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ABSTRACT
The substantia gelatinosa Rolandi (SGR) was first described about two centuries ago. In the following decades an enormous amount of information has permitted us to understand - at least in part - its role in the initial processing of pain and itch.
Here, I will first provide a comprehensive picture of the histology, physiology, and neurochemistry the normal SGR. Then, I will analytically discuss the SGR circuits that have been directly demonstrated or deductively envisaged in the course of the intensive research on this area of the spinal cord, with particular emphasis on the pathways connecting the primary afferent fibers and the intrinsic neurons. The perspective existence of neurochemically-defined sets of primary afferent fibers giving rise to these circuits will be also discussed, with the proposition that a cross-talk between different subsets of peptidergic fibers may be the structural and functional substrate of additional gating mechanisms in SGR. Finally, I highlight the role played by slow acting high molecular weight modulators in these gating mechanisms.

Keywords: substantia gelatinosa, lamina II, gate theory, pain, nociception, primary afferent fibers, dorsal root ganglia, spinal cord, sensory system, neurotransmitters, neuromodulators, anatomy, histology, physiology, neurochemistry

Word count in manuscript excluding boxes, references and figure legends: 30,260

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Contents

2.4.2.1.2. Dynorphins
2.4.2.1.3. Opioid receptors
2.4.2.2. Tachykinins and tachykinin receptors
2.4.2.2.1. Tachykinins
2.4.2.2.1.1. Substance P
2.4.2.2.1.2. Other tachykinins
2.4.2.2.2. Tachykinin receptors
2.4.2.3. Somatostatin and somatostatin receptors
2.4.2.3.1. Somatostatin
2.4.2.3.2. Somatostatin receptors
2.4.2.4. Calcitonin gene-related peptide (CGRP) and CGRP receptor
2.4.2.5. Galanin and galanin receptors
2.4.2.6. Neurotensin and neurotensin receptors
2.4.2.7. Neuropeptide Y (NPY) and NPY receptors
2.4.2.8. Gastrin-releasing peptide (GRP) and GRP receptor (GRPR)
2.4.3. Neurotrophic factors and receptors
2.4.3.1. Neurotrophins and neurotrophin receptors
2.4.3.1.1. NGF and tropomyosin receptor kinase A (trkA) receptor
2.4.3.1.2. BDNF and tropomyosin receptor kinase B (trkB) receptor
2.4.3.1.3. NT3, NT4 and tropomyosin receptor kinase C (trkC) trkC receptor
2.4.3.2. GDNF and GDNF receptors
2.4.4. Transient receptor potential (TRP) channels
2.4.4.1. TRPV1
2.4.4.2. TRPM8
2.4.4.3. TRPA1
2.4.5. Others
2.4.5.1. Calcium-binding proteins
2.4.5.1.1. Calbindin
2.4.5.1.2. Calretinin
2.4.5.1.3. Parvalbumin
2.4.5.2. Protein kinase C (PKC)
2.4.5.3. NO and NO synthesizing enzymes
2.4.5.4. Transcription factors

2.5. Glia

3. Neuronal circuitries in the superficial dorsal horn

3.1. Input to SGR

3.1.1. Input from PAFs
3.1.1.1. Lamina II_o and II_id
3.1.1.1.1. pPAFs expressing SP+BDNF: the excitatory channel
3.1.1.1.2. pPAFs expressing SST+GDNF: the inhibitory channel
3.1.1.2. Lamina II_iv
3.1.2. Input from other laminae
3.1.3. Input from descending fibers

3.2. Output from SGR

3.3. Intralaminar circuits

4. The gate theory and beyond

1. Introduction and general concepts

A thin, gelatinous, semitransparent layer of the gray matter named the substantia gelatinosa of Rolando (SGR) or, less frequently, the gelatinous substance of the posterior horn of the spinal cord caps the apex of the posterior (dorsal)[1] horn and may be visible in fresh transverse sections of the spinal cord. The SGR contains small neurons and glia, an abundant neuropil, but very few myelinated fibers. This scarcity of myelinated fibers, as it became apparent with the development of ultrastructural analysis, is traditionally considered to be the main reason for its jelly-like appearance. The SGR was named after the Italian anatomist Luigi Rolando (Turin, 1773 – 1831) who was the first to describe it (Box 1). In Rexed's laminar subdivision of the spinal gray matter, the SGR corresponds to lamina II, as Rexed himself claimed. However, it was controversial, in the past, whether or not the adjacent part of lamina III also belonged to SGR (Ralston, III, 1965; Réthelyi, 1977; Réthelyi and Szentàgothai, 1973; Scheibel and Scheibel, 1968; Sterling and Kuypers, 1967; Szentàgothai, 1964; Wall *et al.*, 1979)[2].

Although different in shape and extension, lamina II can be detected at all spinal levels and forms most of the dorsal part of the head of the dorsal horn (DH), as the more superficial lamina I is only a very thin layer of gray matter. In the dorso-lateral part of the head of the DH, the SGR directly contacts the white matter at the level of the Lissauer's tract (or dorso-lateral fasciculus of spinal cord). The SGR receives a direct input from the primary afferent fibers (PAFs) originating from the primary sensory neurons of the dorsal root ganglia (DRGs) and reaching the spinal cord through the dorsal roots of the spinal nerves. A substantial fraction of these fibers derive from nociceptors and thermoreceptors, but also low threshold mechanoreceptors (LTMs) reach the SGR. The SGR neurons, for the most, form a fine network inside lamina II. Their short axons make synapses with each other or with neurons in other laminae of the gray matter, including the contralateral lamina II. Notably, rather than directly contributing projection fibers to the ascending anterolateral system or the spinothalamic tracts (STTs) that convey nociceptive information to higher centers, the SGR controls nociception at the interface between first order DRG neurons and second order projection neurons in laminae I and III-V. The pivotal role of SGR in the control of pain was strongly sustained since the publication of the Gate Theory of Pain (Melzack and Wall, 1965), with a significant boost to basic and applied research in the field. More recently, the SGR has also been implicated in the integration of pruritic stimuli.

2. The microscopic anatomy, histology and physiology of the SGR

2.1. Architecture

The most relevant cytoarchitectonic features of lamina II are its high neuronal density, the small size of its neurons, and the paucity of myelinated fibers. In several mammals, including humans (Schoenen, 1982), monkey (Light and Perl, 1979a), cat (Light and Perl, 1979a; Rexed, 1952; Rexed, 1954), rat (Light and Perl, 1979a; Lorenzo *et al.*, 2008; Pan and Pan, 2004) and mouse (Woodbury *et al.*, 2000), the SGR can be further subdivided into two zones (sublaminae), an outer and inner lamina II (lamina II_o and II_i)[3]. At least in rodents (Fig. 2), lamina

[1] In this paper, according to proper anatomical nomenclature, I will use the term posterior only when referring specifically to humans; otherwise, in animals or when not specifically referring to man, I will employ the adjective dorsal.

[2] At beginning of the eighties of the last century a consensus was reached under the auspices of the *Somatosensory Commission of the International Union of Physiological Sciences* that the SGR and lamina II are the same, and quite distinct from other parts of the spinal gray matter.

[3] The subdivision of lamina II into an outer and an inner part originally proposed by Rexed (1952) was an interpretation based on Cajal's description of the limiting cells in the dorsal part of the gelatinosa. Actually, Rexed mentioned that the cells in the outer zone were smaller and most densely packaged than in the inner zone.

II_i further consists of a dorsal (II_id) and a ventral (II_iv) part (Ribeiro-Da-Silva and De Koninck, 2008). The type(s) of interneurons populating these zones, their relationship with PAF endings, and neurochemistry are at the basis of this subdivision.

Besides the neurons and glia, the SGR contains a synaptically dense region (neuropil) that receives input signals from several sources: the aforementioned PAFs from the DRG neurons; the descending fibers from several areas of the brain; and other spinal cord neurons. SGR neurons, in turn, send output signals primarily to neurons in laminae I, III and IV (Bennett *et al.*, 1980; Gobel, 1975, 1978; Light and Kavookjian, 1988), but, at least in rat and monkey, also to the brainstem or the thalamus (Giesler, Jr. *et al.*, 1978; Willis *et al.*, 1978). Inside the neuropil there are also the dendritic arborizations of the antenna neurons (Schoenen, 1982). These are a peculiar type of neurons with cell bodies in laminae III-IV and dendrites that ascend dorsally into lamina II (and lamina I) where they receive input from other neuronal processes in the SGR neuropil. At least some of these neurons have axons that ascend to the brain along the STT (Surmeier *et al.*, 1988) or reaching the dorsal columns (Brown and Fyffe, 1981) - see also Boxes 2 and 3.

2.2. *SGR neurons*

Despite the huge amount of work devoted to understanding the biology of SGR neurons we are very far from defining functionally distinct classes of these cells based on precise sets of anatomical and physiological parameters, as it could be done e.g. for the hippocampal dentate gyrus interneurons (Hosp *et al.*, 2014).

There are several reasons that explain our current inability to do this. First, lamina II neurons may be morphologically very similar but they are heterogeneous in size, physiological properties and function. Second, many of them do not fit into any of the categories used by different authors for classification within and across species. The partial inadequacy of current classifications derives from the fact that they were based (directly or indirectly) on the original attempts to classify lamina II neurons in single section Golgi-stained preparations. However, these preparations provided incomplete pictures of dendrite spreading in comparison to intracellular filling techniques. Third, it appears that dendritic projections outside the SGR are related to the type(s) of PAF input that excites the cells. Fourth, we still miss too much information on the synaptic circuitry of SGR neurons (see 3. Neuronal circuitries in SGR). Therefore, we still cannot draw a fully coherent picture accurately describing their synapses and correlating this type of ultrastructural information with cell function (Todd, 2017).

In the following sections, I analytically review the histological, neurochemical and physiological properties of the SGR neurons. Readers who are familiar with these issues find a summary of these data in Box 5.

2.2.1. Morphology and classification

2.2.1.1. First studies on the morphology of SGR neurons

The morphology of the SGR neurons has been extensively investigated. These neurons have an array of shapes that follows a continuum rather than discrete categories, and their sorting is so complex to the point that numerous authors believe it impossible to frame them into a coherent classification. In addition, the axons of SGR neurons display some unique features that, in some cases, may be intermediate between those of the classical projection neurons with long axons, or the local interneurons whose axons are short and confined to the immediate surroundings of the cell body (Willis, Jr. and Coggeshall, 2004b). A further complication depends on interspecies differences.

Technical advances in single neuron staining, from the Golgi impregnation to horseradish peroxidase (HRP), biotin or Lucifer yellow (LY) filling - usually after electrophysiological characterization, or genetic engineering with fluorescent reporter proteins (FRPs) - have

significantly improved our current view on the functional morphology of SGR neurons (Todd, 2017). Knowledge of technical evolution is important to consider under the right perspective any of today's classification of these neurons. With the Golgi stain or its modifications, Cajal described several different kinds of neurons in the SGR: in his studies, the various shapes and sizes of axonal and/or dendritic arborizations were the main features used to sort silver-impregnated cells. Indeed, Cajal was the first to propose a histological classification of the SGR neurons in two main types, the *central cells* and the *limiting cells* (to which he also referred to as limitrophe or border cells). Such a classification derived primarily from observations in newborn dogs and cats (Cajal, 1909). In cat, these cells (or at least subpopulations of them – see below 2.2.1.1.1 Islet cells) were subsequently called *islet cells* and *stalked cells*, respectively (Gobel, 1975, 1978), and thence described in various papers combining electrophysiological intracellular recordings and HRP filling (Bennett *et al.*, 1980; Gobel *et al.*, 1980; Light *et al.*, 1979). Later, still using the Golgi impregnation, islet and stalked cells were identified also in humans (Schoenen, 1982 - but see below 2.2.1.2. Classification of primate SGR neurons) and rat (Todd and Lewis, 1986).

Gobel's classification of SGR neurons was based on the distribution of their processes in the Rexed's laminae of the gray matter; dendritic and axonal branching pattern and shape; and presence/distribution of dendritic spines. In his descriptions, he showed that the islet and stalked cells were typically multipolar, had highly ramified axonal and dendritic trees, with many thin dendritic shafts departing with different orientations form the perikaryon (Gobel, 1978). Gobel also described three other less represented populations of neurons in SGR: the spiny cells, the arboreal cells and the lamina II/III border cells, each with morphological features different from the islet and the stalked cells (Gobel, 1975, 1978). According to his descriptions, the islet cells projected to lamina II; the stalked cells to lamina I; the arboreal cells to laminae I and II; the border cells to laminae II_o and II_i, and the spiny cells to laminae I-III (Falls and Gobel, 1979; Gobel, 1979; Gobel and Falls, 1979).

Before the introduction of Gobel's classification, Cajal's central cells were further subdivided into *funicular cells* and *short-axoned cells*[4]. In studies with Golgi impregnation methods, the funicular cells corresponded to propriospinal projection neurons that connected adjacent spinal segments. In rat and cat, the axons of these neurons entered the white matter before reentering the SGR (Bicknell and Beal, 1984; Maiskii *et al.*, 1983; Szentàgothai, 1964). Short-axoned cells were, instead, Golgi type II cells with axons totally confined to the gray matter of lamina II.

Today, Gobel's classification is still the basis of the more widely accepted terminology for identifying the principal (*i.e.* mostly represented) neurons in the SGR: the islet and the stalked cells. From these initial observations, the differences in the morphologies of the islet and the stalked cells (and of the other less commonly represented neurons) suggested that *different categories of neurons had different functions* in the (local) neural circuitry of the SGR. Another line of thought claimed that the *sublaminar localization* of the SGR neurons was, instead, the main determinant to discriminate function, in relation to the pattern of termination of PAFs

[4] This type of subdivision refers to a general classification of the spinal cord neurons where three main categories were distinguished: the motor neurons, the funicular (projection - Golgi type I) neurons and the interneurons (short-axoned neurons - Golgi type II), but see (Willis, Jr. and Coggeshall, 2004b) for a discussion on spinal cord "interneurons". However, this subdivision is somewhat confusing. Funicular neurons, in the more general description of the spinal cord anatomy, are neurons whose axons give rise to the tracts of the white matter. Of these, the *propriospinal projection neurons* have axons that re-enter the gray matter travelling along the *fasciculi propri* at the gray-white matter interface. "True" projection neurons, instead, have axons that form the ascending tracts (funiculi) of the white matter and indeed reach the brain.

(Woolf and Fitzgerald, 1983). More recent studies confirmed that sublaminar localization is an important parameter to be taken into consideration to understand the wiring of local and interlaminar circuitries in the SGR (Kato *et al.*, 2007, 2009). These studies employed a very sophisticated technique of glutamate uncaging by laser scanning photostimulation, but morphology still remains important to recognize at least some of the principal cell types in lamina II.

2.2.1.1.1. Islet cells

Islet cells are intralaminar neurons, i.e. their entire axonal and dendritic projections remain within the SGR. In Golgi impregnation studies, islet cells, which were frequently encountered in small groups (islets = small islands), were the prevalent cells in SGR and had a very small round or fusiform cell body (about 7-14 µm diameter in rodents) with not much cytoplasm. Gobel's islet cells, for the most, corresponded to the central cells in Cajal's descriptions[5]. The name "central cell" was confounding because these neurons were sparse throughout the SGR, although their cell bodies preferentially laid in lamina II_i often at the interface with II_o. The dendritic tree displayed very occasional spines (Brown, 1981; Brown *et al.*, 1981), and had a parasagittal orientation (medio-laterally flattened and longitudinally oriented). It considerably spanned rostro-caudally, but very little in the dorso-ventral direction (Cajal, 1909; Pearson, 1952; Scheibel and Scheibel, 1968; Szentàgothai, 1964). Remarkably, although the dendritic tree of the islet cells was pictured as a thin sheet in single Golgi sections, serial reconstructions demonstrated a higher thickness in the medio-lateral (transverse) direction (Sugiura, 1975).

The axon was short and its branches became progressively thinner and displayed several boutons *en passant*. Important differences between the islet cells and all the other types of SGR neurons so far categorized are the confinement of the islet cell axon into the (sub)lamina in which lies the cell body (Gobel, 1979), and the fact that these neurons give rise to presynaptic dendrites (V1 profiles) at glomeruli (Gobel *et al.*, 1980) - see 2.2.4.5. Glomeruli.

2.2.1.1.2. Stalked cells

Stalked cells are interlaminar neurons corresponding to the limiting cells described by Cajal (1909) and to the vertical cells in more recent rodent classifications. The name derived from the short stalk-like appearance of the spines covering their dendrites. After Golgi impregnation, the stalked cells, differently from the islet cells, occurred individually in the SGR. Stalked cells were larger than islet cells and had a cell body located in lamina II_o in close proximity or at the border with lamina I. In HRP filled neurons, the dendritic tree of the stalked cells was cone- or fan-shaped (Bennett *et al.*, 1980; Gobel *et al.*, 1980) with a ventrally oriented base, and travelled deep into lamina II, often being confined to its outer subdivision, but sometimes also running into laminae II_i-IV. After Golgi impregnation, the axon ended in lamina I (Gobel, 1975, 1978), but ramifications in deeper laminae were also reported (Light and Kavookjian, 1988; Schoenen, 1982). Differently from islet cells, stalked cells do not give rise to presynaptic dendrites at glomeruli (Gobel *et al.*, 1980).

2.2.1.2. Classification of primate SGR neurons

In the first Golgi studies on monkey and humans, the morphology of lamina II neurons was principally referred to the islet and stalked cell categorization described in cat (Beal and Cooper, 1978). However, a few years later, four different types of Golgi-impregnated neurons were described in man (Schoenen, 1982). The *stellate cells*, preferentially located in lamina II_i represented the most abundant type (about 40%). Their dendrites were smooth, with only a

[5] A difference between the original description of the central cells by Cajal and the subsequent studies on these neurons is that Cajal reported that the axons of the majority of central cells passed to the white matter, an observation that was not confirmed in less dated investigations. Gobel (1979) has hypothesized that such a difference was related to the use of embryonic or young animals' tissue.

few spines, and formed an elliptical arbor that entered laminae I and III. After giving collaterals to lamina II, the axon travelled deeply into laminae III-IV. About 30% of impregnated neurons were *islet cells* according to Gobel's criteria. Approximately 20% of the stained cells, the *filamentous cells*, had multiple filiform (hence the name), spiny dendrites. Their dendritic tree was trapezoid, dorsally or ventrally oriented in relation to the position of the cell body in lamina II_i or II_o, respectively. The *curly cells* (about 10%) mainly populated lamina II_o had a complex, twisted, spine-rich discoid dendritic tree placed in a parasagittal plane. The axons of the filamentous cells and of the curly cells passed into lamina I or the Lissauer's tract.
In another classification, human SGR neurons were of four different types, named II_1-II_4, out of ten different categories of neurons in laminae I-III. Types II_3 and II_4 were short-axoned Golgi type II cells, whereas some II_3 cells could be short propriospinal neurons (Abdel-Maguid and Bowsher, 1984, 1985; Bowsher and Abdel-Maguid, 1984).
Because the above studies in human are old and from a time in which much less was known of the cell types in rodents, it is likely important to revisit the issue of the classification of lamina II neurons in primates.

2.2.1.3. Classification of rodent SGR neurons

2.2.1.3.1. Hamster

A classification in hamster was proposed that - after biocytin intracellular staining -correlated neuronal morphology and electrophysiological responses (Grudt and Perl, 2002). These authors divided the SGR neurons into five types: islet cells, central cells, vertical cells, medial-lateral cells and radial cells. This classification has been seminal for the subsequent categorization of the rodent SGR neurons.

2.2.1.3.2. Rat

Bicknell and Beal (1984) subdivided the rat propriospinal/projection neurons (with axons entering the white matter) into limiting cells (with a diameter of about 21 µm in postnatal rats), large (about 23 µm) or small (about 14 µm) central cells, and transverse cells. By contrast, the short-axoned cells (with axons confined to the spinal cord gray matter and size around 10-14 µm) were categorized into islet, stalked, inverted stalk-like, and vertical cells. The results of this study are somewhat difficult to fit into other classifications of the SGR neurons, as they took into consideration also the morphological modifications that these cells undergo from the last stage of gestation (E15) up to twenty days postnatally. Thus, e.g. transverse cells were not present in the SGR beyond E19. Nonetheless, these observations are important as they outline that intrinsic interneurons and propriospinal/projection neurons have different modes of development and change substantially their shape from the embryonic period to postnatal life. This needs consideration, as most work with Golgi impregnation or electrophysiology employed very young animals.
Later, after simultaneous whole-cell recordings from pairs of cells, Lu and Perl subdivided the SGR neurons according to their previous classification in hamster (Lu and Perl, 2003, 2005). The extensive dendritic arborization - four-hundred µm or more along the rostro-caudal axis of the cord - characterized the islet cells. Dendrites were, instead, shorter in the central cells, and the vertical cells had dendrites with ventrally oriented arbors. About twenty-five percent of SGR neurons, however, displayed very unusual shapes and could not be classified (Lu and Perl, 2005).
Similarly, islet, central, radial and vertical cells were described by others after blind whole-cell voltage-clamp, intracellular filling with Neurobiotin and according to several morphometric parameters, mainly focused onto dendritic geometry (Yasaka *et al.*, 2007). Another group (Maxwell *et al.*, 2007), using a similar approach, has recognized five morphological types of SGR neurons (islet, vertical, central, radial, and antenna cells). Very interestingly, Maxwell's

classification included the territory of axonal projection as an additional criterion for categorization, thereby coming to conclude that *all lamina II neurons had locally arborizing axon collaterals*.

Similar studies combining patch-clamp recording, biotin filling and confocal microscopy described at least the islet and the vertical cells (Abe *et al.*, 2009; Melnick, 2008). More recently, Todd's group has studied the morphology of SGR neurons in relation to their neurotransmitter content and firing pattern. These authors showed that the islet cells were GABAergic, while the radial cells and most vertical cells were glutamatergic, with considerable heterogeneity among the remaining types, some of which did not fall into any of the most commonly described morphological categories (Yasaka *et al.*, 2010). Up-to-date studies have used adenoviral vectors expressing the green fluorescent protein (GFP) to mark a subpopulation of SGR interneurons connected to the lamina I spino-parabrachial projection neurons (Cordero-Erausquin *et al.*, 2009) – see Box 2. GFP-labeled neurons in SGR were stalked cells, vertical cells, or neurons with pyramidal cell bodies located in lamina II_o not fitting to any precise category, but not islet cells.

