showed that most of the immunostaining was localized to dendrites and cell bodies, although some central terminal elements were also labeled, suggesting that the receptor also occurs in primary sensory neurons. The distribution of $GABA_B$ receptor immunoreactivity by light microscopy was recently described in the spinal cord and was highest in lamina I and II, where it was observed in both cell bodies and neuropil. Unlike GABA receptors, glycine receptors are restricted to dorsal horn neurons. [19]. The specificity of mixed GABA/glycine synapses in the superficial dorsal horn appears to be determined by the expression, properties, and subsynaptic localization of target $GABA_A$, $GABA_B$, and glycine receptors and changes during development.

Other classical transmitters and other neuropeptides

Cell bodies immunoreactive for choline acetyltransferase (ChAT) have also been described in this region of the central nervous system (CNS).

Such cholinergic neurons are found predominantly in laminae III–IV and are located presynaptically to the primary sensory fibers of synaptic terminals and to dorsal horn cells. The study showed that most ChAT-IR neuronal cell bodies and boutons colocalize GABA immunoreactivity. Serotonin comes from cell bodies located in the brain stem. In the cat, retrograde tracing showed that serotonin-IR profiles have direct contact with projection neurons [20]. Despite two ultrastructural studies, very little is known about the synaptic contacts of noradrenergic fibers in the dorsal horn, except that they are presynaptic to dorsal horn neurons. However, the light microscopic distribution of noradrenergic fibers in the dorsal horn and their origin in the brainstem are well known. Neurotensin immunoreactivity occurs in neurons of lamina I and II.

Markers of non-peptidergic primary sensory fibers

Since Hunt and Rossi's seminal paper in 1985, the concept of two populations of sensory fibers conveying nociceptive information has emerged: peptidergic and non-peptidergic. The former express sensory neuropeptides (in particular substance P), while the latter exhibit fluoride-resistant acid phosphatase (FRAP) activity. This concept was largely ignored for a decade, with researchers focusing primarily on fiber terminals expressing sensory neuropeptides, particularly substance P [3]. However, in recent years there has been a resurgence of interest in this concept. It was found that a population expressing FRAP activity, originally described several years earlier by two independent groups, could specifically bind isolectin IB4 and be recognized by the monoclonal antibody LA4. However, real interest in the non-peptidergic population arose with the discovery that the two populations differ in their neurotrophic support in adults. In fact, during development, both populations require nerve growth factor (NGF) for survival, but soon after birth only the peptidergic type continues to respond to NGF, while the non-peptidergic population instead begins to respond to glial cell lineage-derived neurotrophic factor (GDNF). Accordingly, the peptidergic population expresses the high-affinity trkA NGF receptor, whereas the non-peptidergic population expresses GDNF receptors. The latter population has also been shown to express the purinergic receptor $P2X_3$ and the capsaicin receptor VR1. Although the distinction between two populations of primary sensory fibers, peptidergic and non-peptidergic, appears attractive, it is not entirely precise, since the small proportion of sensory fibers that colocalize CGRP and somatostatin are unresponsive to NGF in adults and bind the IB4 lectin. It should also be noted that in all of the above putative nociceptive fibers, the "classical" synaptic transmitter is most likely glutamate or both glutamate and aspartate [1,4]. Of the markers of non-peptidergic nociceptive sensory fibers described above, FRAP is the most studied. At the light microscope level, FRAP activity is localized to a band in the middle third of lamina II, corresponding to sublamina IIBd. At the ultrastructural level, FRAP occurs in C-boutons of type I but not type II synaptic terminals. The physiological role of this enzymatic activity is still unknown, but it is useful as a marker of small diameter sensory fibers. Most recent studies have used IB4 binding as a marker of the nonpeptidergic nociceptive fiber population.

Neurochemistry of synaptic terminals

Boutons C synaptic terminals are probably all immunoreactive for glutamate and possibly for aspartate. Of the neuropeptides in C boutons, CGRP is the most abundant, as it is found in virtually all type Ib C boutons (i.e., dense core vesicles). Substance P immunoreactivity occurs in a subpopulation of type I CGRP-IR C terminal boutons. Somatostatin also occurs in C-bouton terminals, where it colocalizes with CGRP. Regarding the surrounding glomerular profiles, several neurochemicals (D) were found in the "correct" glomerular dendrites: substance P, neurokinin B, enkephalin, somatostatin, GABA, glycine and ChAT. Presynaptic dendrites (V1) contain, among others, the following antigenic sites: somatostatin, enkephalin, GABA and glycine. In the peripheral axons of the glomeruli (V2) the following were found: GABA, glycine, ChAT and enkephalin. Certain colocalizations were found in the surrounding terminal profiles: GABA+CHAT in the V2 profiles and dendrites (D); GABA + enkephalin in profiles V1, D and V2; and enkephalin + substance P in profiles D. GABA + glycine colocalization was demonstrated in cell bodies at the light microscopic level [1,2]. Todd subsequently provided evidence for GABA+glycine colocalization in V2 profiles and some V1 profiles in type II terminals, but not in type I. The occurrence of GABA+glycine colocalization in presynaptic dendrites (V1 profiles) of type II terminals is not surprising, as Spike and Todd found such colocalization in islet cells. However, it should be noted that GABA, not glycine receptors, are found on primary sensory fibers. Therefore, it is likely that only GABA acts on C boutons, whereas glycine targets other glomerular profiles.

Concluding Remarks

In conclusion, since Melzack and Wall's influential theoretical paper introducing the spinal gate control theory, a variety of complex synaptic mechanisms have been postulated in the rat superficial dorsal horn, consistent with or inconsistent with their main hypothesis. Despite recent progress, the fact remains that direct evidence integrating the circuitry of the dorsal horn, the physiological characteristics of the neurons, and the chemical nature and type of synapses involved is still lacking. In rats, the outer two-thirds of lamina II appears to play an important role in modulating nociception. However, details of the modulatory mechanisms and the neurochemicals involved are still not well known.

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