2.2.1.3.3. Mouse

Perl's group has applied its classification to a set of lamina II GABAergic inhibitory neurons tagged by GFP in a transgenic line where expression of the fluorescent tag depended on the mouse prion promoter (Hantman *et al.*, 2004; Hantman and Perl, 2005). For the most, GFP-tagged neurons were located in lamina II_o and corresponded to the central cells described in their previous studies. The lamina II neurons not labeled by GFP displayed similar morphologies to some of the other types described in hamster (Grudt and Perl, 2002).

Another mouse study focused onto a subpopulation of GABAergic neurons (see 2.4.1.3.1. GABA), identified following genetic engineering with a 67 kD glutamic acid decarboxylase (GAD67)-GFP construct (Heinke *et al.*, 2004). In this study, GFP-labeled SGR cells fell into islet (the most numerous), vertical and radial categories.

We have also been able to recognize most of the morphological cell types according to Grudt and Perl's classification after patch clamp recordings and intracellular filling with biocytin (Vergnano *et al.*, 2004) or LY (Ferrini *et al.*, 2007, 2010).

2.2.2. Physiology

Initial studies on the electrophysiological properties of the SGR neurons date back to the last years of the eighties of the previous century. The approaches used at that time were the recording of the receptive fields of individual neurons after stimulation of the (cutaneous) PAFs, or the investigation of the membrane properties and patterns of action potential discharge. Subsequent studies included the analysis of current-voltage relationships, and of the frequency of spontaneous miniature events (Grudt and Perl, 2002). The different approaches thence used to identify single subsets of SGR neurons proved to be of paramount importance for their functional characterization (Graham *et al.*, 2007). However, these neurons have been (and still are) more difficult to record from than many other central neurons, primarily for their relatively small size. An additional issue of complication relates to their complex and variegated morphology (as discussed above). Finally, there are quite limited numbers of recordings from in vivo intracellularly stained neurons belonging, beyond any doubt, to lamina II. Altogether, these issues not only made the functional classification of SGR neurons as complex as the morphological one, but, again, a significant fraction of these neurons escaped any possible classification (Todd, 2017).

The membrane properties of nociceptive SGR neurons in the cat lumbar spinal cord were first investigated in vivo (Iggo *et al.*, 1988). In the subsequent year, another in vivo cat study (Réthelyi *et al.*, 1989), using fine micropipette electrodes filled with HRP, recorded the

responses of the SGR neurons to different types of peripheral stimulation. No clear relationship between the cell morphology and functional response was found, but this study was the first to indicate that neurons responding similarly to different peripheral stimuli do vary in their general geometry and synaptic arrangements. At the same time, Yoshimura and Jessell (1989a, b) succeeded in investigating the membrane properties of SGR neurons in rat, using an in vitro slice preparation. They thus observed that the functional properties of lamina II neurons were heterogeneous and related to voltage-activated ionic conductances of different natures.

A significant number of studies (commented below) were added in the following decades and converged to describe five main types of discharge patterns in SGR neurons: tonic firing, delayed firing, transient firing (or initial bursting), adapting firing (phasic firing) and single spiking. To these, electrically silent neurons in the rat SGR should also be added (Melnick, 2010).

After the seminal work of Rèthelyi *et al.* (1989), the correlation between the functional responses of SGR neurons and their morphology has been the object of numerous studies. Thus, Grudt and Perl (2002) observed that the islet cells and the central cells responded to depolarization with tonic firing (although the latter also displayed transient firing patterns), whereas the radial and vertical cells displayed delayed firing (although the responses of the vertical cells were indeed heterogeneous). In another rat study (Ruscheweyh and Sandkühler, 2002), the SGR neurons displayed delayed or transient patterns but not tonic firing, and it became clear that the different types of neurons differed in their membrane holding potentials and spontaneous activities, e.g. the islet cells had the lowest membrane potential compared to other lamina II neurons. The two studies above were contradictory as to the existence of tonic firing neurons. However, two other contributions in rat were confirmative of their presence, showed that Na^+ and rapidly activating delayed-rectifier K^+ channels were sufficient to generate tonic firing, and considered tonically-firing neurons as excitatory interneurons that could be postsynaptically targeted by μ opioid receptor (MOP) agonists (Melnick *et al.*, 2004; Santos *et al.*, 2004). A few years later, Santos *et al.* (2007) identified synaptically coupled interneurons in rat, using tight-seal whole-cell recordings and a cell-attached stimulation technique. Recorded cells were - in order of relative frequency - tonic, delayed-firing or adapting neurons. In addition, about 10% of these SGR neurons connected monosynaptically to neurons in laminae I and III, and, surprisingly, about 85% of recorded neurons were glutamatergic excitatory interneurons, firing with a tonic or a delayed pattern to evoke inward currents in a postsynaptic cell. The same researchers further studied the electrophysiological properties of the islet cells (Melnick, 2008). They reported that only these neurons responded to hyperpolarization with a slow inward current, presumably a hyperpolarization-activated inward current (h-current - I_h), that was likely responsible of the peculiar membrane and firing responses of this specific type of SGR cells. These results recall those from a previous investigation in mouse, where a specific subpopulation of GABA-immunoreactive (IR) lamina II neurons, morphologically equivalent to the central cells, was genetically tagged with GFP (Hantman *et al.*, 2004). Fluorescence-tagged neurons fired tonically upon depolarization, displayed, for the most part, an I_h and were located in lamina II_o. However, another study on a transgenic mouse line expressing the enhanced green fluorescent protein (EGFP) under the control of the GAD67 promoter (EGFP-GAD76 mouse) to tag the GABAergic neurons was inconclusive about the existence of a correlation between morphology and firing pattern (Heinke *et al.*, 2004).

A subsequent transgenic mouse study, where the voltage dependent potassium channels Kv4.2 were genetically ablated, demonstrated that a Kv4.2-mediated transient inactivating A-type current (I_A) regulated neuronal excitability in the DH, and that the number of tonic firing neurons was increased in *Kv4.2*$^{-/-}$ mice (Hu *et al.*, 2006).

Excitatory DH neurons in BAC transgenic mice expressing EGFP under the control of the vesicular glutamate transporter 2 (VGLUT2) gene (VGLUT2/EGFP mice) have been subject of a recent electrophysiological and morphological characterization (Punnakkal *et al.*, 2014). The same work compared the properties of these neurons to those of the inhibitory GABAergic (GAD67/EGFP tagged) and glycinergic (glycine transporter 2-GLYT2/GFP tagged) neurons. The study has confirmed that excitatory and inhibitory neurons differed in several biophysical properties and in their local circuitry. Specifically, about one-third of all excitatory neurons of the vertical, central, and radial types displayed VGLUT2/EGFP. These neurons had more depolarized action potential thresholds and longer action potential durations than the inhibitory neurons and, for the most, displayed a delayed firing pattern, whereas the inhibitory interneurons were firing tonically. Finally, PAF-evoked inhibitory inputs were found in the majority of glutamatergic and glycinergic cells, but only in less than half of the GABAergic neurons.

Collectively, the above observations lead to the generalization that the response of SGR neurons to depolarization was different between excitatory and inhibitory cells: most excitatory cells displayed transient outward currents with I_A-type firing patterns (delayed, gap- or reluctant-firing), while the majority of inhibitory cells was characterized by I_h inward currents and tonic firing (Yasaka *et al.*, 2010).

On the other hand, spontaneous activity did not differ significantly between excitatory and inhibitory neurons. Yasaka et al. (2007) have recorded the frequencies of the spontaneous excitatory postsynaptic currents (sEPSCs) and inhibitory postsynaptic currents (sIPSCs) from the main types of SGR neurons and observed that the former only had a tendency to be higher in the radial and vertical cells, whereas the latter tended to be higher in the islet cells.

It is worth noting that when SGR neurons were organotypically cultured their electrophysiological features were substantially unchanged. Development of SGR neuron phenotypes and physiological properties in organotypic cultures obtained from rat fetuses with attached DRGs was comparable to age-related acute slices, if one excludes the appearance of giant sEPSCs and increased network excitability in vitro (Lu *et al.*, 2006). Similarly, we have characterized the electrophysiological properties of mouse SGR neurons in organotypically cultured deafferented spinal cord slices (Ferrini *et al.*, 2010). Despite the use of a remarkably different approach in another species, after LY injection neurons showed passive and active membrane properties, firing patterns and morphologies fully consistent with the results of Lu and co-workers (2006) and with the above reviewed data in intact animals or acute slices.

In parallel to the electrophysiological observations summarized above, other in vivo studies aimed to functionally characterize individual SGR neurons according to their responses to different sensory stimuli (mainly) applied to the skin or to the dorsal roots. However, electrophysiological properties could not always be significantly associated with the type of stimulus (Bennett *et al.*, 1980; Cervero *et al.*, 1979; Cervero and Iggo, 1980; Furue *et al.*, 1999; Graham *et al.*, 2004; Iggo *et al.*, 1988; Lopez-Garcia and King, 1994; Réthelyi *et al.*, 1989; Wall *et al.*, 1979; Woolf and Fitzgerald, 1983; Yoshimura and Jessell, 1989b). Further functional information was obtained by recording simultaneously from pairs of SGR neurons. With this approach it was shown that unidirectional excitatory and inhibitory synaptic connections existed between specific morphological types of neurons (Lu and Perl, 2003, 2005; Zheng *et al.*, 2010).

Information on SGR neuron morphology and physiology is summarized in Box 5.

2.3. *Neuropil organization*

2.3.1. Initial studies on the SGR neuropil

Kölliker was the first to report the existence of PAF collaterals reaching the SGR (Kölliker, 1890). Subsequently, Cajal described two layers of these collateral fibers that entered the SGR: a thinner, more superficial layer consisting of unmyelinated fibers, and a thicker, deeper layer of myelinated fibers (Cajal, 1909). To put these observations under the right perspective, one has to keep in mind that, at that time, unmyelinated fibers were very difficult to visualize (Table 1 in Box 1). Fortunately, a few years later Ranson developed the pyridine silver method that allowed to better stain the thin fibers (Ranson, 1913, 1914). Thus, the idea that collaterals of the central processes of the DRG neurons, after travelling in the Lissauer's tract (Lissauer, 1886), were one of the sources of the fine fibers that reached the SGR was reinforced. With the introduction of axonal degeneration studies it was then possible to show that a substantial contribution to the superficial plexus also came from axon collaterals of the propriospinal neurons (Szentàgothai, 1964). This conclusion was reached after a period of considerable debate. The interpretation of axonal degeneration studies after dorsal rhizotomy was, in fact, made difficult for several reasons, among which the diverse rate of degeneration among PAFs of different diameters (Heimer and Wall, 1968). In addition, at least in cat, it was reported that degenerated synapses in the SGR were somewhat replaced by collateral sprouting from the propriospinal neurons (Murray and Goldberger, 1986). Even so, degeneration techniques (Carpenter *et al.*, 1968; Ralston, 1965, 1968; Shriver *et al.*, 1968; Sterling and Kuypers, 1967) and anterograde tracing of single PAFs (Light and Perl, 1979a; Mense and Prabhakar, 1986; Ralston *et al.*, 1984; Réthelyi *et al.*, 1982; Sugiura *et al.*, 1986, 1989) eventually confirmed that the superficial plexus contained both unmyelinated PAFs and propriospinal axons.

The coarse myelinated PAF collaterals of the deep plexus described by Cajal, instead, entered the SGR from the bottom, and formed a series of typical arborizations referred to as flame-shaped arbors (Cajal, 1909; Scheibel and Scheibel, 1969; Szentàgothai, 1964). In discussing the localization of the flame-shaped arbors in the DH, it is important to recall here that their first descriptions were before Rexed's laminar subdivision, and when the SGR was considered to comprise both laminae II and III (Willis, Jr. and Coggeshall, 2004b). Subsequent observations reported that the flame-shaped arbors, indeed, entered lamina II_i, but interspecies differences were substantive (Willis, Jr. and Coggeshall, 2004b). Another issue of attention about the flame-shaped arbors is that they originate from hair follicle afferents (Brown, 1981). Today most studies consider that hair follicle afferents terminate in laminae III-IV and *not* in SGR, although at least two tracing studies have confirmed the existence of myelinated afferents in lamina II_i (Hughes *et al.*, 2003; Woodbury *et al.*, 2000). The overall picture regarding the myelinated input to SGR is further complicated by observations of medio-lateral and segmental differences (Woodbury *et al.*, 2000), and by the significant plasticity of the SGR over the first weeks after birth, as cutaneous LTMs project extensively throughout the superficial dorsal horn (SDH)[6] during the first phases of postnatal development.

2.3.2. Types of processes and synaptic contacts

The abundance of the SGR neuropil is due the conspicuous presence of synapses of heterogeneous sources. A very distinctive ultrastructural feature is the abundance of complex multisynaptic arrangements made by the PAFs: these multisynaptic complexes were originally described by Ralston in cat (1965) that referred to them as "complex synaptic arrays", and subsequently named synaptic glomeruli. Glomeruli appeared to be relatively few in number with respect to simple axo-axonic synapses when individual sections of the SGR were observed at TEM. However, Duncan and Morales (1973) stated that 75% of synapses in lamina II occurred in glomeruli. Systematic serial sections are indeed needed to have an idea of the high density of

[6] I will use SDH to indicate laminae I-III

glomeruli and their synaptic connections. However, quantitative studies of this type were very few and mainly focused to establish the relative numbers of different types of synaptic configurations in SGR. In the lumbar spinal cord of cat deprived of glomeruli after dorsal rhizotomy, 96.5% of synapses were axo-dendritic, the remaining ones being about equally divided among axo-axonic, dendro-dendritic and dendro-axonic synapses (Duncan and Morales, 1978). In rat, out of 6,045 synaptic contacts only 54 occurred between axon terminals, and 10 between dendrites (Zhu *et al.*, 1981). Whereas glomeruli had a central axonal bouton of primary afferent origin, terminals in non-glomerular axo-dendritic synapses might as well rise from PAFs, but also from intrinsic spinal neurons or descending fibers. Dendro-dendritic and dendro-axonic contacts derived from intrinsic spinal cord neurons having their cell bodies in lamina II or other laminae of the SDH (see Boxes 2 and 3).

2.3.2.1. Types of PAFs terminating in the SGR

Stimuli of heterogeneous nature collected from different tissues and organs reach the SGR, and, according to their origin, these stimuli are broadly subdivided into somatic or visceral. Somatic afferents originate from the skin, muscles, bones or joints. They are classified based on their response properties to stimulation.

The pattern of termination of PAFs in SGR, particularly those originating from the skin, has been extensively investigated with tract-tracing techniques, electrophysiological recordings and dye filling, electron microscopy and immunocytochemistry (ICC). These approaches were at times used in combination with each other.

Touch in the mammalian hairy skin and mechanosensation in deeper somatic tissues are mediated by morphologically and physiologically distinct classes of LTMs whose fibers are sorted, according to conduction velocities and presence/absence of a myelin sheet, into types Aβ, Aδ and C (Abraira and Ginty, 2013). Temperature is transmitted along Aδ (cold) and C (warm) fibers. Temperatures above 43°C and below 12°C give rise to pain, which is transmitted by both Aδ and C fibers. Noxious temperatures are also transmitted by polymodal C fibers (Nomoto *et al.*, 2004). Mechanical pain is instead mediated by Aδ and C high threshold mechanoreceptors (HTMs)[7], whereas additional sets of C fibers generate itch (Hoon, 2015) or pleasant touch sensations (Olausson *et al.*, 2002).

The central projections of visceral sensory fibers reaching the SGR, for the most, derive from Aδ and C polymodal LTMs that code both non-noxious and noxious stimuli[8]. Therefore, considering their ability to code noxious stimuli, most visceral afferents should be regarded as nociceptors (Christianson and Davis, 2010).

In general terms, the first observations on the arborization of PAFs in SGR indicated that axonal arbors were distributed along parasagittal planes with typically enlarged, club-shaped terminal endings (Réthelyi, 1977; Scheibel and Scheibel, 1968, 1969). Subsequent studies succeeded in analyzing the distribution of different functional types of PAFs in individual laminae of the SDH. It was thus evidenced that the SGR was the main site of termination of cutaneous unmyelinated C PAFs, but also contained terminals of visceral origin, which were sparser and

[7] LTMs (D-hair cells) were originally described about half a century ago in hairy skin of every species examined including humans. It is commonly assumed that all Aδ fibers belong to nociceptors, but Aδ LTMs exist in several species, and are neurochemically and functionally different from the Aδ HTMs (Djouhri, 2016).

[8] It should here be remembered that viscera, differently from somatic structures, receive a double sensory innervation. More precisely, visceral structures of the head and neck, thoracic and abdominal viscera are innervated by cervical, thoracic and upper/more cranial DRG neurons as well as the sensory neurons of the inferior vagal and glossopharyngeal ganglia; the distal colon and the pelvic viscera, instead, receive a double innervation from the lower/more caudal thoraco-lumbar and lumbo-sacral segments of the spinal cord (Christianson and Davis, 2010).

spanned over a larger number of spinal segments than the cutaneous afferents (Sugiura *et al.*, 1989).

Starting from the seventies of the last century, there was debate about which type(s) of PAF endings terminated in the SGR, in particular as regarding the possibility that finely myelinated Aδ nociceptive fibers indeed existed in lamina II. In fact, from one side, several morphological (LaMotte, 1977; Ralston and Ralston, 1979) and functional studies (Light and Perl, 1979a; Mense and Prabhakar, 1986; Réthelyi *et al.*, 1982) converged to demonstrate that these fibers did not enter the SGR, but other investigations reached opposite findings (Ataka *et al.*, 2000; Chen and Sandkuhler, 2000; Gobel and Binck, 1977; Gobel and Falls, 1979; Ikoma *et al.*, 2007; Lao *et al.*, 2004; Lu and Perl, 2005; Sandkühler *et al.*, 1997). Today we accept that nociceptive and thermoreceptive Aδ and C afferents are distributed throughout the entire lamina II, whereas the axons of LTM Aβ fibers and some fine myelinated Aδ hair follicle afferents (LTMs) arborize only in lamina II_i.

2.3.2.2. Glomeruli

Glomeruli (Fig. 3) are considered to be pivotal to the process of presynaptic modulation of peripheral information conveyed to the SDH (Ribeiro-Da-Silva, 2015; Willis, Jr. and Coggeshall, 2004b). The SGR is particularly enriched in glomeruli, but they are also found in laminae I and III. In all species studied so far, glomeruli are formed by a central axonal bouton (C bouton) of primary afferent origin, which is surrounded by several dendrites and a few axon terminals. An incomplete glial envelope separates this set of neuronal processes from the surrounding tissue. Glomeruli were initially described in cat and named synaptic arrays (Ralston, 1965, 1968), central synaptic complexes or large synaptic complexes (Réthelyi and Szentagothai, 1969). There are no ultrastructural observations of glomeruli in humans. However, in monkeys of several species descriptions also referred to glomeruli or their C boutons as ansiform axonal complexes (Beal and Fox, 1976) or scalloped endings (Knyihar-Csillik et al., 1982). Subsequent studies adopted the more widely employed term glomeruli (DeLanerolle and LaMotte, 1983; Knyihar-Csillik *et al.*, 1989; Réthelyi *et al.*, 1982) that, at the same time, started to be used in rodents (see below). Studies in primate embryos indicated that glomeruli appeared in this order much earlier than in all other mammals (Knyihar-Csillik et al., 1999). The ultrastructure of glomeruli in monkeys is somewhat different from that in rodents, as three types of glomeruli were described in the primate SGR, whereas there are four types in rodents (for comparison see Fig. 1 in Merighi et al. 2008). Rat glomeruli have been characterized in depth (Coimbra et al., 1974; Ribeiro-Da-Silva et al., 1985; Ribeiro-Da-Silva and Coimbra, 1982) and differences with mice were minimal (Hiura *et al.*, 1991; Marker *et al.*, 2006; Salio *et al.*, 2005, 2014). Ribeiro-da-Silva and Coimbra (1982) were the first to divide rat glomeruli in two types (I and II) on ultrastructural and neurochemical grounds. Rodent type I glomeruli were further subdivided into non-peptidergic type Ia (Fig. 3F-G - corresponding to monkey glomeruli with a dense sinusoid axon terminal) or peptidergic type Ib (Fig. 3B, E - corresponding to monkey glomeruli displaying a central terminal with large granular vesicles). Rodent type II glomeruli (Fig. 3C-D - homologous to monkey glomeruli with terminals containing "regular"[9] synaptic vesicles) were also subdivided in type IIa and type IIb. In monkeys, the SGR contained mainly glomeruli with dense sinusoid axon terminals.

As regarding intralaminar distribution and relative abundance in rat, type Ia glomeruli were particularly abundant in lamina II_id, followed by lamina II_iv and II_o; type Ib glomeruli were the

[9] Regular is the term used by Knyihar-Csillik and co-workers (1989) to describe this type of glomeruli that were thus indicated as RSV (regular synaptic vesicle) glomeruli. The *regular* vesicles are indeed the small clear agranular vesicles storing low MW transmitter amino acids.

least represented and confined, in order of laminar incidence, to lamina II_id and II_o; type IIa were most abundant in lamina II_iv, a few being also present in II_id; type IIb were confined to lamina II_iv (for review see Ribeiro-Da-Silva and De Koninck, 2008).
Glomeruli in rodents and monkeys contained two different types of axonal endings, the C boutons and the peripheral V2 terminals (Fig. 3C), and two types of peripheral dendrites, the plain dendrites (devoid of synaptic vesicles) and the vesicle-containing V1 dendrites (Fig. 3 B-C, G). The unmyelinated PAFs of the C type generated the C boutons in type I glomeruli, whereas poorly myelinated Aδ and myelinated Aαβ PAFs gave rise to the C boutons in type IIa and IIb respectively (Coimbra et al., 1984). Dendrites in glomeruli derived from the antenna cells of deeper laminae or from other lamina II interneurons (Réthelyi and Szentagothai, 1969). The large majority of them were plain dendrites postsynaptic to the C bouton. In cat, it was calculated that 97% of glomerular synapses were axo-dendritic (Duncan and Morales, 1978). However, V1 dendrites, belonging to the islet cells or possibly to the antenna cells in deeper laminae, were presynaptic to the C bouton and thus formed dendro-axonic synapses (Carlton and Hayes, 1990; Spike and Todd, 1992). Axo-axonic synapses made by the V2 axons that contacted the C bouton were mainly observed in type II glomeruli (Ribeiro-Da-Silva and Coimbra, 1984; Zhu *et al.*, 1981).

2.3.2.3. Non-glomerular synapses

According to several studies, about 95% of synapses in SGR were simple asymmetric excitatory synapses or symmetric inhibitory synapses (Duncan and Morales, 1973, 1978; Ralston, 1971; Ralston and Ralston, 1979). Axons engaged in these synapses contained agranular round (generally in excitatory terminals) or flat (generally in inhibitory terminals) vesicles of small size (SSVs) and, at times, large dense core vesicles (LDCVs)[10]. Non-glomerular synapses had different origin and could be differentiated among each other only after tracing procedures or, in certain cases, neurochemical labeling (see below sections 2.3.2.5. Synapses made by the descending fibers and 2.4. Neurochemistry of SGR).
In a rat study (Zhu *et al.*, 1981), axo-axonic non-glomerular synapses were divided into two subpopulations: a more represented AA-type that contained SSVs, and a less abundant AAgr-type enriched in LDCVs that preferentially accumulated in the postsynaptic axons. AA-type synapses were further subdivided into AA1 and AA2 subtypes. The pre- and postsynaptic terminals of AA1 had more prominent postsynaptic densities and a larger synaptic cleft than AA2, and contained spherical SSVs. The presynaptic axons of AA2 synapses mainly contained flattened SSVs, while spherical vesicles were confined to the postsynaptic boutons. The same authors also described DD1 and DD2 dendro-dendritic synapses that contained pleomorphic synaptic vesicles at both the pre- and postsynaptic compartments, and concluded that the thickness of the postsynaptic density was the most reliable feature to differentiate between the two groups (Zhu *et al.*, 1981).

2.3.2.4. Synapses made by the intrinsic spinal cord neurons

Although it is widely demonstrated that certain types of SGR neurons (also referred to as local circuit neurons) have short axons that ramified within the same lamina II and that the latter contained axons or dendrites originating from other spinal cord neurons, there is a limited number of TEM studies that analyzed the synaptic circuitry of the intrinsic spinal cord neurons. The first ultrastructural observations on the synaptic arrangements of presumed propriospinal fibers in cat SGR led to hypothesize that contacts made by these fibers were of the *en passant* type (Réthelyi and Capowski, 1977; Réthelyi, 1977; Scheibel and Scheibel, 1968). Subsequent

[10] These vesicles were also named p (peptidergic)-type vesicles once it became clear that they stored neuropeptides. Today it is accepted that they store high MW neurotransmitters in a more general sense.

work in the same species showed that the axons of nocireceptive and mechanoreceptive HRP-labeled neurons formed simple axo-dendritic synapses, whereas dendrites were engaged in dendro-dendritic contacts (Réthelyi *et al.*, 1989). These authors have shown that only some mechanoreceptive neurons, but not the nocireceptive neurons, displayed presynaptic vesicle containing dendrites. However, a few nocireceptive neurons received synapses from presynaptic dendritic profiles.

As mentioned, paired recordings have functionally demonstrated the occurrence of synaptic connections between SGR neurons (Lu and Perl, 2003, 2005; Zheng *et al.*, 2010). The existence of excitatory and inhibitory local synaptic inputs to the islet cells from within lamina II was also demonstrated by laser scanning photostimulation (Kato *et al.*, 2007). Connections between the GABAergic SGR neurons (islet cells) were also confirmed in paired recording experiments on spinal cord slices obtained from EGFP-GAD67 mice (Labrakakis *et al.*, 2009). Collectively, these observations supported and extended the initial ultrastructural studies on the synapses made by the intrinsic spinal cord neurons. Therefore, it is today accepted that these synapses not only contribute for a significant fraction of the axo-dendritic contacts in lamina II but are functionally relevant to explain the SGR circuitry.

2.3.2.5. Synapses made by the descending fibers

The rostroventral medulla (RVM)[11] is the main source of several descending pathways that reach the SGR and modulate nociceptive information in the SDH, for the most in an inhibitory fashion. Of these, the most important is the raphespinal tract (Fields and Basbaum, 1994), originating from the neurons of the raphe magnus nucleus (RMN); other descending fibers originate from neurons of the gigantocellular complex (Martin *et al.*, 1985) or locus coeruleus (West *et al.*, 1993).

2.3.2.5.1. Serotoninergic fibers

RMN neurons are serotoninergic and send their axons to the DH ipsilaterally. In two rat studies, serotonin (5HT) -IR axons made synaptic and non-synaptic associations with neurites in the SGR neuropil (Marlier *et al.*, 1991; Maxwell *et al.*, 1985). 5HT-IR varicosities did not form - in most cases - identifiable synaptic specializations, but were simply closely apposed to cell bodies, dendrites or axons (Miller and Salvatierra, 1998) – see Box 4. When present, synapses were of the axo-dendritic or axo-somatic types only. In the rat lumbosacral spinal cord, 5HT-IR varicosities were in close proximity to about 50% of the enkephalin (ENK) - or neurotensin-IR neurons (Miller and Salvatierra, 1998). In another rat study, however, a spino-bulbo-spinal loop comprising the RMN serotoninergic neurons was shown to be pro-nociceptive and to intervene in some forms of central sensitization (Suzuki *et al.*, 2002).

In cat, by combining HRP tracing, ICC and the electrical stimulation of RMN (Miletic *et al.*, 1984), a positive correlation was observed between the number of 5HT-IR contacts onto the dendritic shafts (rather than spines) of lamina II stalked cells and the suppression of responses to noxious stimuli that followed the RMN stimulation. Serotoninergic synapses on nociceptive stalked cells were eight times those on the nociceptive lamina II_o or non-nociceptive lamina II_i islet cells. Both types of neurons were unaffected by electrical stimulation. In another cat study (Ruda *et al.*, 1982), 5HT-IR axons were observed in lamina II_i. These axons contained clusters of synaptic vesicles of heterogeneous morphologies and made synapses primarily on small caliber dendritic shafts, and, less frequently, larger shafts, dendritic spines, and neuronal cell bodies. However, other authors reported that 5HT-IR terminals were, instead, more numerous in cat

[11] The RVM includes several raphe nuclei (raphe magnus, raphe pallidus, and raphe obscurus) and adjacent nuclei of the reticular formation (reticularis gigantocellularis, and reticularis gigantocellularis pars α). All these nuclei have been implicated in descending inhibition of SGR neurons.

lamina II_o, similarly to rat (Light *et al.*, 1983). Immunolabeled terminals were of two types: the small dome-shaped boutons with sparse small dense core vesicles (SDCVs) and round or pleomorphic clear vesicles, and the less represented larger scalloped or egg-shaped boutons, which contained many mitochondria and SDCVs. The first type was seen throughout SGR whereas the second was only found lamina II_o; both were engaged in symmetric synapses with distal dendritic arborizations of the SGR neurons, rarely with proximal dendrites or perikarya, and never with axon terminals.

In monkeys, 5HT-IR terminals were described in lamina II_o (LaMotte and de Lanerolle, 1983b). These terminals contained many SSVs and a few LDCVs; in most cases, they made symmetrical synapses with large and small dendrites, but not dendritic spines. Axo-somatic contacts were rare. 5HT-IR terminals were also observed to be engaged in axo-axonic contacts with LDCV-type terminals in glomeruli (see 3.1.1.2. Glomeruli).

2.3.2.5.2. Noradrenergic fibers

The axons of several groups of brainstem noradrenergic neurons reach the SGR where they regulate nociception in a facilitatory or inhibitory fashion (Monhemius *et al.*, 1997; Suzuki *et al.*, 2002; Zhuo and Gebhart, 1997). The initial studies on noradrenergic terminals on rat SGR were carried out with glyoxylic acid-$KMnO_4$ fixation. With this approach, labeled terminals with SDCVs were observed in lamina II_o and appeared to be simply opposed to small-caliber dendrites/spines or vesicle-containing axons, whereas synaptic specializations were rarely seen – see Box 4. After dorsal rhizotomy, there were no contacts between the noradrenergic terminals and the degenerated axon terminals of PAFs (Satoh *et al.*, 1982), suggesting that interactions were of an indirect type. Subsequent immunocytochemical studies were carried out using specific antibodies against noradrenaline or dopamine-beta-hydroxylase, its key synthesizing enzyme, and showed IR terminals in lamina II_o (Hagihira *et al.*, 1990). Here, more than 50% of positive terminals were engaged in synapses with small dendrites or dendritic spines, but there were no synaptic contacts with PAFs. A less dated study has localized the adrenergic α2C receptor to the distal dendrites of the neurokinin receptor 1 (NK1) -IR projection neurons (antenna neurons) in lamina III (Olave and Maxwell, 2003). As the dendritic arbor of these neurons spanned in ventro-dorsal direction along the entire thickness of SGR, it seemed reasonable to hold that the antenna neuron dendrites were an important target of the descending noradrenergic system.

Functionally, noradrenaline, acting through α1 adrenoceptors, dose-dependently increased the frequency and amplitude of miniature IPSCs (mIPSCs) in rat GABAergic and glycinergic SGR neurons, but not of miniature EPSCs (mEPSCs) (Baba *et al.*, 2000a, 2000b). A subsequent study has confirmed that stimulation of α1 adrenoceptors reduced the primary afferent glutamatergic synaptic input to GABAergic lamina II neurons by acting onto $GABA_A$ receptors and T-type Ca^{2+} channels (Yuan *et al.*, 2009).

2.4. Neurochemistry of SGR

The neurochemistry of the SGR has been widely investigated in relation to the continuing progress in ICC that had a tremendous boost starting from the eighties of the last century, as testified by several reviews published in that period (see for example Gibson and Polak, 1986; Hunt, 1983; Todd and Spike, 1993).

The distribution of most neurochemicals is very complex, as many of them not only can be present in different compartments of the SGR neurons, but also in the terminals deriving from PAFs or the descending fibers, as well as the neurites of neurons having their cell bodies in other laminae of the gray matter.

A very detailed description of the chemical neuroanatomy of the DH has been published by Willis, jr. and Coggeshall (2004) in their authoritative book on the sensory mechanisms in spinal

cord. As immediately apparent to readers, the list of neurochemicals in lamina II is impressive and it is beyond the purpose of this contribution to address this issue beyond reasonable details.

Notably, most of the work on the neurochemistry of the DH dates back to more than three decades. Therefore, it must be taken into account that there were many instances in which the quality and specificity of primary antibodies and/or hybridization probes used in localization studies had been poorly documented. In addition, many neurochemicals have been described only occasionally, a large number of studies were simply qualitative, often with no subsequent confirmations of the initial observations. Finally, a particular mention should be made to the continuously expanding diversity of membrane receptors subtypes, particularly for the "classic" excitatory and inhibitory neurotransmitters, that are expressed in the SGR neuropil and on the plasma membrane of the SGR neurons.

Thus, in the following I will only address the most important and widely studied neurochemicals so far localized in SGR, with particular emphasis for those whose presence/function has been also confirmed by functional and pharmacological studies.

I will also consider other classes of substances which do not have a direct or indirect role in neurotransmission but have been useful as selective neuronal markers.

To better put things under the right perspective and help readers to navigate across the neurochemical complexity of the SGR, some pivotal concepts on the modalities of neuron-to-neuron communication in CNS are summarized in Box 4.

A final general issue of attention regards the intralaminar organization of the SGR in relation to its Aδ and C PAF input (Light, 1992), as most authors agree that the peptidergic fine afferent input is distributed to lamina II_o whereas the non-peptidergic input reaches lamina II_i, but full consensus has not been reached yet.

2.4.1. Fast transmitter aminoacids, transporters, and receptors

2.4.1.1. Glutamate

Glutamate is the main excitatory neurotransmitter of central neurons. Immunoreactivity in rat SGR was initially described with different light and electronic immunocytochemical approaches (Kai-Kai and Howe, 1991; Miller *et al.*, 1988). Using high resolution immunogold TEM ICC and a specific anti-glutamate antiserum that did not cross-react with GABA or aspartate and specifically recognized the vesicular glutamate pool, we offered the first reliable immunocytochemical demonstration for the existence of glutamatergic neurons in SGR (Merighi *et al.*, 1991). As later reviewed (Todd and Spike, 1993), identification of these neurons was initially difficult as a consequence of the lack of suitable immunocytochemical markers for the glutamatergic axons, cross-reactivity of anti-glutamate antisera with other excitatory and inhibitory aminoacids, and concurrent presence of a diffuse network of glutamatergic PAFs and terminals. Fortunately, glutamatergic neurons were subsequently more easily and specifically labeled by the use of antisera against VGLUT2 (Todd *et al.*, 2003), albeit the transporter was not detected in cells bodies, but only in axons (Oliveira *et al.*, 2003). By using a combination of different neuropeptide markers together with VGLUT2, Todd et al. (2003) provided evidence that in the rat DH most of the VGLUT2 staining was associated to the axons of local glutamatergic interneurons, whereas VGLUT1 and VGLUT3 were expressed in PAFs. In PAFs, VGLUT1 stained the terminals of A LTMs, while VGLUT3 was present in non-peptidergic C LTMs (Seal *et al.*, 2009). Other studies then showed that physiologically characterized glutamatergic neurons were vertical, radial or antenna cells (Maxwell *et al.*, 2007), but some islet cells that did not stain for GABA were also hypothesized to be glutamatergic (Spike and Todd, 1992). A very recent and excellent review addressing the functional identification of the interneurons in the

SDH has considered in detail, among others, the neurochemical and functional features of the SGR glutamatergic interneurons (Todd, 2017).

2.4.1.2. Glutamate receptors

The diversity of glutamate receptor subtypes in central neurons is impressive and the SGR makes no exception to this (Bleakman *et al.*, 2006). Somewhat surprisingly if one considers the amount of functional data, localization studies have been relatively few, particularly at ultrastructural level.

2.4.1.2.1. Ionotropic glutamate receptors

There are three families of ionotropic glutamate receptors: the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, the N-methyl-D-aspartate (NMDA) receptors, and the kainate receptors.

2.4.1.2.1.1. AMPA receptors

Glutamate receptors of the AMPA type open very quickly and thus mediate fast excitatory neurotransmission. AMPA receptors (AMPARs) consist of four subunits (named GluR1-4), and the subunit composition determines their channel properties. The GluR2 subunit is particularly important as it makes the receptor impermeable to calcium ions, its expression thereby governing the permeability of the AMPAR to the cation. Numerous localization studies in the nineties of the last century converged to demonstrate that GluR1 and GluR2/R3 were highly concentrated in the SDH (Willis, Jr. and Coggeshall, 2004a).

In situ hybridization (ISH), has shown that in rat lamina II there were high numbers of GluR2 strongly labeled cells, a moderate number of cells expressing GluR1 or GluR3, while the expression of GluR4 was very low (Furuyama *et al.*, 1993). A later study in vitro was the first to show that calcium-permeable AMPARs were, for the most, localized on GABAergic inhibitory interneurons and neurons immunostained for the preferred substance P (SP) receptor NK1 (Albuquerque *et al.*, 1999). More recent immunocytochemical studies have confirmed that most SGR neurons displayed immunoreactivity for GluR2, many also expressed GluR1 and/or GluR3, but none GluR4 (Polgár *et al.*, 2008), which was, instead, detected in lamina III antenna neurons (Todd *et al.*, 2009) and some lamina I projection neurons (Polgár *et al.*, 2010). Ultrastructurally, the GluR2/3 subunits were detected in small dome-shaped terminals, likely of intrinsic origin (Popratiloff *et al.*, 1996). Functionally, postsynaptic AMPARs regulate much of glutamate neurotransmission that is not regulated by NMDA receptors at the synapses made by PAFs onto NK1-IR neurons in laminae I and III/IV (Chen *et al.*, 2016; Tong and MacDermott, 2006; Yasaka *et al.*, 2009). It is therefore unfortunate that we still don't exactly know which type(s) of SGR neurons express these receptors.

In SGR, AMPARs also have a presynaptic localization at C boutons of type I and type II glomeruli (Popratiloff *et al.*, 1996, 1998).

2.4.1.2.1.2. NMDA receptors

NMDA receptors (NMDARs) are ion channels made up of four subunits, including at least one GluN1 (previously referred to as NR1), and combinations of four (A–D) GluN2 (previously NR2) and two (A or B) GluN3 subunits.

Activation of NMDARs in the SDH neurons is a key process related to nociception in normal and pathological conditions (Ren and Dubner, 2007). Nonetheless, also for these receptors localization studies are comparatively few.

Using ISH with probes against GluN1 or the NMDAR complex as a whole, SGR neurons were labeled diffusely with low to moderate intensity (Furuyama *et al.*, 1993; Tolle *et al.*, 1993; Watanabe *et al.*, 1994a, 1994b). Later, with an immunocytochemical approach, we specifically showed the presence of GluN1 in cell bodies of nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase stained islet cells in rat lamina II_i (Aimar et al., 1998). With ICC and an

antigen-unmasking procedure, a preferential expression of the GluN2B subunit was described in laminae I-II, with GluN2A being found in deeper laminae (Nagy *et al.*, 2004).
With pan NMDAR probes virtually all DRG neurons were labeled, indicating that receptors are also present in PAF terminals (Watanabe *et al.*, 1994a).

2.4.1.2.1.3. Kainate receptors

Kainate receptors are made of multimeric assemblies of GluK1-3 and GluK4,5 subunits[12]. GluK1-3 subunits can form functional complexes with a low affinity for kainate, and GluK5 subunits are retained in the endoplasmic reticulum unless assembled with GluK1-3 to form the high affinity receptor (Gallyas *et al.*, 2003).
In rat DH (Henley *et al.*, 1993) and human SGR (Kalb and Fox, 1997; Shaw and Ince, 1994), expression of kainate receptors was initially studied with [^{3}H] kainate autoradiography to conclude that they were highly concentrated in these areas of the spinal cord. However, labeling of the rat DH was low in the first study using a GluK1-specific ISH probe (Furuyama *et al.*, 1993). In keeping with this initial localization, GluK1 was later demonstrated have a presynaptic localization at PAF glomerular C boutons (Lucifora *et al.*, 2006). In the same paper it was also shown a presynaptic localization of GluK4 and GluK5 at type II glomeruli, whereas GluK2-3 was predominantly postsynaptic to PAFs.
Subsequent functional studies on mice lacking GluK1 and/or GluK2 have demonstrated that kainate receptors were indeed expressed in SGR and that their activation could either suppress or facilitate excitatory synaptic transmission in spinal cord slices (Xu *et al.*, 2006; Youn *et al.*, 2005; Youn and Randic, 2004).

2.4.1.2.2. Metabotropic glutamate receptors

There are eight different types of metabotropic glutamate receptors (mGluRs), named $mGluR_1$-$mGluR_8$. Receptors are divided into groups I-III based on structure and physiological activity, and further split into subtypes.
Group I comprises $mGluR_1$ and $mGluR_5$, which increase the NMDAR activity and are mainly postsynaptic. Both receptors have been localized to the SGR albeit at different levels of expression. Specifically, immunostaining for $mGluR1_B$ was faint and no staining was obtained for $mGluR1_A$ (Jia *et al.*, 1999), but, functionally, $mGluR_1$ was reported to be activated postsynaptically and upregulated after spinal nerve injury (Aira *et al.*, 2012). Immunoreactivity for $mGluR_5$ was, instead, very strong (Alvarez *et al.*, 2000; Jia *et al.*, 1999), and calretinin-containing neurons expressed the receptor in another localization study (Aronica *et al.*, 2001).
Quantitative ultrastructural data showed that only a small fraction of presumptive type IIa glomeruli made by the Aδ fibers were labeled for either $mGluR1_B$ or $mGluR_5$, and that immunoreactivity was most frequently restricted to mGluR5 positive plain dendrites (Jia *et al.*, 1999). The absence of mGluR5 in the C boutons of glomeruli needs to be confirmed as the receptor was previously demonstrated to be expressed in DRG neurons (Valerio *et al.*, 1997).
$mGluR_5$ was also detected in cultured DH neurons (Hu *et al.*, 2007), and work in vitro has demonstrated that $mGluR_5$ was not expressed in GAD67-GFP tagged inhibitory DH neurons, but regulated the excitability of excitatory neurons (Hu and Gereau, 2011).
Group II mGluRs ($mGluR_2$ and $mGluR_3$) decrease the NMDAR activity and are mainly presynaptic. Immunoreactivity for $mGluR_2/_3$ was concentrated in lamina II_i (Jia *et al.*, 1999), most frequently in calbindin-containing neurons (Aronica *et al.*, 2001). Jia and co-workers (1999)

[12] In 2009 the kainate receptor subunits have been renamed to correspond with their gene name. GluR5-7 are now GluK1-3 and KA1 and KA2 are GluK4 and GluK5, respectively (Collingridge *et al.*, 2009). The new terminology is adopted here.

have shown that vesicle-containing profiles IR for $mGluR_{2}/_{3}$ were characteristically encountered in presumptive type IIa, but not type I glomeruli.
Group III comprises five different receptors that also decrease the activity of NMDAR and have a prevailing presynaptic localization. Of the receptors of this group, only mGluR4 was localized in the inner part of SGR (Vilar *et al.*, 2013).

2.4.1.3. GABA and glycine

GABA and glycine are the main inhibitory neurotransmitters in CNS. Both are found in SGR neurons, and very recent findings have demonstrated that GABA dominantly inhibited excitatory interneurons and projection neurons in lamina I and II_o while glycine preferentially inhibited those in lamina II_i and III (Takazawa *et al.*, 2017).

2.4.1.3.1. GABA

GABA-containing SGR neurons were initially identified by autoradiography (Ribeiro-Da-Silva and Coimbra, 1980) or antisera against GAD, the key GABA biosynthetic enzyme (Barber et al., 1978; Hunt et al., 1981; McLaughlin et al., 1975). The GAD-IR neurons were of large size and predominantly found in lamina II_o or at the limit between lamina II_i and III (Hunt et al., 1981). The subsequent discovery that GAD existed in two isoforms of different MWs (65 and 67 kD referred to as GAD65 and GAD67, respectively) prompted some investigators to use isoform-specific antisera for a more precise detection. Initial observations confirmed that GAD65 and GAD67 immunoreactivities were both prominent in rat lamina II_i (Mackie et al., 2003), and later studies employed these isoform-specific antisera to better characterize the SGR inhibitory neurons after electrophysiological recordings (Maxwell et al., 2007). The lamina II_i localization of GAD65- and GAD67-IR neurons was also confirmed in mouse, where these neurons were hypothesized to have distinct responses to peripheral noxious stimulation (Nowak *et al.*, 2011). In rat (Barber *et al.*, 1982; Maxwell *et al.*, 2007; Todd and McKenzie, 1989) or mouse (Hantman *et al.*, 2004; Hantman and Perl, 2005; Heinke *et al.*, 2004), many GABAergic SGR cells were islet cells. In rat, initial localization studies reported that only the large islet cells were immunostained for GABA, whereas the smaller ones were not (Spike and Todd, 1992; Todd and McKenzie, 1989; Todd and Spike, 1993)[13]. Todd and McKenzie (1989) have observed that more than 25% of the SGR neurons in their Golgi preparations were GABA-IR, and, for the most, displayed the typical morphology of the islet cells. Stereological analysis has afterwards confirmed these observations, as approximately 30% of SGR neurons were GABAergic (Polgár et al., 2003). In humans, the filamentous cells besides the islet cells were GABAergic (Waldvogel et al., 1990). Several other studies then extended these data and led to conclude that GABAergic neurons in SGR included the islet, radial, central and vertical cells, but not the stalked cells (Hantman *et al.*, 2004; Heinke *et al.*, 2004; Maxwell *et al.*, 2007).
At the ultrastructural level, initial studies to localize GABA-IR employed the peroxidase-anti-peroxidase (PAP) pre-embedding technique with antibodies against GAD. The first among these studies was carried out in rat by McLaughlin *et al.* (1975) who, notably, reported that IR terminals not only were presynaptic to dendrites and cell bodies, but to other axons, and highlighted the importance of these axo-axonic synapses as substrates of presynaptic inhibition. About a decade later, two other PAP studies were published: one in cat (Basbaum *et al.*, 1986), again reporting the localization of GAD[14], and the other in rat, describing the distribution of GABA immunostaining (Magoul *et al.*, 1987). Both were in agreement as

[13] There was some confusion in earlier literature as regarding the classification of the “small islet cells” that sometimes were referred to central cells. Here, I will refer to the inhibitory "large islet" cells simply as “islet cells”, while the smaller ones will be referred to as “central cells” independently of their glutamatergic (probably the most represented) or GABAergic phenotype.
[14] This study was, indeed, carried out in the spinal nucleus of the trigeminal nerve, not in spinal SGR.

regarding the relative paucity of axo-axonic synapses and the occurrence, in GABAergic contacts, of certain cytological features that did not totally fit to the classic description of Gray type II inhibitory synapses. Thus, somehow, these studies opened a debate as regarding the cytology of the GABAergic synapses and the diffusion of axo-axonic synapses in the SGR. We have been the first to show, by means of post-embedding immunogold labeling, that the rat SGR contained GABA-IR cell bodies, axons, and dendrites (Merighi *et al.*, 1989). Subsequent immunogold observations in monkey were consistent with our study (Carlton and Hayes, 1990; Hayes and Carlton, 1992). In these three studies, GABA-IR axon terminals were seldom presynaptic in axo-axonic synapses and not only contained flattened but also round SSVs. In addition, some vesicle-containing dendrites, at times, formed reciprocal synapses with the C boutons in glomeruli (V1 profiles see 2.3.2.2. Glomeruli). However, in a different rat study Mitchell et al. (1993) reported that the axons containing the gold particles indicative of GABA-immunoreactivity could often be seen to form symmetric synapses (Gray type II), whereas axons that formed asymmetric synapses (Gray type I) were not labeled. In more recent times, we have extended our previous observations showing that GABA-IR V1 dendrites, mainly filled with round vesicles, were detected only in type Ia and IIa rat glomeruli (Bardoni et al., 2007).

An unequivocal definition of the ultrastructural features of GABAergic synapses in SGR is today perhaps less substantial than in the nineties of the last century, when many of the above papers were published. In fact, we now have realized that vesicles' shape did not always correlate with the excitatory or inhibitory function of the synapse, as well as that GABA-IR synapses not always displayed the canonical ultrastructure of Gray's type II (Klemann and Roubos, 2011)[15]. In addition, we have understood that cation-chloride cotransporters also regulated Cl^- homeostasis with fundamental implications for the direction and magnitude of anion flow through $GABA_A$Rs (Price *et al.*, 2005).

Another important issue of discussion has been the incidence of the axo-axonic GABAergic synapses that are traditionally the structural substrate of presynaptic inhibition. In SGR, an important mechanism producing PAF presynaptic inhibition occurs via a channel-mediated depolarization (primary afferent depolarization - PAD) of their terminals. Minimally trisynaptic pathways mediated PAD of LTMs, with GABAergic interneurons forming last-order axo-axonic synapses onto the PAF terminals (Rudomin, 2009). However, the $GABA_A$R-mediated PAD was not fully abolished when neurotransmission was principally limited to monosynaptic actions. This suggested that several types of synaptic configurations and not only axo-axonic synapses could be involved in PAD (Shreckengost *et al.*, 2010). If so, as the functional importance of PAD in SGR is indubitable, the debate on the relative abundance of the different types of synapses made by the GABAergic interneurons would lose much of its interest.

As it is the case of glutamate, a vesicular GABA transporter (VGAT) is responsible for the uptake of GABA into synaptic vesicles (Chaudhry *et al.*, 1998). Thus, it is possible to use VGAT as a marker of the SGR inhibitory neurons, but because VGAT also loads vesicular glycine, this approach did not allow differentiating between the GABAergic and the glycinergic neurons.

[15] It may be important to remember here that the morphology synapses is strictly dependent on the quality of fixation and the appropriateness of the subsequent steps that are needed to prepare the spinal cord for TEM observation. Post-embedding immunogold localization with primary antibodies against GABA usually requires fixation with high concentrations of glutaraldehyde and is compatible with osmium post-fixation. This offers a quasi-optimal tissue preservation where inhibitory synapses display pleomorphic vesicles and are clearly symmetric, at least when examined in series. A recent quantitative 3D analysis of mouse hippocampal synapses using data from focused ion beam scanning electron microscopy (FIB-SEM) has demonstrated that vesicles in excitatory synapses appeared to be of oblate ellipsoid shape whereas in inhibitory synapses they assumed the form of a cigar ellipsoid shape (Khanmohammadi et al., 2015). These observations highlight the difficulty of a proper recognition of the shape of synaptic vesicles at functionally different synapses.

Immunocytochemical studies on GABAergic neurons demonstrated that they might contain several other neuroactive substances, including acetylcholine and glycine, ENK and neuropeptide Y (NPY), nitric oxide synthase (NOS) or parvalbumin (Laing *et al.*, 1994; Rowan *et al.*, 1993; Spike *et al.*, 1993).

2.4.1.3.2. Glycine

The initial attempts to stain the spinal cord with anti-glycine antibodies resulted in successful staining of a subpopulation of lamina II neurons (Todd et al., 1996; Todd and Sullivan, 1990). The colocalization of glycine with GABA was established from the very first immunocytochemical studies on SDH (Todd and Sullivan, 1990). Glycinergic neurons consistently represented a subpopulation of GABAergic neurons, as there were no pure glycinergic cells. After quantitative analysis, they resulted about 50% of the total population of GABAergic neurons (Polgár et al., 2003). Glycinergic neurons also contained the glycine transporter 2 (GLYT2) mRNA (Zafra et al., 1991) and protein (Spike et al., 1997) after ISH studies and/or immunocytochemical labeling. Much of what we today know about glycinergic neurons in CNS has come from the work of Zeilhofer's laboratory, in particular through the use of their GlyT2-EGFP mouse (Zeilhofer *et al.*, 2005). As far as this discussion is concerned, they have demonstrated a remarkable correspondence of EGFP and GlyT2 immunocytochemical staining in the spinal cord. The EGFP-positive neurons were low in number in SGR that, instead, displayed a dense network of GlyT2+EGFP double labeled axons and terminals. In the same paper, Zeilhofer and colleagues have also investigated the active and passive membrane properties of the glycinergic SGR interneurons in acute slice preparations. They did not find differences between the functional properties of EGFP-positive and negative neurons, stressing the need of a genetic tag to identify the glycinergic neurons and functionally investigate them in whole-cell patch-clamp recordings.

2.4.1.4. GABA and glycine receptors

2.4.1.4.1. GABA receptors

There are two classes of GABA receptors named $GABA_A$ and $GABA_B$. $GABA_A$ receptors ($GABA_A$Rs) are ligand-gated ion channels (ionotropic). The assembly of five subunits (named with the Greek letters α-ε), selected from nineteen different isoforms, forms the receptor complex that surrounds a central Cl^- channel (Sigel and Steinmann, 2012). $GABA_B$ receptors ($GABA_B$Rs) are, instead, G protein-coupled receptors (metabotropic), existing in two subtypes named $GABA_{B1}$ and $GABA_{B2}$ (Kaupmann *et al.*, 1998).

Staining with two monoclonal antibodies for the β2-β3 subunits of $GABA_A$R showed intense immunoreactivity in small-sized cell bodies of rat and cat lamina II_i (Alvarez *et al.*, 1996). Another study in rat analyzed the distribution of the $GABA_A$R subunits α1, α2, α3, α5, β2-β3 and γ2 (Bohlhalter *et al.*, 1996). In lamina II, these authors were able to find immunoreactivity for α2, α3, β2-β3 and γ2, but reported that cellular elements were difficult to recognize even by confocal laser microscopy. In mouse, some neurons in lamina II_o expressed α2 and α3, whereas others in lamina II_i had only a single subunit (Paul *et al.*, 2012). As in rat, it was difficult to assess with certainty the presence of positive cell bodies. RT-PCR and Western blot studies in mouse have very recently demonstrated expression of the $GABA_A$R subunit δ in SGR (Iura *et al.*, 2016).

Using ICC, it was well documented that rat SGR neurons expressed $GABA_B$Rs (Margeta-Mitrovic *et al.*, 1999) or, specifically, its $GABA_{B1}$ subunit (Charles *et al.*, 2003).

A large amount of work has confirmed that GABARs were fully functional in SGR and had an important role in nociception (Choi *et al.*, 2008; Fukuhara *et al.*, 2013; Grudt and Henderson, 1998; Grudt and Williams, 1994; Iura *et al.*, 2016; Kato *et al.*, 2006; Wakita *et al.*, 2016; Yowtak *et al.*, 2011). Notably, several electrophysiological studies demonstrated that the exogenous or endogenous activation of presynaptic $GABA_B$Rs inhibited the release of glutamate and peptides

from the terminals of Aδ and C PAFs and decreased the release of GABA and glycine from the spinal inhibitory interneurons (Ataka *et al.*, 2000; Yang and Ma, 2011; Yang *et al.*, 2015). In rat, we have very recently shown that laminae III-IV neurons synaptically connected to Aβ LTMs expressed functional $GABA_BRs$ at their dendrites, and discussed the possibility that some of these neurons projected to SGR and might be involved in allodynia (Salio *et al.*, 2017).

2.4.1.4.2. Glycine receptors

Glycine activates a transmembrane chloride channel that is selectively blocked by strychnine. The selectivity of the strychnine block at these glycine receptors is very important, as NMDARs also possess a glycine binding site. Strychnine-sensitive glycine receptors exist in four α subunits (α1-α4) and one β subunit (Zeilhofer *et al.*, 2012).

One of the first autoradiographic studies on glycine receptors was carried out in post-mortem samples of human spinal cord and reported that binding sites were uniformly distributed in the gray matter (Gillberg and Aquilonius, 1985), but a subsequent human survey reported highest densities in the SGR (Probst *et al.*, 1986). However, in two rat studies SGR neurons were not immunostained with antisera directed against the purified glycine receptor (Basbaum, 1988; Mitchell *et al.*, 1993). After ISH, the α1 and β subunit mRNAs were abundant in the DH, with comparatively low levels of expression of α2 (Malosio *et al.*, 1991). Although the immunocytochemical localization of glycine receptors may have been difficult for technical reasons, numerous reports converged to demonstrate that these receptors are indeed present and functional in SGR (see for example Grudt and Henderson, 1998; Grudt and Williams, 1994; Wakita *et al.*, 2016).

2.4.1.5. Acetylcholine

Cholinergic neurons have been unequivocally detected in the SDH of both rodents and primates. In rat, cholinergic cell bodies were demonstrated after choline acetyltransferase immunostaining and resulted to be mostly located in lamina III, but some of their terminals were in lamina II and might participate in synaptic glomeruli and be presynaptic to PAFs (Ribeiro-da-Silva and Cuello, 1990a). In a subsequent study combining immunocytochemistry and retrograde tracing, these neurons were shown to make contacts with both myelinated and unmyelinated PAF terminals (Olave et al., 2002). Lamina III cholinergic neurons also contained GABA, but not glycine (Todd, 1991; Ribeiro-da-Silva, 2015). In primate, cholinergic cells bodies have been found in lamina II as well, and terminals also participated in synaptic glomeruli where they co-localized with GABA immunoreactivity (Pawlowski *et al.*, 2013).

2.4.1.6. Acetylcholine receptors

Acetylcholine receptors (AchRs) are transmembrane receptors of two main kinds: nicotinic and muscarinic (mAchR).

2.4.1.6.1. Nicotinic acetylcholine receptors (nAchRs)

nAChrRs are ionotropic Na^+-, K^+- and Ca^{2+} channels that modulate membrane potential. Each receptor is a pentamer composed of different combinations of subunits (α1-10, β1-4, δ, ε and γ). Neuronal nAChR are heteropentameric and homopentameric assemblies of subunits of a gene family where specified combinations of α and β subunits form the functional receptors (Fasoli and Gotti, 2015).

In SGR, using affinity-purified specific antibodies, the α3, α4, α5, β2, and β4 subunits were all demonstrated on PAF terminals binding the isolectin B4 (IB4 - Khan *et al.*, 2003). nAchRs containing α2-β2 subunits were demonstrated to facilitate glycine release in rat (Kiyosawa *et al.*, 2001), whereas, in mouse, the α4-β2 subtype exerted a tonic inhibitory control on nociceptive transmission (Rashid *et al.*, 2006).

2.4.1.6.2. Muscarinic acetylcholine receptors (mAchRs)

mAChRs are metabotropic G protein-coupled receptors of five subtypes (M1-M5) that are widely expressed in the nervous system (Haga, 2013). A significant proportion of neurons in SDH expressed the M2 subtype; these M2+ neurons were about one third of those immunoreactive for GABA, NOS or the somatostatin receptor 2a (Stewart and Maxwell, 2003). The M2 receptor was also localized to PAFs, as demonstrated after dorsal rhizotomy and immunocytochemistry (Li *et al.*, 2002).

mAchRs are known to intervene in the regulation of fast neurotransmission in DH and to exert antinociceptive effects; the underlying mechanisms derive from direct and indirect inhibitory effects onto lamina II neurons, but are still not fully understood, as interspecies differences have emerged, specifically following observations on knockout mice.

Initial functional studies described a muscarinic facilitation of GABA release in rat SGR: it was thus hypothesized that this facilitatory effect was at the basis of the antinociceptive action produced by the intrathecal administration of muscarinic agonists and acetylcholinesterase inhibitors (Baba *et al.*, 1998). In the following decade, the Pan's group has devoted many efforts to unravel the mechanisms of action of mAchRs in rodent SGR using patch clamp electrophysiological recordings. They first demonstrated that presynaptic mAchRs inhibited glutamate release onto rat lamina II neurons (Li *et al.*, 2002). In a subsequent mouse study, they established that the M2 mAChR expressed by PAFs played a critical role in reducing the glutamatergic input to SGR neurons, whereas the M3 and M2/M4 subtype expressed by a subpopulation of SGR interneurons reduced glutamate release from excitatory lamina II interneurons (Zhang *et al.*, 2007a). Later, they reported that M5 was, instead, the predominant receptor subtype that potentiated glutamatergic synaptic transmission in mouse spinal cord (Chen *et al.*, 2010).

The Pan's group also showed that the M3 subtype was localized on glycinergic interneurons in rat where it was the principal responsible for the muscarinic potentiation of glycine release (Wang *et al.*, 2006), and mouse (Zhang *et al.*, 2007b). They also observed that the concurrent stimulation of rat mAChRs on adjacent GABAergic interneurons attenuated glycine release from the M3+ interneurons after activation of presynaptic $GABA_BRs$ (Wang *et al.*, 2006). Using different strains of mAchR knockout mice this group also reported that M3 increased GABA release from the lamina II interneurons, whereas presynaptic M2/M4 mainly attenuated the GABAergic inputs to lamina II neurons. The latter effect was opposite to that of M2/M4 in rat (Zhang *et al.*, 2006, 2009), where acetylcholine enhanced GABAergic (but not glycinergic) transmission also after activation of pronociceptive phospholipase A2 (Liu et al., 2011).

Finally, in knockouts, stimulation of presynaptic M2/M4 reduced the glycinergic input to SGR neurons (Zhang *et al.*, 2007b), and the M3 and M2/M4 subtypes, expressed on a subpopulation of lamina II excitatory interneurons reduced glutamate release from these neurons (Zhang et al., 2007b).

2.4.2. Neuropeptides and neuropeptide receptors

The SGR is an area of the spinal cord gray matter that is particularly enriched in neuropeptides of different origins, and the SGR interneurons are themselves an important source of neuropeptides. The results of the first immunocytochemical studies on peptide containing neurons in SGR converged to demonstrate that - with few remarkable exceptions (see below) - these neurons were short-axoned interneurons (for review see Todd and Spike, 1993). Another generalization that can be drawn from the existing literature is that SGR neurons very often - and not differently from most central and peripheral neurons - synthesize a fast excitatory or inhibitory neurotransmitter together with one of more neuropeptide(s) - see Box 4. Finally, with the exception of the ENKs (Marvizón *et al.*, 2009; Todd *et al.*, 1992b, 2003, 2006; Todd and Spike, 1993) and dynorphin (Sardella *et al.*, 2011b), which may be expressed by both the

glutamatergic and the GABAergic neurons, all other peptides appear to have a mutually exclusive localization in excitatory or inhibitory neurons.

2.4.2.1. Enkephalins, dynorphins and opioid receptors

2.4.2.1.1. Enkephalins

ENKs are two endogenous opioid pentapeptides that differ for a single aminoacid (Met-enkephalin and Leu-enkephalin) and display slight differences in opioid receptor preference[16]. The immunocytochemical distribution of the two neuropeptides has been extensively studied from the very beginning of the ICC era, showing intense labeling of the SGR (Simantov *et al.*, 1977). The interest on ENKs grew rapidly after the seminal demonstration that they hyperpolarized the SGR neurons in vitro (Yoshimura and North, 1983). Differently from other widely investigated neuropeptides, ENK seemed to be only intrinsic to the spinal cord, as experiments in which PAFs were depleted of their peptide content by neonatal administration of capsaicin or surgical manipulations (Pohl *et al.*, 1990) had not effect on immunoreactivity within the SGR. There were discrepancies in the observations on the prevalence of ENK in the different laminae (sublaminae) of the SDH, but it should be noted that most studies were carried out when ICC was indeed very challenging, and that not all these studies were based on appropriate statistical analyses (for a list of relevant references referring to laminar/sublaminar distribution across species see Willis, Jr. and Coggeshall, 2004a).

In humans (DeLanerolle and LaMotte, 1982), cat (Bennett *et al.*, 1982) and rat (Todd *et al.*, 1992b), both the islet and the stalked cells were reported to contain ENK. However, another study in humans indicated that were, instead, the stellate cells to express the pentapeptide (Schoenen *et al.*, 1985). In rat, ENK coexisted with GABA in the islet cells, which are inhibitory for the most, but the ENK-positive stalked cells were not IR for GABA (Todd *et al.*, 1992b). This result was in line with the observations indicating that the majority of ENK-expressing neurons in SGR were glutamatergic (Marvizón *et al.*, 2009; Todd and Spike, 1992). However, ENK-positive terminals represented only a small fraction of the total number of glutamatergic terminals in SGR (Marvizón *et al.*, 2009). Preproenkephalin-GFP transgenic mice were also developed to better identify the ENK-expressing neurons (Huang *et al.*, 2010). The proportion of GFP-tagged neurons in SGR was around 40%, and GABA was found to co-exist with ENK in about 43% of laminae I-III neurons. Some of the SGR neurons expressing ENK also contained SP (Ribeiro-Da-Silva *et al.*, 1991) or somatostatin (SST - Marvizón *et al.*, 2009).

The first ultrastructural observations on ENK localization demonstrated that the peptide was selectively stored in LDCVs, leading to put forward the hypothesis that ENK could act as a neuromodulator in SGR (Pelletier and Leclerc, 1979).

Studies in monkey with pre-embedding ICC showed that ENK immunoreactivity was concentrated in lamina II_o (LaMotte and de Lanerolle, 1983a). The pre-embedding approach did not permit to adequately recognize the subcellular site of localization, but confirmed that LDCVs were present in positive terminals together with SSVs. ENK-IR terminals often formed asymmetric synapses, most frequently with dendrites of various sizes and dendritic spines. Presynaptic ENK-IR axons were also observed to be part of glomeruli, corresponding to the V2 profiles of subsequent classifications. LaMotte and de Lanerolle (1983a) described the occurrence of ENK-IR terminals in monkey LDCV glomeruli (corresponding to type Ib peptidergic glomeruli in rodents) and RSV glomeruli (corresponding to type II non-peptidergic glomeruli in rodents). In the latter, the ENK-IR axon terminal was, in turn, presynaptic to a glomerular dendrite. The same authors have observed that the central bouton of the LDCV

[16] As it has been difficult to discriminate between Met- and Leu-enkephalin in localization studies I will use the term ENK in a more general sense.

glomeruli with ENK-IR V2 profiles had the same ultrastructural features of the SP-IR central boutons described in a different study (DeLanerolle and LaMotte, 1983).
In cat, ultrastructural analysis revealed that ENK-IR profiles were more heterogeneous than in monkeys (Glazer and Basbaum, 1983). In this study, ENK-IR dendrites were seen to be, for the most, postsynaptic to unlabeled axons of presumptive primary afferent origin, but also to ENK-IR axons at dendro-axonic synapses. The majority of ENK-IR axons were engaged in axo-dendritic synapses, but some axo-axonic synapses with unlabeled axons containing flat vesicles were also seen, as well as a few axo-somatic synapses. Analysis of serial ultrathin sections revealed a triad composed of an ENK-IR axon and an unlabeled axon with flat vesicles both contacting an unlabeled dendrite. An additional type of ENK-IR profile was found in glomeruli of lamina II_i (Glazer and Basbaum, 1983). It was characterized by its content in SSVs, but it was impossible to ascertain whether it was a V1 dendrite or a V2 axon.
In rat glomeruli, ENK-IR was mainly detected in V2 profiles and, much less frequently, in plain dendrites or V1 profiles (Ribeiro-Da-Silva *et al.*, 1991). Ribeiro-da-Silva and colleagues also observed some ENK+SP double labeled plain dendrites that were part of type Ib glomeruli and reported that ENK (and SP)-IR axonal varicosities and dendrites were most commonly not engaged in glomeruli all across the SGR.

2.4.2.1.2. Dynorphins

Dynorphins (DYNs) are opioid peptides widely distributed in several areas of CNS including the spinal cord, and derived from the precursor protein prodynorphin. Cleavage of prodynorphin generates DYN A, DYN B, and α/β-neo-endorphin (Day *et al.*, 1998).
Initial studies on DYNs were carried out by radioimmunoassay (RIA) and gel permeation chromatography to characterize IR species. With this approach DYN and α-neo-endorphin were described to be highly concentrated in the human thoracic SGR (Przewéocki *et al.*, 1983). In rat and rabbit, DNY was also detected in spinal cord extracts using RIA, and dorsal rhizotomy or mid-thoracic spinal cord transection demonstrated an intrinsic origin of immunoreactivity (Botticelli *et al.*, 1981; Pohl *et al.*, 1990). Initial localization studies in cat reported that, differently from ENK, DYN was mainly detected in laminae I and V of the spinal cord and not in SGR (Cruz and Basbaum, 1985). In keeping with this observation, antibodies microprobes detected local release of IR DYN A1-8 in rat laminae I and IV-V (Riley *et al.*, 1996).
DYN A and prodynorphin were more recently reported to be present in some SGR neurons (Marvizón *et al.*, 2009). Later, about eighty percent of DYN A-IR neurons, identified by the presence of preprodynorphin, were shown to contain GABA and galanin, and to be about 11% of the total population of the inhibitory neurons in SGR; the remaining twenty percent were not IR for GABA and thus, presumably, glutamatergic (Sardella *et al.*, 2011b). A succeeding study has confirmed the existence of prodynorphin-IR excitatory neurons that responded to nociceptive stimuli, and made synapses onto projection neurons having their cell bodies in lamina III (Baseer *et al.*, 2012).
The central projections of the DRG neurons may be another (less conspicuous) source of DYN in SGR (Botticelli *et al.*, 1981; Marvizón *et al.*, 2009).
Functionally, whole-cell patch clamp recordings from isolated spinal cord neurons showed that Ca^{2+} currents were inhibited by DYN A (Sah, 1990), and initial electrophysiological studies in intact animals or spinal cord slices converged to demonstrate that DYN A, by binding to opioid receptors, had an inhibitory effect onto PAF neurotransmission. After extracellular recordings of cutaneous sensory responses, DYN A1-13 caused a selective reduction of the nociceptive responses of cat spinocervical tract neurons if given in proximity of these cells, but not into the SGR (Fleetwood-Walker *et al.*, 1988). In rat, DYN A1-17 reduced the synaptic responses of SGR neurons by inhibiting the AMPAR-mediated excitation from PAFs (Randic *et al.*, 1995).

More recently, in a very elegant work with intersectional ablation of excitatory and inhibitory neurons in spinal cord, SGR neurons expressing DYN were shown to be necessary to gate Aβ fibers from activating SST nociceptive SGR neurons (Duan *et al.*, 2014). Also, current evidence has accumulated that DYN, released by inhibitory SGR neurons, can reduce itch (Kardon *et al.*, 2014).

2.4.2.1.3. Opioid receptors

Opioid receptors are a group of inhibitory G protein-coupled receptors. ENKs are not the only endogenous ligands of these receptors that also bind DYNs, endorphins, endomorphins and nociceptin. There are four major classes of opioid receptors: the three classical receptors (μ, δ, κ) and the non-classical (nociceptin) receptor. They should be properly named MOP, DOP, KOP and NOP, respectively (McDonald and Lambert, 2005). Each is further subdivided in subtypes. Opioid receptors are almost 40% identical to SST receptors. ENK have the highest preference for DOPs, a lower preference for MOPs and little or no effect on KOPs.

The first localization of SGR opioid receptors was carried out in rat using autoradiography with [^{3}H]diprenorphine, a potent opiate antagonist (Pert *et al.*, 1976). Then, it was demonstrated that opioid receptors played a role in morphine analgesia (Johnson and Duggan, 1981). Other autoradiography studies in monkey (Wamsley *et al.*, 1982), humans (Czlonkowski *et al.*, 1983) and rat (Waksman *et al.*, 1986) confirmed the abundance of opioid receptors in SGR.

Limited information was, instead, obtained using ICC. MOPs have been detected in small size SGR neurons, preferentially within lamina II$_i$, in humans (Abbadie *et al.*, 2000) and rat (Ding *et al.*, 1995). In the latter species, ultrastructural observations suggested that MOPs were localized to neurons, but had extrasynaptic distributions (Cheng *et al.*, 1996; Moriwaki *et al.*, 1996). Small unmyelinated axons and axon terminals constituted 48% and 15%, respectively, while dendrites were 36% of IR profiles. Such a pattern of quantitative distribution was compatible with the idea that immunoreactivity in SGR derived from propriospinal projection neurons and/or short axoned cells. In double labeling experiments for MOP and Leu-ENK, 21% of the MOP-IR dendrites were closely apposed or postsynaptic to Leu-ENK-IR axon terminals (Cheng *et al.*, 1996). Together with the aforementioned extrasynaptic distribution of MOPs, these observations suggested that ENK volume transmission occurs in SGR, but data are still too few to draw a conclusion.

Physiologically, MOPs have been shown to inhibit glycine receptor- and GABA$_A$R-mediated inhibitory neurotransmission (Grudt and Henderson, 1998), whereas postsynaptic KOPs have been demonstrated to inhibit AMPAR-mediated PAF signaling (Randic *et al.*, 1995). More recent studies from the Basbaum's group demonstrated a selective control of heat and mechanical pain by MOPs and DOPs (Scherrer *et al.*, 2009).

2.4.2.2. Tachykinins and tachykinin receptors

2.4.2.2.1. Tachykinins

The tachykinins are one of the largest family of neuropeptides that comprises, among others, neurokinin A (NKA - formerly named substance K), neurokinin B (NKB), neuropeptide K (or neurokinin K), and SP (Steinhoff *et al.*, 2014).

2.4.2.2.1.1. Substance P

SP was the first neuropeptide to be implicated in nociception (Lembeck, 1953; Pernow, 1953). Therefore an impressive amount of literature dealt with its localization in the spinal cord, where the peptide concentrates in laminae I-III and, less intensely, V (Willis, Jr. and Coggeshall, 2004a). Many of the SGR neurons expressing SP have been identified as stalked cells in rat (Ribeiro-Da-Silva *et al.*, 1991). In this species, virtually all neurons that expressed SP co-expressed ENK (Ribeiro-Da-Silva *et al.*, 1991; Senba *et al.*, 1988) and VGLUT2 (Todd *et al.*, 2003),

and thus were considered to be excitatory. In mouse, the SP precursor preprotachykinin A was as well detected in lamina II_o excitatory neurons (Gutierrez-Mecinas *et al.*, 2017).
Besides the intrinsic neurons, peptidergic PAF (pPAF) terminals were another important source of SP (and other tachykinins) in SGR (Tuchscherer and Seybold, 1989). These terminals, which have been extensively characterized at the ultrastructural level (Merighi *et al.*, 1991; Ribeiro-Da-Silva, 1995), originated from peptidergic small- to medium-sized dark neurons in DRGs that give rise to nociceptive thin-myelinated Aδ or unmyelinated C fibers (see also Fig. 3A). Functionally, these fibers were Aδ cutaneous HTMs (Nagy and Hunt, 1983) or C high threshold nociceptors, and preferentially terminated in lamina II_o (Lawson *et al.*, 1997).
Quantitative light microscopy studies in normal and rhizotomized rats were carried out to demonstrate the source(s) of SP immunoreactivity in the DH. One of these studies proved that more than one third of the SP-IR varicosities in SGR disappeared after dorsal rhizotomy, and that the varicosities double labeled for SP and calcitonin gene-related peptide (CGRP), a recognized marker of pPAFs, or galanin were the ones to be depleted (Tuchscherer and Seybold, 1989). Another quantitative study demonstrated that, in PAF terminals, SP also colocalized with cholecystokinin (CCK - Tuchscherer *et al.*, 1987). Thus, the coexistence of SP with CGRP, galanin or CCK in SGR varicosities testified their primary afferent origin. Ultrastructurally, in PAF terminals SP was segregated within LDCVs where it steadily colocalized with CGRP (Merighi *et al.*, 1991), galanin (Zhang *et al.*, 1993) or the brain-derived neurotrophic factor (BDNF - Merighi *et al.*, 2008a, 2008b). In rats and mice, we have demonstrated the occurrence of triple-labeled LDCVs containing SP+CGRP+BDNF in SGR terminals (Merighi *et al.*, 2008a). It is highly probable that at least some of these LDCVs also stored galanin, but there is not a direct ultrastructural evidence of this. Multiple peptide-containing terminals also contained glutamate, instead stored in SSVs, as their main neurotransmitter (Merighi *et al.*, 1991), and were engaged in simple axo-somatic synapses or formed the C boutons of type Ib glomeruli (Merighi *et al.*, 2008b).

2.4.2.2.1.2. Other tachykinins

Localization studies on NKA have been less numerous and received minor attention than those on SP. It is worth noting that, in sensory neurons, NKA colocalized with SP (Dalsgaard et al., 1985) and both peptides were demonstrated to be co-stored in individual LDCVs in neuronal cell bodies of the DRGs (Merighi *et al.*, 1988) and their central projections in SGR (Merighi *et al.*, 1989). In rat spinal cord slices, NKA and SP enhanced the release of glutamate (and aspartate) after electrical stimulation of a dorsal root (Kangrga and Randic, 2001) and a subsequent functional study has indicated that NKA not only bound the NK-1 receptor (see below) but had a more relevant function than SP as a pronociceptive primary afferent neurotransmitter (Trafton et al., 2001).
The distribution of NKB has been the subject few studies in which the tachykinin was localized using an antibody raised against the "peptide 2" portion of its precursor sequence, a peptide that is cleaved off during formation of mature NKB (McLeod et al., 2000), or an antibody against the NKB precursor preprotachykinin B (Gutierrez-Mecinas *et al.*, 2016; Polgár *et al.*, 2006). These studies demonstrated that NKB immunoreactivity occurred throughout the SDH, with the highest density in lamina II_i. Immunoreactive profiles were axon terminals, dendrites and neuronal cell bodies. Colocalization experiments showed that NKB+ profiles were not immunoreactive for SP or GABA, suggesting that the circuits made by NKB were separated from those involving SP (McLeod *et al.*, 2000).

2.4.2.2.2. Tachykinin receptors

There are three G protein-coupled tachykinin receptors named NK1, NK2 and NK3, preferentially binding SP, NKA and NKB, respectively. Receptors are not fully specific, but have different affinity for single tachykinins (Steinhoff *et al.*, 2014).
The distribution of NK1 was extensively studied in DH once it became evident that the receptor undergoes a remarkable plasticity after nociceptive stimulation (Trafton *et al.*, 2001). Although the SGR contained IR dendrites, light microscopy localization studies demonstrated that NK1-IR cell bodies were, for the most, outside SGR, in laminae I and III-V (Willis, Jr. and Coggeshall, 2004a), and that the population of SP-responsive SGR neurons in rat was very limited (Bleazard *et al.*, 1994). In mouse, using [Sar^9, $Met(O_2)^{11}$]-SP, a ligand that specifically binds SP, we showed the existence of inhibitory (purely GABAergic or mixed GABAergic/glycinergic) agonist-responsive SGR neurons of heterogeneous morphology (Vergnano *et al.*, 2004). After immunocytochemical staining, we also confirmed the presence of NK1 in oval-to-fusiform neurons with the typical morphological features of the islet cells. At the ultrastructural level, NK1-IR was initially detected independently form synaptic specializations at simple axo-dendritic contacts (Zerari *et al.*, 1998). Subsequently, NK1-IR was shown to be present together with TRPV1-IR in a fraction of SGR dendrites (Doly *et al.*, 2004).
NK3 has also been detected in SGR neurons, and 86% of the NO synthase (NOS)-IR neurons in lamina II were NK3-IR (Seybold *et al.*, 1997).
Functionally, Bleazard and colleagues (1994) reported that only 10% of SGR neurons responded to the application of a selective NK1 receptor agonist, and that SGR neurons were equally insensitive to specific NK2 and NK3 agonists. However, in another study it was demonstrated that a large majority of lamina II neurons responded to NK1, fewer to NK2 and none to NK3 agonists (Bentley and Gent, 1995). The same study also showed that some neurons responded to both NK1 and NK2 agonists and that, in most cases, responses were excitatory, although inhibitory effects were also observed.

2.4.2.3. Somatostatin and somatostatin receptors

2.4.2.3.1. Somatostatin

SST, also known as somatotropin release inhibiting factor, is a tetradecapeptide (SST-14). A second active endogenous form is SST-28, which consists of SST-14 extended by another 14 aminoacid residues at the N-terminal. SST-14 is the predominant form in neurons (Viollet *et al.*, 2008)[17].
SST expressing SGR neurons were described in humans (Schoenen *et al.*, 1985) and rat (Alvarez and Priestley, 1990; Mather and Ho, 1992; Ribeiro-Da-Silva and Cuello, 1990b). Most of these initial studies converged to indicate that IR neurons were islet cells, but, after Golgi-impregnation, some were stalked cells or cells with other morphologies (Mather and Ho, 1992). On these bases it was hypothesized that the majority of SST-IR SGR neurons were inhibitory. Subsequent studies in rat have, instead, shown that most SST-IR boutons of non-primary afferent origin were IR for VGLUT2 and formed synapses at which AMPARs were also expressed; these observations demonstrated the glutamatergic nature of SST-IR SGR neurons (Todd *et al.*, 2003). In a combined light and pre-embedding ultrastructural study (Ribeiro-Da-Silva and Cuello, 1990b), SST-IR neurons, of small size and with rostro-caudally oriented dendrites, were reported to be concentrated in lamina II_i. Their axons contained a mixture of round SSVs and less numerous LDCVs, were presynaptic to dendrites or neuronal cell bodies and postsynaptic to dendrites in dendro-axonic contacts. SST-IR dendrites were also described,

[17] Antisera used in immunocytochemical localization studies not always had a well-characterized specificity enabling to discriminate between the two forms of SST. Therefore, in the following description I will simply refer to SST in general terms.

sometimes, as V1 profiles in glomeruli. Altogether, these observations were supportive for the identification of SST-IR neurons as central and large vertical cells.

As for SP, a second SST-containing system of primary sensory origin also exists in SGR, initially demonstrated following dorsal rhizotomy (Tuchscherer and Seybold, 1985, 1989). SST-IR PAF terminals derived from the small- to medium-sized dark neurons in DRGs that gave rise to nociceptive thin-myelinated Aδ or unmyelinated C fibers (see also Fig. 3). These neurons were also IR for CGRP, but not SP, after quantitative studies (Salio and Ferrini, 2016). At ultrastructural level, SST immunoreactivity was specifically localized to LDCVs after post-embedding gold labeling procedures (Merighi *et al.*, 1989). In their TEM pre-embedding study, Ribeiro-da-Silva and Cuello (1990b) have observed that, in the middle third of SGR, a small number of SST-IR axon terminals were the C boutons of type Ib glomeruli, and thus of primary afferent origin. These observations were subsequently confirmed in my laboratory by showing SST+CGRP costorage in LDCVs within simple SGR axonal varicosities or C boutons in type Ib glomeruli (Merighi *et al.*, 2011). We also showed that SST-IR profiles were about 25% of the total number of CGRP immunolabeled profiles and, by triple immunogold labeling, we furthermore demonstrated costorage of SST, CGRP and the glial-derived neurotrophic factor (GDNF) in the LDCVs herein contained (Salio *et al.*, 2014). Altogether these observations reinforced knowledge that the SST-IR PAF terminals originated from a subset of peptidergic DRG neurons (Salio *et al.*, 2014; Salio and Ferrini, 2016).

2.4.2.3.2. Somatostatin receptors

In humans, rat and mouse, there are five cloned SST receptor subtypes that were originally classified into two groups. The two groups (SST1 and SST2) were differentiated by their affinities for the SST analogues octreotide (SMS-201,995) and seglitide (MK-678): SST1 receptors have high affinity for both analogues, whereas the affinity is lower for members of the SST2 group. Later, the SST1 group was found to contain three receptor subtypes, now named sst2, sst3 and sst5, and the SST2 group was found to comprise two subtypes, today named sst1 and sst4. All receptor subtypes bind SST-14 and SST-28 with similar high affinities, except sst5, which has a much greater affinity for SST-28 than SST-14 (Møller *et al.*, 2003). sst2 further exists in two isoforms named sst2a and sst2b that bind SST with similar high affinity, but differ in resistance to agonist-induced desensitization (Vanetti *et al.*, 1993).

Initial localization studies, mainly in rat, demonstrated that sst2 and sst2a were expressed by spinal neurons concentrated in the superficial laminae of the DH, whereas sst2b was localized sparsely in the entire spinal gray matter (Willis, Jr. and Coggeshall, 2004a). In rat, sst2a expression was restricted to GABA-IR neurons that, for the most, were identified as islet cells (Todd *et al.*, 1998). In mouse, after ICC and patch clamp recording, we have confirmed that some SGR neurons expressed sst2a and functionally responded to octreotide (Bencivinni *et al.*, 2011). In the same study, by ultrastructural immunogold labeling we showed that sst2a not only was localized to axons (v2) and postsynaptic dendrites in type II glomeruli, but also in the C boutons of type Ia glomeruli, thus confirming the existence of an extraspinal DRG source of sst2a. By light microscopy ICC, it was later estimated that around half of the inhibitory interneurons in SGR expressed sst2a in rat (Polgár *et al.*, 2013b) and mouse (Iwagaki *et al.*, 2013; Polgár *et al.*, 2013a). Another recent study was confirmatory of receptor localization in mouse and provided additional information on sst2a expression in human SGR and DRG neurons (Shi *et al.*, 2014). By acting on sst2a, SST activated K^+ channels and postsynaptically hyperpolarized the SGR neurons (Jiang *et al.*, 2003; Kim *et al.*, 2002; Nakatsuka *et al.*, 2008).

2.4.2.4. Calcitonin gene-related peptide (CGRP) and CGRP receptor

CGRP is produced by alternative processing of the primary transcript of the calcitonin gene (Amara et al., 1985). The predominant form of the peptide in sensory pathways is α-CGRP

(Amara et al., 1985). To the present discussion CGRP is of particular relevance because it is highly expressed in DRG neurons and is, thus, a very useful and specific marker of PAF terminals in SGR. Despite the well know functions of the peptide peripheral projections in neurogenic vasodilatation, inflammation and modulation of the immune responses (Aggarwal *et al.*, 2012; Holzmann, 2013), the central effects of CGRP remain somewhat elusive, although it has been long known that the peptide is released in the DH as a consequence of nociceptive PAF stimulation (Duggan *et al.*, 1987; Morton and Hutchison, 1989) and evidence sustained its pronociceptive effects (Sun *et al.*, 2003, 2004).

From the very beginning, immunocytochemical localization studies confirmed the intense labeling of the SGR and the primary afferent origin of CGRP-IR fibers herein distributed in numerous mammals, including humans (Gibson *et al.*, 1981; Gibson and Polak, 1986; Wall *et al.*, 1981). Ultrastructural studies subsequently demonstrated that CGRP was selectively localized to LDCVs in glutamatergic terminals of the rat SGR (Merighi *et al.*, 1991). Work in monkeys, then, analyzed in detail the ultrastructure and synaptic circuitry of physiologically-identified CGRP-IR C fiber terminals in lamina II (Alvarez *et al.*, 1993).

In the course of the following decades, full consensus was reached that CGRP in DH exclusively originated from PAFs: this allowed establishing the primary afferent origin of simple axo-dendritic synapses where the axon was CGRP-IR and that the peptide was specifically localized in C boutons of type Ib glomeruli in rat (Ribeiro-Da-Silva, 2015) and several other species. Work from various laboratories has now led to the demonstration that LDCVs in CGRP-IR terminals also store additional neurochemicals such as SP (Merighi *et al.*, 1991; Salio *et al.*, 2007), galanin (Zhang *et al.*, 1993), BDNF (Merighi *et al.*, 2008a; Salio *et al.*, 2007), SST (Ribeiro-Da-Silva, 1995), GDNF (Salio *et al.*, 2014) and, very probably, more others. One of these studies has also quantitatively estimated the ratio of costorage of CGRP, SP and BDNF in individual LDCVs (Salio *et al.*, 2007).

Two types of CGRP receptors have been initially described, following investigations mainly in humans and rats (Quirion et al., 1992; Yashpal et al., 1992). Subsequent studies have demonstrated that the CGRP1 receptor was a complex between the calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1); also, the pharmacological profile of type 2 CGRP receptors ($CGRP_2$) has been clarified by showing that it may be generated by other receptors of the adrenomedullin or the amylin families (Hay et al., 2008). Therefore, the term CGRP2 should be abandoned and CGRP1 should be the sole CGRP receptor.

Autoradiography quantitative studies of αCGRP binding sites in rat and monkey demonstrated that the SGR contained relatively lower, but still significant, densities of [^{125}I]αCGRP binding sites, compared to other DH laminae (Yashpal *et al.*, 1992). Subsequent immunocytochemical observations in rat indicated that the CGRP receptor was colocalized with CGRP in primary sensory neurons and their central terminals in SGR (Ma *et al.*, 2003). However, another study, in which ICC and electrophysiology were combined together, demonstrated a colocalization of CGRP receptors and AMPARs in DH neurons with an evoked discharge frequency typical of the wide dynamic range (WDR) neurons (Gu and Yu, 2007). Thus localization of CGRP receptor in SGR still needs to be clarified in full.

2.4.2.5. Galanin and galanin receptors

Galanin is a neuropeptide of 29 aminoacids (30 aminoacids in humans) whose function is still largely unknown. The peptide has a role in nociception and is subjected to remarkable plasticity in primary afferent pathways (Landry *et al.*, 2005; Liu and Hökfelt, 2002).

In the normal SGR, galanin immunoreactivity derived from the interneurons as well as the PAF terminals (Arvidsson *et al.*, 1991; Ch'ng *et al.*, 1985; Kordower *et al.*, 1992; Melander *et al.*, 1986;

Merighi *et al.*, 1990). Galanin-IR interneurons were reported to have small cell bodies (Todd and Spike, 1993), and subsequent observations proved that they were also GABA-IR (Simmons *et al.*, 1995). More recent work has shown that the galanin-IR neurons formed a small (3%) but distinct population of rat SGR neurons (Tiong *et al.*, 2011), were concentrated in lamina II_o but also detected in lamina II_i, albeit less numerous. The same authors have calculated that the galanin-IR neurons represented about 10% of the GABAergic interneurons and that their boutons were minimal fractions of the total number of GABA-IR terminals in the superficial laminae of the DH.

The type(s) of SGR neurons that contain galanin is still a matter of discussion: a study from the Hökfelt's laboratory reported that positive neurons in lamina II were often islet cells (Zhang *et al.*, 1995), but this observation remains to be fully acknowledged as derived from an ultrastructural study primarily addressing the coexistence of galanin with other peptides in the spinal DH rather than the morphology of IR cells.

The primary afferent origin of the dense plexus of galanin IR fibers in the SDH was proved following dorsal rhizotomy, in a combined light and electron microscopic study in cat (Arvidsson *et al.*, 1991). In the same survey, coexistence of galanin and SP was demonstrated in the light microscope, and electron microscopic observations revealed that galanin IR terminals were engaged in axo-dendritic or axo-axonic contacts or formed the C bouton of type Ib peptidergic glomeruli. In keeping with these ultrastructural observations, all galanin-IR DRG neurons were of small to medium size, and CGRP-IR.

Galanin acts onto three different receptors named GalR1, GalR2 and GalR3 (Lang *et al.*, 2014). Receptors were originally detected in the SGR by autoradiography and ISH. In adult rats, the GalR1 and GalR2 mRNAs were localized in SGR and DRG neurons, whereas the GalR3 mRNA was rare in the spinal cord and absent in DRGs (Burazin *et al.*, 2000; O'Donnell *et al.*, 1999; Shi *et al.*, 2006). More recent work on knock-in mice expressing fluorescently tagged GalR1 and GalR2 was not confirmative of the presence of GalR2 mRNA in SGR, and only faint levels were detected in DRG neurons (Kerr *et al.*, 2015). The GalR1 protein was instead expressed in neuronal perikarya of DRGs and SGR, albeit at levels lower than in laminae III-IV neurons (Kerr *et al.*, 2015).

Galanin modulates spinal nociception in a complex biphasic fashion and in a dose-dependent manner. Functional work has first demonstrated that the peptide interacted with GalR1 and GalR2, with a diverse array of actions on inhibitory and excitatory SGR neurons, respectively (Alier *et al.*, 2008; Xu *et al.*, 2010). Subsequently, it was shown that peptide activation of GalR2/R3 modulated the Ca^{2+}-dependent spontaneous release of glutamate from PAFs of the Aδ and C types positively (low concentration) or negatively (high concentration), whereas activation of GalR1 produced hyperpolarization of excitatory SGR neurons (Yue *et al.*, 2011). In keeping with this latter observation, the selective destruction of GalR1 expressing neurons in the SDH interneurons resulted in heat hypoalgesia (Lemons and Wiley, 2011). Some of the effects of galanin that are mediated by GalR1 were also related to a mode of action consistent with a transient receptor potential (TRP) channel-dependent mechanism (Hulse *et al.*, 2012).

2.4.2.6. Neurotensin and neurotensin receptors

Neurotensin is a 13 aminoacid neuropeptide distributed throughout CNS, with highest levels in the hypothalamus, amygdala, nucleus accumbens and SGR (Gibson *et al.*, 1981; Uhl *et al.*, 1977). In rat, neonatal administration of capsaicin, which selectively depletes the Aδ and C PAFs, had no effects on neurotensin immunoreactivity in SGR, indicating a source intrinsic to the spinal

cord (Priestley *et al.*, 1982). Another immunocytochemical study - albeit after colchicine treatment[18] - showed that neurotensin-positive neurons were concentrated at the external and internal edges of lamina II and were morphologically similar to the islet cells (Seybold and Elde, 1982). However, Todd's group has later proved that neurotensin IR neurons did not stain for GABA (Todd *et al.*, 1992a). In addition, a subsequent study from the same researchers demonstrated that 94% of the neurotensin-IR axons in the rat DH expressed VGLUT2 (Todd *et al.*, 2003) and, thus, were excitatory interneurons.

The neurotensin receptor 1 was localized to neuronal cell bodies and fibers of lamina II_i (Fassio *et al.*, 2000; Uhl, 1982).

2.4.2.7. Neuropeptide Y (NPY) and NPY receptors

NPY is a 36 aminoacid peptide expressed by a group of SGR neurons (Hunt *et al.*, 1981; Krukoff, 1987; Sasek and Elde, 1985), where it was reported to coexist with GABA, but seldom with glycine and never with ENK (Rowan *et al.*, 1993). Subsequent studies have demonstrated that the NPY-IR interneurons in SGR represented a notable fraction of the total population of inhibitory neurons (18% in laminae I-II) and, in rat, constituted a subpopulation distinct from those of the galanin-, nNOS- or parvalbumin-IR cells (Polgár *et al.*, 2011; Tiong *et al.*, 2011). These four populations of inhibitory neurons were functionally distinct as to expression of sst2a receptors and responses to nociceptive stimulation, with those expressing NPY acting to inhibit nociceptive projection neurons whose cell bodies were located outside SGR (Polgár *et al.*, 2013b). In mouse, many NPY expressing SGR cells also responded to noxious stimuli, and likely had an antinociceptive function (Iwagaki *et al.*, 2016).

There are five known NPY receptors named Y1 to Y5 (Larhammar and Salaneck, 2004). In localization studies, the Y1 receptor was detected in lamina II neurons most of which were also SST-IR (Brumovsky *et al.*, 2006; Zhang *et al.*, 1999). The postsynaptic localization of Y1 in spinal neurons was also confirmed by the presence of IR dendrites after TEM observations (Zhang *et al.*, 1999). Y1-IR neurons were presumably glutamatergic (Todd *et al.*, 2003), *i.e.* they were excitatory but maintained under an Y1 inhibitory influence (Brumovsky *et al.*, 2006).

The Y2 receptor was, instead, localized to peptidergic and non-peptidergic DRG neurons and their central SGR projections (Brumovsky *et al.*, 2005).

Evidence has been provided that NPY presynaptically affected neurotransmitter release in SGR by two concurrent, apparently opposing mechanisms: predominantly on Y1 receptors to attenuate glycinergic/GABAergic IPSCs, but also via Y2 receptors to attenuate glutamatergic EPSCs (Moran *et al.*, 2004). The effects of NPY were more recently shown to be related to the firing pattern of the different types of SGR neurons (Melnick, 2012). In this study, the majority of tonic firing neurons, morphologically resembling the central or some of the radial cells, but not the delayed firing neurons, *i.e.* the vertical or other radial cells, responded in vitro to NPY challenge with an Y1 receptor-activated hyperpolarizing postsynaptic current of the GIRK type. In all cell types, NPY moderately depressed the frequency of both mEPSCs and mIPSCs after binding to Y2 and Y1 receptors, respectively.

2.4.2.8. Gastrin-releasing peptide (GRP) and GRP receptor (GRPR)

GRP was originally discovered as a regulatory peptide that promoted gastrin release in the stomach. Several ISH studies from independent laboratories have shown that neurons expressing the GRP mRNA were largely restricted to lamina I and lamina II_o (Fleming et al.,

[18] During the course of the initial localization of neuropeptides in the nervous system, it was a rather common procedure to use colchicine to block their axonal transport to terminals. By this way, peptides accumulated into the perikaryon, making it easier to detect immunoreactive neurons. It was then understood that, in certain instances, colchicine could interfere with gene expression and transcription, thereby altering the pattern of expression of neuropeptides in neurons subject to the effects of the alkaloid.

2012; Mishra and Hoon, 2013; Sun and Chen, 2007). Very recently, GRP expressing cells in SGR were identified as a distinct subset of excitatory interneurons (Gutierrez-Mecinas *et al.*, 2016). The lamina II interneurons expressing GRPR were excitatory and conveyed itch (Aresh *et al.*, 2017; Mishra and Hoon, 2013; Sun and Chen, 2007).

2.4.3. Neurotrophic factors and receptors

Several neurotrophic factors are known to be involved in the development, differentiation and maintenance of the DRG neurons and in the plasticity of these cells and their projections under several conditions of altered nociception. Nonetheless, evidence is accumulating that some also have a role in the normal SGR once maturity is attained. Therefore, in the following I will resume current knowledge on distribution and physiological actions of those molecules that, in mature animals, may function as modulators of sensory inputs to SGR. Thus, in the present context, the interest for neurotrophic factors lies exclusively on their putative transmitter function, and it is important to stress that such a function is only made possible for factors that undergo a regulated secretion and can be anterogradely transported to the axonal terminals of their parent neurons (Conner *et al.*, 1998; von Bartheld *et al.*, 2001).

2.4.3.1. Neurotrophins and neurotrophin receptors

There are four neurotrophins in mammals (Huang and Reichardt, 2001): nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4 (NT4). They all intervene in spinal cord nociceptive pathways (Merighi *et al.*, 2004; Siniscalco *et al.*, 2011). However, the role of NT3 as a modulator of nociception remains to be unraveled in full, and that played by NT4 is even more unclear (Siniscalco *et al.*, 2011).

2.4.3.1.1. NGF and tropomyosin receptor kinase A (trkA) receptor

NGF was initially characterized for its biological effects on sensory neurons (Levi-Montalcini and Angeletti, 1968). These effects are exerted upon binding to the high-affinity trkA receptor that is expressed in approximately 40% of the rat adult DRG neurons (Richardson et al., 1986; Verge et al., 1989). At the end of development, there is a change in the biological actions of this growth factor that shifts from promoting the survival of the DRG neurons to sustaining their phenotypic differentiation (Carroll *et al.*, 1992; Ruit *et al.*, 1992).

NGF is deeply involved in the plasticity of the adult spinal nociceptive pathways under physiopathological conditions: to simplify, a reduction of its supply to DRGs in neuropathic pain states leaded to a reduced production of SP (and other peptides), whereas an accumulation of NGF in inflammatory pain states resulted in increased SP (and other peptides) synthesis (Lewin and McMahon, 1991). These and numerous other changes cause a phenotypic shift in DRG neuron peptide expression pattern, which reflects at their central SGR projections (Merighi et al., 2004).

There was no evidence for the presence of NGF-IR neurons or processes in the SGR. Lamina II_o was, instead, intensely stained after immunocytochemical labeling with anti-trkA antibodies in humans (Kerwin *et al.*, 1992) and rat (Merighi *et al.*, 2004; Molliver *et al.*, 1995). In rat and mouse, the source of immunoreactivity were the terminals of myelinated PAFs (Molliver *et al.*, 1995) deriving from small and medium sized DRG neurons (Molliver *et al.*, 1995; Salio *et al.*, 2005). Functionally, we have been able to show that a small number of neurons in acute rat spinal cord slices responded to exogenous NGF with an increase in their intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, indicating that trkA receptors might be functional in SGR (Merighi *et al.*, 2004). However, still there is not a convincing demonstration that NGF is anterogradely transported in neurons (Conner *et al.*, 1998). Thus, we do not know if the neurotrophin travels along the central projections of the DRG neurons and, if so, reaches physiologically relevant levels in SGR. The current view that NGF is not anterogradely delivered is an issue that may still not be considered completely set, as recent work has proved that the neurotrophin underwent

an activity-dependent anterograde delivery and modulated synaptic activity in the mouse hippocampo-septal system (Guo *et al.*, 2012).

2.4.3.1.2. BDNF and tropomyosin receptor kinase B (trkB) receptor

My group has devoted several publications to investigate the role of BDNF in the normal spinal somatosensory system (Bardoni *et al.*, 2007; Bardoni and Merighi, 2017; Merighi *et al.*, 2008a; Salio *et al.*, 2005, 2007; Salio and Ferrini, 2016), and we have also reviewed in the past the evidence in support for the intervention of BDNF in the modulation of nociceptive transmission (Merighi *et al.*, 2004, 2008b; Merighi, 2017).

Several decades after the discovery of NGF, BDNF was the second member of the neurotrophin family of growth factors to be found: it was first shown to promote the survival of a subpopulation of DRG neurons, and subsequently purified from the pig brain (Barde *et al.*, 1982). Since then, it became evident that BDNF exerted numerous important functions affecting both central and peripheral neurons (Binder and Scharfman, 2004).

BDNF has high affinity for the trkB receptor that also binds NT4 and, with a lesser affinity, NT3 (Binder and Scharfman, 2004).

The rodent lamina II_o displayed intense BDNF immunocytochemical staining. Labeling derived from the central terminals of a subpopulation of peptidergic small- to medium-sized dark primary sensory neurons (Luo *et al.*, 2001; Salio *et al.*, 2005; Salio and Ferrini, 2016; Wetmore and Olson, 1995) that were demonstrated to anterogradely transport the neurotrophin along their central projections to the spinal cord (Zhou and Rush, 1996). A subset of the DRG neurons expressing BDNF, and, hence, their central terminals in SGR, contained a cocktail of neuropeptides among which CGRP and SP (Salio *et al.*, 2007; Salio and Ferrini, 2016). Quantitative studies at the light level showed that BDNF-IR neurons in DRGs belonged to two distinct subpopulations: the first (41%) was peptidergic and also stained for CGRP and SP; the other was made by non-peptidergic neurons and still remained to be phenotypically characterized in full (Salio and Ferrini, 2016). At the ultrastructural level, BDNF was selectively localized in LDCVs within SGR axonal terminals. Positive LDCVs also stored CGRP and SP in stoichiometric ratios (Salio *et al.*, 2007), this being a classic example of multiple messengers' costorage (Merighi, 2017). The BDNF-IR terminals in SGR were engaged in simple axo-dendritic synapses or formed the C boutons in type Ib glomeruli (Merighi *et al.*, 2008b; Salio *et al.*, 2005).

The adult CNS contains full-length trkB (fl-trkB) receptors and two truncated trkB (tr-trkB) receptor forms that can be generated by alternative splicing of the trkB mRNA (Barbacid, 1994; Klein *et al.*, 1990; Middlemas *et al.*, 1991). Tr-trkB receptors lack the signal transducing intracellular tyrosine kinase domain, but still are active signaling molecules with regulatory effects on neurons and astrocytes (Deinhardt and Chao, 2014; Fenner, 2012).

Fl-trkB receptor mRNA and protein were expressed in SGR. To date, available data refer to rat and mouse. At beginning, light microscopic studies on the whole spinal cord showed a more or less uniform distribution of the mRNA (Ernfors *et al.*, 1992; Widenfalk *et al.*, 2001) and protein (Zhou *et al.*, 1993) in neurons and glia, including a small subset of SGR neurons in a later study (Garraway *et al.*, 2003). Notably, ISH experiments showed the existence of many SGR neurons expressing the trkB mRNA, but ICC revealed that the protein was absent or significantly less abundant (Mannion *et al.*, 1999; Michael *et al.*, 1999)[19]. In subsequent studies we have confirmed this apparent discrepancy as the trkB mRNA was very abundant in SGR (Merighi *et*

[19] It should be recalled here that expression of fl-trkB in spinal cord and SGR undergoes a remarkable plasticity during development and after injury, activity or inflammation of the somatosensory pathways (for review see Merighi et al. 2008b). This, together with potential problems in the specificity of hybridization probes and/or primary antibodies (Salio et al., 2005), possibly explains the differences in the results obtained by the groups that have investigated the expression of the receptor in the spinal cord.

al., 2004), but there were no IR cell bodies after light microscopic ICC (Salio et al., 2005). Salio and coworkers (2005) have been the first to provide a comprehensive study on the localization of fl-trkB in the normal SGR from rats and mice. Using two different primary antibodies that specifically recognized fl-trkB, this study produced a detailed map of receptor localization at perikaya (20% of immunostained profiles[20]), dendrites (50%) and axon terminals (30%) in lamina II. Notably and irrespectively of the type of synapses, fl-trkB was always located in a mutually exclusive fashion at the pre- *or* postsynaptic membranes.

About 90% of fl-trkB IR axon terminals in SGR originated from PAFs of the C and Aδ types and were engaged in type I and II glomeruli, respectively; the remaining 10% formed plain axo-dendritic synapses (see Figure 5 in Merighi et al. 2008b). There is no evidence in the literature for descending sources of fl-trkB and a substantial fraction of the fl-trkB IR axons in axo-dendritic synapses contained LDCVs that were immunostained for CGRP and SP (Salio et al., 2005). Therefore, the primary afferent contribution to the total population of fl-trkB-IR axons in SGR can be estimated to reach 95%, the remaining 5% very likely deriving from SGR-IR interneurons or intrinsic spinal cord neurons in other (deeper) laminae (see below). All fl-trkB IR C boutons in type Ib glomeruli were BDNF- (plus CGRP- and SP-) IR, such a finding demonstrating the presence of fl-trkB autoreceptors in these peptidergic C fibers.

Fl-trkB IR dendrites and cell bodies represented a substantial fraction (about 70%) of the immunostained neuronal profiles in SGR (Salio *et al.*, 2005). This observation confirmed the existence of a population of spinal cord neurons that synthesized the receptor and delivered it to their processes. As mentioned above for IR-axons, fl-trkB IR dendrites in SGR may derive from populations of neurons in SGR or the antenna cells in deeper laminae (see Box 3). Some antenna cells are STT neurons and others project to the dorsal columns (see 2.1 Architecture). In keeping with the possibility that some of the fl-TrkB IR dendrites in SGR belonged to STT neurons, not only it was demonstrated that these neurons expressed trkB but that, once activated by an inflammatory stimulus, they phosphorylated the extracellular signal-regulated kinase ERK (Slack *et al.*, 2005).

In double immunogold studies for fl-trkB and GABA, co-expression of the two markers was only observed in V1 profiles engaged in type Ia, IIa, or IIb glomeruli, i.e. the glomeruli of the non-peptidergic types (Bardoni *et al.*, 2007). These glomeruli also displayed fl-trkB-IR plain dendrites. Considering the glomerular ultrastructure (see 2.2.4.5. Glomeruli), it seemed very likely that the GABA+fl-trkB-IR V1 profiles belonged to islet cells (Bardoni *et al.*, 2007), whereas fl-trkB IR plain dendrites might derive from other SGR neurons (excitatory?) and/or the STT antenna neurons in deeper laminae.

Functionally, BDNF produced hyperalgesia in normal mice (Groth and Aanonsen, 2002), and sensitized the response of SGR neurons to high threshold PAF inputs (Garraway *et al.*, 2003). Very recently, it was demonstrated that BDNF also potentiated the NMDAR responses of lamina I neurons (Hildebrand *et al.*, 2016). In addition, noxious stimulation induced trk receptor and downstream ERK phosphorylation in DH (Pezet *et al.*, 2002). By real time $Ca2^{+}$ imaging, we have demonstrated that PAFs' fl-trkB autoreceptors mediated the release of excitatory neurotransmitters in the rat SGR by a presynaptic mechanism (Merighi *et al.*, 2008a). In addition, after combined electrophysiological and ultrastructural studies, we have observed that the neurotrophin modulated the release of GABA and glycine in the SGR neurons receiving a synaptic input from GABAergic islet cells (Bardoni *et al.*, 2007). Therefore, BDNF may exert a dual action (pronociceptive and antinociceptive) on SGR neurons (Merighi et al., 2008b) - but

[20] Original data in Salio et al. (2005) provide separate figures for rat and mouse. For simplicity, they have been averaged and rounded here.

see 4.1.1.1.1. pPAFs expressing SP+BDNF: the excitatory channel, for discussion on underlying circuitry.
Finally, functional studies have converged to demonstrate that BDNF released from C PAFs induced a long term potentiation of these fibers following the activation of the spinal microglia (Zhou *et al.*, 2011).

2.4.3.1.3. NT3, NT4 and tropomyosin receptor kinase C (trkC) receptor

In SGR, NT3 primarily acted on the trkC receptors expressed by large caliber myelinated Aβ fibers, but the receptor has been localized also in descending serotoninergic axons (Zhou and Rush, 1994). A centripetal (anterograde) transport of NT3 along the central projections of the DRG neurons to the intact DH remains to be demonstrated (Distefano *et al.*, 1992), although it might occur after damage of the sensory neurons (Koltzenburg *et al.*, 1999). Functional evidence indicated that NT3 participated to the regulation of the monosynaptic stretch reflex after birth, and that the physiological role of NT3 could possibly not only be restricted to muscle afferents (Mendell *et al.*, 2001). However, information is still too fragmentary for allowing any type of conclusion to be drawn. Nonetheless, NT3 antinociceptive effects have been shown after behavioral analysis in the isolated spinal cord (Malcangio *et al.*, 1997; Siuciak *et al.*, 1994), and one of these studies demonstrated that NT3 inhibited the release of SP onto DH interneurons via a $GABA_AR$-dependent mechanism (Malcangio *et al.*, 1997).
Albeit with lower affinity, trkA and trkB receptors also bind NT3, thus offering a theoretical substrate for additional effects of this neurotrophin in lamina II.
The NT4 mRNA appeared to be synthesized by some SGR and DRG neurons (Heppenstall and Lewin, 2001). As the trkB receptor also binds NT4, it is possible that the effects of NT4 in lamina II are wider than so far hypothesized.

2.4.3.2. Glial-derived neurotropic factor (GDNF) and GDNF receptors

GDNF was the first member of a family of closely related neurotrophic factors (Lin et al., 1993) others than the neurotrophins. At present, the family comprises GDNF, neurturin, artemin, and persephin that are collectively referred to as GDNF family ligands (GFLs). To exert their biological actions, GFLs need a receptor complex consisting of a main receptor and a ligand-specific co-receptor (Treanor et al., 1996). The main receptor is the transmembrane receptor tyrosine kinase rearranged during transfection (RET) that binds all GFLs (Knowles et al., 2006). The co-receptor is a cell surface-bound GDNF family receptor α (GFRα), GFRα1 in the case of GDNF, that preferentially binds one of the GFLs (Airaksinen and Saarma, 2002). In vitro, GDNF also binds with lower affinity to GFRα2 and GFRα3 but it remains unclear whether these ligand-receptor interactions are of relevance in vivo (Airaksinen and Saarma, 2002).
Similarly to BDNF, data on GDNF and GFL receptor distribution in SGR are available for mouse and rat. In mouse, GDNF-IR fibers in SGR originated from a subpoplation of small-to medium-sized DRG neurons totally separated and numerically much restricted (about one third) than that expressing BDNF (Salio and Ferrini, 2016). In turn, virtually all GDNF-IR DRG neurons also expressed CGRP, and a substantial fraction of them (about 70%) was SST-IR (Salio and Ferrini, 2016). Immunoreactivity occurred in lamina II_i. This sublamina, at least in rodents, contains the central terminals of non-peptidergic nociceptors that bind the IB4 extracted from the seeds of the tropical African legume *Griffonia simplicifolia* (Lorenzo et al., 2008), but GDNF-IR terminals were not labeled after IB4 staining (Salio *et al.*, 2014; Salio and Ferrini, 2016). Ultrastructurally, immunoreactivity was specifically localized to the C boutons of Ib glomeruli; these boutons contained variable numbers of LDCVs that were also stained for CGRP and SST (Salio *et al.*, 2014). CGRP-+GDNF-+SST-IR axons were about one-fourth of the total population of the CGRP immunostained C boutons, the remaining three-fourths being, instead, IR for CGRP+BDNF+SP (Salio *et al.*, 2014). GDNF-IR neurons in DRGs were also very recently described in rat and

reported to decrease with the onset of chronic pain (Ding *et al.*, 2017). Consistently with the observation that the percentage of DRG neurons expressing the GDNF protein is low (less than 10%) it was not totally surprising that two recent transcriptomic studies (see also Box 6) failed to detect significant levels of expression of the mRNA (Usoskin *et al.*, 2015) or did not report it (Li *et al.*, 2016).

The two components of the GDNF receptor complex displayed a variegated pattern of distribution (Bennett *et al.*, 1998; Salio *et al.*, 2014). At light level, RET and GFRα1 were coexpressed at high degree in DRG neurons of heterogenous sizes and in the mid portion of SGR, corresponding to lamina II_id (dorsal part of inner lamina II) in the rodent SGR tripartite subdivision (Ribeiro-Da-Silva, 2015). A very limited number of CGRP-IR neurons in DRGs expressed RET or GFRα1, but those expressing SP were negative. In parallel, a fraction of the IB4 labeled DRG neurons was IR for both components of the GDNF receptor complex. The pattern of distribution of IR fibers in the SGR was consistent with expression data in DRGs.

At the ultrastructural level, GFRα1 was detected in the central boutons of type Ia and II glomeruli, and in plain dendrites of type Ib glomeruli (for a schematic representation see Fig. 12 in Salio et al. 2014).

Functionally, we have shown that, after capsaicin is given as an acute noxious stimulus to young adult animals, GDNF was released from peptidergic CGRP-+SST-IR DRG nociceptors and tonically inhibited the glutamate excitatory drive of laminae I-II neurons (Salio et al., 2014). These and other observations under conditions of inflammatory and neuropatic pain strongly implicated GDNF as a modulator of normal and pathologic pain (Merighi, 2016). The underlying circuitry is discussed in 4.1.1.1.2. pPAFs expressing SST+GDNF: the inhibitory channel.

2.4.4. Transient receptor potential (TRP) channels

The TRP superfamily of genes encodes a number of integral membrane ion channels, further subdivided into seven subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin) and TRPN (NOMPC-like) (Nilius and Owsianik, 2011). TRP receptors are involved in numerous physiological functions and have a widespread distribution in cells and tissues. Most TRPs are non-selective cation channels; and only few are highly selective for Ca^{2+}. The most relevant to the present discussion belong to the TRPV, TRPM, and TRPA subfamilies.

2.4.4.1. TRPV1

TRPV1 is very important in the context of SGR as it is involved in somatic and visceral peripheral inflammation, in the central modulation of nociceptive inputs, as well as the integration of diverse painful stimuli (Frias and Merighi, 2016). TRPV1 was cloned in 1997 from rat DRGs (Caterina *et al.*, 1997). Studies on isolated cells demonstrated that capsaicin, a vanilloid derived from the plants of the genus *Capsicum*, and other natural substances, as well as some physical activators and protons activated TRPV1 (Caterina *et al.*, 1997; Hayes *et al.*, 2000). Cation (Ca^{2+}) influx through TRPV1 resulted in neuronal depolarization, with opening of voltage-gated sodium channels and generation of an action potential. In SGR, TRPV1 was mainly localized to the central projections of polymodal C and mechano-heat Aδ cutaneous nociceptive PAFs of the peptidergic type and increased the spontaneous release of glutamate from their terminals (Kumamoto *et al.*, 2014). It is relevant to recall here that TRPV1 expression differed between rat and mouse, since the receptor is down-regulated in most non-peptidergic C nociceptors in the mouse (Cavanaugh *et al.*, 2011). Capsaicin and several, but not all, TRPV1 agonists enhanced glutamatergic spontaneous excitatory transmission in SGR (Jiang *et al.*, 2009; Yang *et al.*, 2011). Responses to capsaicin were preferentially localized to the SDH (Merighi, 2002) and regarded about 50% of excitatory and inhibitory SGR neurons (Punnakkal *et al.*, 2014). The fact that capsaicin was capable to selectively activate the peptidergic nociceptive fibers in SGR is of

relevance because the vanilloid can be used to mimic the effects of peripheral inflammation on isolated neurons and/or spinal cord slices (Frias and Merighi, 2016).
Besides PAFs, some glutamatergic SGR neurons expressed TRPV1 (Zhou *et al.*, 2009). Existence of a functional population of these neurons was demonstrated in organotypically cultured spinal cord slices, where PAFs degenerated as a consequence of the rhizotomy necessary to the explant (Ferrini *et al.*, 2010). Given that TRPV1 is expressed in PAF terminals and SGR neurons, understanding of its functions in lamina II was made difficult. Most TRPV1 agonists are well known to evoke the release of the neurochemicals contained in PAF terminals, thus one expects that an excitation of SGR neurons occurs in the course of spontaneous inflammation (Treede *et al.*, 1992). However, we have provided evidence that when acute (Ferrini *et al.*, 2007) or organotypically cultured (Ferrini *et al.*, 2010) spinal cord slices were challenged with capsaicin an activation of inhibitory neurotransmission also occurred. I will discuss in another section the possibility that parallel channels of sensory information are activated in SGR (see 3. Neuronal circuitries in SGR). Notwithstanding, it is worth recalling here that a correlation between the SGR cell morphology and the increase of sIPSCs was not evident in our preparations. Capsaicin-responsive neurons - after LY injection - were islet/central cells, radial cells, medio-lateral cells, and stalked cells, *i.e.* they were either inhibitory or excitatory.
Another important effect of the activation of TRPV1 (and TRPA1) in lamina II neurons might be the regulation of itch (Ross *et al.*, 2014).

2.4.4.2. TRPM8

TRPM8 mediates innocuous cold sensations and was expressed by a subpopulation of trkA+ DRG neurons (Kobayashi *et al.*, 2005). These channels are activated by several chemicals, chiefly plant derivatives (Jiang *et al.*, 2016; Kumamoto and Fujita, 2016). The PAFs with TRPM8 (and TRPA1) profiles targeted functionally distinct subpopulations of SGR neurons (Wrigley *et al.*, 2009).

2.4.4.3. TRPA1

TRPA1 receptors are activated by noxious cold, several natural chemicals of vegetal origin and anesthetics (Bandell *et al.*, 2004; Piao *et al.*, 2009). Similarly to TRPV1, activation of TRPA1 channels, which were expressed by specific subsets of Aδ and C fibers, enhanced the SGR release of glutamate and facilitated excitatory synaptic transmission (Kobayashi *et al.*, 2005; Kosugi *et al.*, 2007). More recently, the TRPA1 agonist cinnamaldehyde was demonstrated to elicit a barrage of EPSCs in vertical and radial, but not islet or central neurons that also responded to the TRPV1 agonist capsaicin (Uta *et al.*, 2010). Notably, Uta and co-workers showed that the effect of cinnamaldehyde was presynaptic. Intriguingly, the agonist had a selective inhibitory action only on monosynaptic C-fiber-evoked EPSCs, but not on those mediated by the Aδ PAFs (Jiang *et al.*, 2016; Uta *et al.*, 2010).

2.4.5. Others

All neurochemicals and receptors so far considered are somehow implicated in SGR neurotransmission. Other biologically active molecules do not directly intervene in neuron-to-neuron communication but they will be considered in this section as useful markers to identify certain functional sets of SGR neurons.

2.4.5.1. Calcium-binding proteins

Calbindin, calretinin and parvalbumin are three calcium-binding proteins localized to the general cytoplasm of CNS neurons. They have been long ago reported in SDH (Ren and Ruda, 1994). Here, calbindin and calretinin were expressed in the majority of the excitatory interneurons, sometimes in combination, whereas parvalbumin was localized to at least certain inhibitory interneurons. Recent work has additionally localized another calcium-binding protein, secretagogin, in rat, mouse and human SDH neurons (Shi *et al.*, 2012).

2.4.5.1.1. Calbindin

Calbindin is a 28kD calcium-binding protein widely distributed in the nervous system (Celio, 1990). Although a population of DRG neurons could be immunolabeled for calbindin, initial localization studies in rat SGR converged to demonstrate that immunoreactivity in lamina II derived exclusively form intraspinal sources (Antal *et al.*, 1990; Yamamoto *et al.*, 1989; Yoshida *et al.*, 1990)[21]. These studies indicated that calbindin containing neurons were distributed in laminae I-III and formed a separate subpopulation from that of the parvalbumin-IR cells (Yoshida *et al.*, 1990). In addition, TEM observations localized immunoreactivity to dendrites, cell bodies or, less frequently, axons (Antal *et al.*, 1990; Yoshida *et al.*, 1990). IR cell bodies in SGR might not all belong to interneurons, as indicated by at least one retrograde tracing study (Menétrey *et al.*, 1992). Other studies have shown that the lamina II neurons containing calbindin were not stained for GABA in rat (Antal *et al.*, 1991), nor they were fluorescent in transgenic mice with GFP-tagged GABAergic neurons (Hantman and Perl, 2005). In rat, a subpopulation of calbindin-IR SGR neurons also expressed preprotachykinin B (Polgár *et al.*, 2006). In mouse, a large fraction of calbindin-IR SGR neurons was enkephalinergic (Huang *et al.*, 2010).

Expression of calbindin was also reported in neurons of cat lamina II (Anelli and Heckman, 2005; Merkulyeva *et al.*, 2016).

2.4.5.1.2. Calretinin

Calretinin is a 29 kDa calcium binding protein originally isolated from chick retina, with 58% homology to mammalian 28 kDa calbindin (Rogers, 1987). Initial observations in rat demonstrated that a small fraction (from 5 to 10%) of medium-to-large sized DRG neurons expressed the calretinin mRNA (Heppelmann and Emson, 1993) or protein (Ren *et al.*, 1993). In these two studies, calretinin and its mRNA were also detected in SGR, with very intense staining of small neuronal cell bodies and fibers. A later study in cat confirmed that IR neurons were of small size and a very small minority of those expressing calbindin (Anelli and Heckman, 2005). Calretinin-IR neurons were subsequently shown to express the A-type K^+ channel alpha subunit Kv 4.3 in rat (Huang *et al.*, 2005), or SST in a transgenic mouse line (Gutierrez-Mecinas et al., 2016). In another transgenic mouse line, two distinct populations of calretinin-expressing neurons were identified and termed typical - with central, radial or vertical cell morphologies and the characteristic properties of the excitatory interneurons - and atypical - with the morphology of the islet cells and the electrophysiological properties of the inhibitory interneurons (Smith et al., 2015).

2.4.5.1.3. Parvalbumin

Parvalbumin is a calcium-binding albumin protein of 9-11 kDa widely expressed in GABAergic fast-spiking interneurons of the cerebral cortex (Hu et al., 2014). Initial localization studies in rat spinal cord reported that IR neurons were restricted to a band at the border between lamina II and III and had a morphology closely resembling that of the islet cells (Yamamoto *et al.*, 1989; Yoshida *et al.*, 1990). Ultrastructurally, somatic or dendritic positive profiles were observed, and, at times, dendrites were filled with synaptic vesicles (Antal *et al.*, 1990; Yoshida *et al.*, 1990). Not surprisingly - see note 21 - in several studies, dorsal rhizotomy did not affect the pattern of immunostaining in SDH (Medici and Shortland, 2015; Yamamoto *et al.*, 1989; Yoshida *et al.*, 1990). However, it is today well established that parvalbumin defines a subpopulation of proprioceptive DRG neurons unreactive for CGRP (Medici and Shortland, 2015). The protein was

[21] Although calcium-binding proteins may be expressed by subsets of DRG neurons, lack of expression in PAFs should not be regarded as unexpected as these proteins do not undergo anterograde transport. A remarkable exception is represented by neuronal calcium sensor-1 (Averill *et al.*, 2004).

reported to be present in the GABAergic neurons of rat lamina II (Antal *et al.*, 1991; Laing *et al.*, 1994). However, in transgenic mice where these neurons were made fluorescent by genetic engineering Hantman and Perl (2005) observed that not all GABAergic neurons in SGR were parvalbumin-IR. Very recently, it was reported that the IR neurons accounted for ~10% of inhibitory interneurons in lamina II (Boyle *et al.*, 2017). Functionally, parvalbumin-IR neurons in lamina II_i were confirmed to be predominantly inhibitory and to have a tonic or phasic fast axon potential discharge pattern (Hughes *et al.*, 2012, 2013). In mice, optoactivation of parvalbumin SGR neurons expressing the blue light-sensitive cation channel channelrhodopsin-2 evoked a release of GABA, which was modulated by presynaptic $GABA_BRs$ (Yang *et al.*, 2015). In the same species, it was also reported that 25% of the enkephalinergic SGR neurons contained parvalbumin (Huang *et al.*, 2010). A subpopulation of parvalbumin-IR interneurons in lamina II_i, with the morphology of the islet cells or the central cells of the smaller size, was recently reported to be the source of the inhibitory synapses regulating non-noxious tactile input to the spinal cord (Hughes *et al.*, 2012). Their axon terminals made synaptic contacts (V2 profiles) onto the central terminals of type II glomeruli, and, at these glomeruli, some parvalbumin-IR vesicle-containing dendrites (V1 profiles) were also observed. In addition, parvalbumin-IR axons made simple axo-axonic synapses onto unreactive axons, or simple axo-dendritic synapses onto parvalbumin-IR dendrites.

Parvalbumin was not detected in cat SGR neurons (Anelli and Heckman, 2005).

2.4.5.2. Protein kinase C (PKC)

The term protein kinase C indicates a family of proteins that are essential in signal transduction. In rat and mouse, its gamma-isoform (PKCγ) was reported to be restricted to a population of excitatory interneurons with the morphology of the central cells in lamina II_i many of which also express neurotensin or SST (Gutierrez-Mecinas *et al.*, 2016; Polgár *et al.*, 1999a; Todd, 2017).

2.4.5.3. Nitric oxide (NO) and NO synthesizing enzymes

NO is a gas that has diverse effects on neural signaling (Vincent, 1994). The neuronal form of its synthesizing enzyme, nNOS, is the neuronal NADPH diaphorase. There were numerous nNOS/NADPH diaphorase-labeled neurons in the SDH (Valtschanoff *et al.*, 1992). In SGR, most of these neurons were GABA- and/or glycine-IR (Spike *et al.*, 1993), and initial studies converged to indicate that nNOS was not present in glutamatergic neurons (Laing *et al.*, 1994; Reuss and Reuss, 2001; Spike *et al.*, 1993), but more recently two thirds of nNOS-IR SGR neurons were reported not to be GABAergic (Sardella *et al.*, 2011a). We have characterized the ultrastructure and functional responses of NADPH diaphorase-stained SGR neurons and demonstrated that NO-producing islet cells modulated the release of sensory neuropeptides in rat lamina II_i (Aimar *et al.*, 1998). More recent studies proposed that NO could be also released from glia to mediate long-term presynaptic facilitation in SGR (Ikeda and Murase, 2004).

2.4.5.4. Transcription factors

In the adult SDH, transcription factors' immunoreactivity is often used to define the excitatory or inhibitory phenotype of neurons (Cheng *et al.*, 2005). In SGR, the transcription factor Pax2 was shown to be persistently expressed in the inhibitory interneurons (Larsson, 2017), whereas the transcription factor Bhlhb5, which is transiently expressed in the DH of the developing spinal cord, appeared to play a role in the formation and regulation of pruritic circuits (Ross *et al.*, 2010).

It should also be mentioned that, during embryonic development, there is a precisely orchestrated temporal pattern of expression of several transcription factors that play an important role in defining the lamination of the DH – see 5. Spinal gray matter lamination in the third millennium: has the SGR come to age?

2.5. *Glia*

Very little work has been devoted to unraveling the physiological role of glial cells in SGR, as much of current research was carried out to understand the intervention of glia in pathological conditions mainly related to inflammation. It is, in fact, widely accepted that while glia have a limited role, if any, in basal nociceptive processing, pain is amplified when glia become activated (Biggs *et al.*, 2010; Watkins *et al.*, 2007). As this contribution focuses onto the normal SGR, I will only briefly mention here some findings on the participation of unstimulated glia in the modulation of neuron-to-neuron communication. Current views, in fact, corroborate the existence of a neuron-to-astrocyte bidirectional cross talk in the regulation of central synapses that relay nociceptive signals to the brain (Old and Malcangio, 2012).

In culture, it was demonstrated that SGR astrocytes expressing the P2X purinoceptor 7 (P2X7) were capable to release glutamate and reactive oxygen species (Ficker *et al.*, 2014; Gao *et al.*, 2017). Under these in vitro conditions, astrocytic glutamate induced a cationic current in the neighboring neurons, following activation of NMDARs and AMPARs.

Work in the more complex slice setting substantiated the occurrence of a glutamate-mediated astrocyte-to-neuron signaling. In slices of the rat SDH, activation of P2X7 receptors by the agonist BzATP or low extracellular Ca^{2+}, both triggering intracellular Ca^{2+} elevations in astrocytes, induced slow inward currents in lamina II neurons (Bardoni *et al.*, 2010). In the same paper, calcium imaging showed that an increase of $[Ca^{2+}]_i$ was responsible for glutamate release from astrocytes and that astrocytic glutamate evoked NMDAR-mediated episodes of synchronous activity in clusters of SGR neurons. Another study has suggested that the astrocytic glutamate aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) were actuated by synaptic activity (Zhang *et al.*, 2009). The same authors claimed that recording glutamate transporter currents may provide a means of examining in real time the physiological responses of glial cells during sensory processing, sensitization, hyperalgesia and chronic pain.

In addition to being capable of releasing glutamate, SGR astrocytes have been hypothesized to also release NO that, in turn, interacted with the NMDAR-dependent signaling of second order projection neurons in other laminae of SDH (Dauch *et al.*, 2012). Finally, a number of chemokines released by SGR astrocytes have been implicated as regulators of the activity of the nearby interneurons (Cao *et al.*, 2014; Gao *et al.*, 2009).

Astrocytes may not be the only type of glia that intervenes in SDH neurotransmission as a very recent slice work has involved the microglia in regulation of NMDAR but not AMPAR signaling in SGR neurons (Sonekatsu *et al.*, 2016).

3. Neuronal circuitries in the superficial dorsal horn

Despite the considerable amount of work that has been devoted to the study of SGR, neuronal circuitries in this and other laminae of the SDH remain, for the most, elusive. Not only one of the main reasons for this substantial lack of information is our present difficulty to identify functionally distinct populations of interneurons - this issue has been very recently and authoritatively reviewed (Todd, 2017), but also the impossibility to provide a comprehensive reconstruction of the precise wiring of the SGR neurons has an indubitable impact. In this section I will try to synthesize the data of the literature into a coherent framework. I will also propose a hypothetical circuitry of parallel processing of information in SDH that will be hopefully useful to prompt further investigations on the matter.

3.1. Input to SGR

3.1.1. Input from PAFs

PAFs derive from neurochemically and functionally heterogeneous subpopulations of DRG neurons (Fig. 3 and Box 6). Those of the C and Aδ types provide the most important input to SGR. Today, we have relatively good markers for C fibers and their peptidergic (CGRP and

others) or non-peptidergic (IB4/LA4) subtypes. Before IB4 came into use, fluoride-resistant acid phosphatase (FRAP) was among the differentiation antigens proposed to distinguish functional subclasses of primary sensory neurons (Dodd and Jessel, 1985; Jessell and Dodd, 1985). Labeling with these three markers displayed substantial overlap, but FRAP was historically an important marker of the now identified population of afferents which are named non-peptidergic nociceptive C fibers, before it was discovered that they bind IB4 and express the P2X purinoceptor 3 (P2X3 – Vulchanova *et al.*, 1998) and other markers like MRGD (Liu *et al.*, 2008; Wang and Zylka, 2009). FRAP is now known to represent an isoform of prostatic acid phosphatase and to suppress pain by dephosphorylating extracellular adenosine monophosphate (AMP) to adenosine that activates A1-adenosine receptors in DH (Zylka *et al.*, 2008).

On the other hand, we still experience considerable difficulties to specifically label the Aδ fibers of the nociceptive (HTMs) or non-nociceptive (LTMs) types (Djouhri, 2016), although Aδ LTMs could be identified e.g. in a trkB-EGFP mouse line (Li *et al.*, 2011). This severely hampers our capability to understand in full the circuitries made by thinly myelinated fibers in SDH. Fortunately, however, different functional sets of C and Aδ PAFs terminate in an ordinate fashion, thus making it possible to subdivide the SGR in neurochemically and functionally distinct sublaminae along a dorso-ventral gradient.

All PAFs release glutamate alone or in combination with other transmitters/modulators at glomeruli and/or simple axo-dendritic contacts. Evidence has been provided that the flow of excitatory input to SGR mainly had an upward direction whereas the inhibitory input zones of lamina II neurons appeared to be dorsoventrally centered on the perikaryon of the target neurons (Kato *et al.*, 2009). This is very important in relation to the peptidergic input to SGR, as peptides and other high MW transmitters may act via volume transmission – see also Box 4. In the reason of these observations, I will consider separately the PAF input to laminae II_o-II_id from one side and that to lamina II_iv from the other.

3.1.1.1. Lamina II_o and II_id

These two regions of the rodent SGR are the site of termination of thermoreceptive and nociceptive C fibers and contain type I glomeruli (Ribeiro-Da-Silva and De Koninck, 2008). Differences between mouse, cat and primates, from one side, and rat, from the other, may be relevant, as lamina II_o glomeruli are by far less numerous in this latter species. It is also unclear if, at least at the border between lamina II_o and II_id, some type IIa glomeruli exist, originating from thermoreceptive and HTM (nociceptive) Aδ fibers (Fig. 3). The C boutons of type Ia and type IIa glomeruli provide a "pure" glutamatergic excitatory drive to SGR neurons. The situation is more complicated for Ib glomeruli because their glutamatergic C boutons also release slow-acting high MW positive or negative modulators of neuronal excitability. At least, four different groups of Ib glomeruli can be recognized in laminae II_o-II_id based on the neurochemistry of their C boutons. This observation is obviously in line with the neurochemistry of their parent peptidergic DRG neurons that form two quantitatively more relevant populations expressing CGRP, TRPV1 and trkA (Fig. 3) - *i.e.* the better markers of the nociceptive C fibers. In addition to these neurochemicals, one of these two populations of DRG neurons also contains SP and BDNF (red in Fig. 3), the other SST and GDNF (orange in Fig. 3). Another important difference is that only the C fibers expressing SP and BDNF form glomeruli *and* simple axo-dendritic contacts in SGR, whereas those containing SST+GDNF appear to be exclusively engaged in glomeruli.

3.1.1.1.1. pPAFs expressing SP+BDNF: the excitatory channel

PAFs of this group can be further divided into two subgroups each characterized by the absence (red in Fig. 3 and 4) or presence (pink in Fig. 3 and 4) of fl-trkB at the axolemma.

Glutamatergic PAFs expressing SP and BDNF but devoid of fl-trkB receptors may be pivotal to trigger a long lasting excitation in the dorsal part of SGR following two different circuits that converge onto the large vertical/stalked cells (A1 and A3 in Fig. 4). Note that circuit A1 is made of a chain of excitatory (red in Fig. 4) synapses, whereas circuit A3 includes two islet cells in sequence so that a reduction of the inhibitory drive onto the large vertical/stalked cell is eventually attained. Therefore, the resulting effect onto the large vertical cells (the output SGR neurons that connect to lamina I projection neurons) is excitatory (Fig. 4 and 7).

As these PAFs contain a mix of messengers with different MW, release can occur selectively in dependence of the frequency and firing pattern of activation (Merighi, 2017) – see also Box 4. In keeping with this notion and with the view that intensity of noxious stimuli is encoded by nociceptive fibers in their firing pattern (rather than just in their firing rate - Fig. 7), a concurrent increase of SP release, NK1 receptor activation and C fibers' firing frequency was demonstrated in rat SDH (Adelson *et al.*, 2009).

SP released in the SDH has been long known to be a positive modulator of nociception and to intervene in the central sensitization of nociceptors (Seybold, 2009). However, NK1 receptors are mainly distributed *outside* the SGR in neurons of laminae I and III-IV (Fig. 4 and Boxes 2 and 3). Thus it is currently accepted that the peptide's modulatory effects in SDH are primarily extrasynaptic (Liu and Sandkühler, 1995).

SP could also be released by some large vertical/stalked cells, but the relative number of SP-IR axons deriving from these neurons is relatively small (about one fifth) compared to that of SP-IR PAFs (Ribeiro-Da-Silva *et al.*, 1991). Although it remains to be established how functionally relevant (if any) may be the contribution of SP released by the spinal neurons (Todd, 2017), interaction with the lamina I glutamatergic NK1+ projection neurons can occur at axo-dendritic synapses or by volume transmission, to reinforce excitation once the system is overstimulated (Fig. 4).

In slices, pharmacological activation of NK1 receptors also enhanced inhibitory neurotransmission in SGR neurons of heterogeneous morphology, among which the large vertical (stalked) cells and the islet cells (Vergnano *et al.*, 2004). In a subsequent study, where NK1 expressing neurons were specifically destroyed in vivo by injection of SP-saporin (Rahman *et al.*, 2007), the $GABA_AR$ antagonist bicuculline lost its facilitatory effect on the electrically evoked Aδ and C fiber post-discharge, confirming a recruitment of NK1 receptors in the modulation of GABAergic inhibition in SDH. As the early hypothesis that some GABAergic neurons in laminae I-III also expressed the NK1 receptor was not confirmed in subsequent studies, it seems likely that inhibitory effects of SP in SDH are conveyed through an indirect polysynaptic pathway. Thus GABAergic inhibitory interneurons would provide a feed-forward inhibition of the A1 circuit along a yet unknown pathway.

Similarly to SP, BDNF also displays a combination of excitatory (Merighi *et al.*, 2008a) and inhibitory (Bardoni *et al.*, 2007) effects onto SGR neurons. The A1 and A3 polyneuronal chains depicted in Fig. 4 can also explain these effects. More precisely, as fl-trkB receptors are not simultaneously expressed pre- and post-synaptically at the first-to-second order sensory neuron synapses (Salio *et al.*, 2005), BDNF can: *i.* Trigger an indirect pathway along a minimally bi-neuronal chain of excitatory SGR neurons, a central and a vertical cell in sequence (circuit A1 in Fig. 4, Fig. 7 in Lu and Perl 2005 and 7B in Yasaka et al., 2007). This pathways implies the activation of dendritic *postsynaptic fl-trkB receptors* at simple axo-dendritic synapses or type Ib glomeruli made by pPAFs (Salio *et al.*, 2005); *ii.* Directly act along a parallel pathway with the activation of *presynaptic fl-trkB auto receptors* at different Ib glomeruli (circuit A3 in Fig. 4). As the C fibers that initiate circuit A1 express TRPV1, they release BDNF following capsaicin stimulation, *i.e.* a condition that mimics inflammation (Merighi *et al.*, 2008a). Notably and

consistently with the electrophysiological data obtained after recordings from pairs of SGR neurons or challenging inhibitory SGR neurons with the neurotrophin, the long lasting excitation of these neurons that occurs after the capsaicin-stimulated release of BDNF may follow the same polyneuronal chain of circuit A3. This is consistent with the concurrent observation of an increase in frequency of GABAergic/glycinergic mIPSCs but a reduction of eIPSCs in SGR neurons after BDNF challenge, when excitatory neurotransmission was blocked (Bardoni *et al.*, 2007). Therefore, the A3 circuit provides the structural substrate for a disinhibitory (*i.e.* excitatory) pathway where SP and BDNF synergistically act as co-transmitters. Likely, this pathway may be important in the long lasting central sensitization of SGR synapses (Fig. 7) under certain inflammatory conditions and/or neuropathic pain (Merighi *et al.*, 2008b).

3.1.1.1.2.pPAFs expressing SST+GDNF: the inhibitory channel

pPAFs containing SST+GDNF originate from a subpopulation of DRG neurons that are IR for LA4 and were hypothesized to have peculiar neurochemical and functional features (Averill *et al.*, 1995)[22]. These neurons (orange in Fig. 3) are roughly equivalent in number to those expressing SP+BDNF.

Remarkably, their central projections contain glutamate as a main excitatory neurotransmitter together with SST and GDNF that negatively modulate nociception. In keeping with the previously observed concurrent presence of transmitters with opposing function in these fibers, a very recent study has provided evidence that SST-expressing PAFs in the mouse are pruritoceptive, rather than nociceptive, and that SST released from these afferents has an anti-nociceptive effect (Huang et al., 2018).

Yasaka and co-workers (2007) have postulated that the C fibers provide a direct excitatory input to islet, central, radial and vertical cells in SGR (see their Fig. 7). If one holds that SST+GDNF pPAFs (orange in Fig. 5) are an important inhibitory channel to tonically control the outflow of information from SGR (Fig. 7), then an inhibitory central cell, activated by PAF fast glutamatergic neurotransmission, is the more likely candidate to link these fibers with the large vertical cells transferring information outside lamina II (circuit B1 in Fig. 5). A second parallel inhibitory pathway might also converge onto the vertical cells and puts in the game SST and GDNF. Once co-released in SGR, the two neurochemicals could hyperpolarize the C boutons of IB4+ non-pPAFs (light green in Fig. 5) that express sst2a and GFRα1 at Ia glomeruli (circuit B2 in Fig. 5). The following experimental evidences support the B2 circuit*: i.* SST has well-established central analgesic effects (Pintér *et al.*, 2006) that arise as a consequence of the tonic inhibition of C and Aδ PAFs originating from the rat skin (Guo *et al.*, 2008; Wang *et al.*, 2009) and reduction of the glutamate-evoked activation of these fibers (Luo *et al.*, 2010); *ii.* GDNF tonically inhibits the glutamatergic excitatory drive of SGR neurons upon binging to GFRα1 (Salio *et al.*, 2014); *iii.* The large vertical cells receive input from non-pPAFs at type Ia glomeruli (Maxwell *et al.*, 2007), and may thus be primary targets of IB4+ non-pPAFs. This does not exclude that synaptic interactions are much more complex, as the vertical cells, in turn, give rise to V1 profiles at the same type of glomeruli.

A third additional way (B3 in Fig. 5) by which the SST+GDNF pPAFs may provide inhibition implicates the same Ib glomeruli of lamina II_id that initiate the A1 pathway (red in Figs. 4 and 5), as they possess GFRα1-IR peripheral dendrites (Salio *et al.*, 2014). The latter derive from laminae I-II neurons that express GFRα1 after genetic tagging the receptor with EYFP (Ortega-de San Luis and Pascual, 2016). Their morphology and functional properties remain to be established, but it is tempting to speculate that these neurons are transient or tonic central cells (see circuit

[22] This work was published long before GDNF started to be implicated in nociception and thus refers to somatostatinergic neurons only.

A1 in Fig. 4). In keeping with this possibility, intrathecal injections of GDNF inhibited the thermal hypersensitivity induced by SP (Malcangio *et al.*, 2002), and GDNF presynaptically reduced SP/CGRP release from capsaicin-challenged PAFs (Salio *et al.*, 2014).

3.1.1.2. Lamina II_iv

The lamina II_iv is another site of termination of the IB4+ non-pPAFs of the C type as the type Ia glomeruli made by these fibers are equally distributed in the two halves of lamina II_i (Salio *et al.*, 2014). Lamina II_iv is also the main region where Aδ LTM make synapses at type IIa glomeruli. Finally, at least in rodents, lamina II_iv contains a relatively (to lamina III) small number of type IIb glomeruli made by the Aβ fibers (Ribeiro-Da-Silva and De Koninck, 2008).

Central boutons of IIa glomeruli in this sublamina express GFRα1. Therefore, GDNF released at Ib glomeruli in the more superficial sublaminae may provide inhibition of Aδ HTM through volume transmission (Circuit B4 in Fig. 5). Tentatively, the postsynaptic target of the Aδ HTMs in this circuit may be the II_m vertical cells, which have more ventrally located perikaya at the border between lamina II_o and IIi_d and receive an input from lamina III-IV neurons (Kato *et al.*, 2009).

As mentioned, a small contingent of Aβ LTM enters lamina II_iv although the majority of these fibers reach laminae III-IV. Several lines of evidence indicate that the Aβ LTM input to deeper laminae reaches laminae I (Torsney *et al.*, 2006) and II (Baba *et al.*, 2003) along a polyneuronal chain that very likely recruits the large vertical cells (Kato *et al.*, 2009). The cross-talk between Aβ fibers, from one side, and the Aδ/C fibers, from the other, is at the basis of the previously mentioned gate theory of pain (Melzack and Wall, 1965). Briefly, the theory postulates that projection neurons transmitting pain to the brain, i.e. the STT neurons in laminae I and III-V, receive input from both nociceptors (Aδ/C) and Aβ LTM, and that the Aβ inputs, before reaching the brain, are gated by inhibitory interneurons in SGR.

Recent work (Duan *et al.*, 2014) has highlighted the importance of SST excitatory and DYN inhibitory neurons in the generation of a microcircuit that processes and gates mechanical pain (C1 in Fig. 6), with partial revisiting of the gate theory. Based on neurochemical data, an SST+ excitatory central cell receiving input from an Aβ LTM at V1 glomerular profiles is the first element of the C1 microcircuit. SST, in this case released from other SGR neurons, could also act at V2 (axo-axonic) profiles of IIb glomeruli. However, other neurochemicals may participate to the modulation of mechanical information at these glomeruli, considering their complex pattern of receptor expression.

The C1 circuit conveys increased Aβ inputs to SDH neurons that may be very important in the generation of mechanical allodynia (Chen *et al.*, 2014; Kato *et al.*, 2009).

3.1.2. Input from other laminae

Kato et al. (2009) have demonstrated that excitatory transmission flows in a ventro-dorsal (upward) direction along the SDH. As mentioned, neurons in laminae III-V provide excitatory input to the II_m vertical cells that may be pivotal neurons in the transfer of information to lamina I (Fig. 5).

Parvalbumin-IR neurons in lamina III (see Figure 5 in Todd, 2017) have been reported to make synapses into the PKCγ-IR neurons in SGR (Petitjean *et al.*, 2015). These neurons likely correspond to the excitatory SST-containing central cells of circuit C1 in Fig. 6. However, these parvalbumin positive cells probably respond to tactile stimuli as they were not activated by nociceptive stimuli (Polgár *et al.*, 2013b).

3.1.3. Input from descending fibers

Supraspinal input to SGR has in part been considered in section 2.2.3.3.2. Synapses made by the descending fibers. Based on the response to nociceptive input, the RVM system consists of three types of neurons: ON-cells (pronociceptive), OFF-cells (antinociceptive), and neutral cells

(Morgan *et al.*, 2008; Urban and Gebhart, 1999). The ON/OFF system is triggered by opioids that excite the OFF cells and inhibit the ON cells.
As mentioned, 5HT descending fibers target primarily the large vertical/stalked cells that receive input at their proximal dendritic shafts (Miletic *et al.*, 1984). However, the RVM system not only consists of serotoninergic neurons but also comprises GABAergic and glycinergic neurons that participate to supraspinal modulation of SGR nociceptive circuitries (Antal et al., 1996). It was suggested that the RVM-mediated antinociceptive effects may be preferentially conveyed by GABAergic/glycinergic pathways: responsive neurons in SGR were located in the IB4+ lamina II_i and had (for the most) the morphology of the large vertical/stalked cells (Kato *et al.*, 2006). Based on Fig. 1 in Kato et al. (2006) these neurons may be the lamina II_m vertical cells. It is thus reasonable that neurochemically distinct descending fibers target diverse classes of vertical cells at the border with lamina I (5HT) or in a more ventral position (GABA/glycine). Very recently it was demonstrated that a population of GABAergic RVM neurons control the ENK^+ neurons in lamina II_i to modulate mechanonociception (François *et al.*, 2017).

3.2. Output from SGR

The outflow of information from SGR reaches excitatory projection neurons in lamina I (Figs. 4-6) or deeper laminae (III-V). Interlaminar SGR neurons sending their axons to other DH laminae comprise all the various cell types so far described in different species with the exception of the islet cells and the central cells. These neurons are the large vertical/stalked cells projecting to lamina I (Fig. 4-6) and the radial cells projecting to laminae III-V. Thus, the SGR sends excitatory signals to other regions of the DH, with the possible exception made by the inhibitory DYN-IR small vertical cells that have been postulated to directly contact lamina I neurons (C2 in Fig. 6 - dashed line).
The existence of interlaminar connections between the SGR and lamina I was initially inferred after experiments of cutaneous PAF stimulation in vivo (Tapper and Wiesenfeld, 1980). The underlying circuitry has been at least partly dissected out in the following decades and described above in section 3.1.1.1. Lamina II_o and IIi_d.
Nociceptive projection neurons in lamina I are remarkably low in number (Spike *et al.*, 2003), with a fraction of them responding to pruritic stimuli (Davidson, 2016). About eighty percent of lamina I projection neurons in rodents project to the parabrachial nucleus[23] and express the NK1 receptor (Todd *et al.*, 1998) – see also Box 2. Itch-processing neurons in lamina I have been identified as STT neurons in primates (Davidson *et al.*, 2012). These neurons responded to both mechanical and chemical itch (see Fig. 1 in Davidson, 2016). In SGR, mechanical itch follows a bi-neuronal chain of an inhibitory neuron expressing NPY and an excitatory neuron, presumably a large vertical/stalked cell; chemical itch is instead gated by a *Bhlhb5*+inhibitory interneuron followed by GRPR+ and natriuretic polypeptide receptor subtype A (NPRA)+ excitatory interneurons (Bautista *et al.*, 2014).
Connections between SGR and lamina III-IV (see also Box 3) were reported to originate from a small subset of NPY-expressing interneurons and hypothesized to be important in reducing the excitatory drive to lamina III projection neurons (Iwagaki *et al.*, 2016; Polgár *et al.*, 1999b, 2011).

3.3. Intralaminar circuits

All types of SGR neurons make synapses inside lamina II: their processes may be totally intralaminar (islet cells) or give rise to axons or axon collaterals inside lamina II (vertical, radial and central cells) before leaving SGR. Thus intralaminar circuitries are very complex and far from being completely understood. These circuitries have been at least in part described above

[23] In primates they project to the thalamus.

and very recently discussed in another publication (Todd, 2017). Here, I will only briefly mention those not considered in the previous sections.

A series of studies were dedicated to a limited set of SGR neurons that expressed GFP under the control of the prion promoter (PrP). These GABAergic neurons were initially reported to have homogenous morphological and functional features, to receive a C PAF input, and to respond to noxious stimulation (Hantman *et al.*, 2004). Subsequently, they were, instead, recognized to be morphologically and neurochemically heterogeneous and to represent a subset of inhibitory interneurons that expressed nNOS and/or DYN and galanin (Ganley *et al.*, 2015; Polgár *et al.*, 2013b). These cells received synaptic input from a variety of PAFs, were reciprocally connected with the islet cells and sent their axons to NK1-IR projection neurons and the giant lamina I neurons (see Figure 5 in Todd, 2017). As PAF input is heterogeneous, mouse PrP-GFP neurons expressing nNOS may correspond to the rat lamina II_i NADPH diaphorase stained islet cells that produce NO and modulate the release of neuropeptides from pPAFs (see Fig. 8 in Aimar et al., 1998).

Todd's group has also studied the NPY-expressing cells in a NPY-EGFP mouse line (Iwagaki *et al.*, 2016). Fluorescent neurons corresponded to about one-third of NPY-IR neurons in laminae I-II, were directly connected to C PAFs and, after pharmacological challenge, supposed to be devoid of TRPV1 or TRPM8. This latter observation is puzzling since C PAFs were reported to express one or the other of the two TRP channels, at least in rat (Kobayashi et al., 2005). Input to the NPY-EGFP SGR neurons was reckoned to derive from C^{MrgD} nociceptors, a specific class of mouse skin nociceptors that, for the most, were devoid of TRPV1[24] and monosynaptically connected to several types of SDH neurons, including the radial, tonic central, transient central, and vertical cells of lamina II and the antenna cells of laminae III-IV (Wang and Zylka, 2009).

4. The gate theory and beyond

A discussion on the importance, the merits and pitfalls of the gate theory goes over the purpose of this contribution, and readers should refer to existing literature on these issues (Braz *et al.*, 2014; Mendell, 2014; Moayedi and Davis, 2012). Along the last decades, a continuous addition of information has partly enlightened the complex circuitry that extends beyond the inhibitory neuron depicted in their original scheme by Melzak and Wall (1965). Hidden by the new complexity, at least some of the ideas of the gate theory resisted along time and have been very important, as stressed by Wall himself to *"provoke discussion and experiment"*. Some of the basic concepts of the theory are nowadays under profound revision. A recent paper has e.g. suggested that mechanical pain transmission neurons may be the SGR somatostatinergic cells (Duan *et al.*, 2014). Even more recently, a computational model was proposed to revisit the theory, based on the notion that, indeed, all PAFs are excitatory (Ropero Peláez and Taniguchi, 2016).

To my knowledge, a point that has not been addressed yet in discussing the gate theory is the contribution of neuropeptides and other high MW modulators to the gate (Merighi *et al.*, 2011). In 1965, these neuroactive substances were poorly characterized or, for the most, yet to be discovered. This issue is closely related to the still ongoing debate on the possibility that pain specificity relates to specialized nociceptive pathways (Braz *et al.*, 2014), a hypothesis that conflicts with the concept of nociceptor's polymodality. The contribution of high MW transmitters/modulators to nociceptive processing has been (and still is) difficult to ascertain with certainty. As widely discussed in previous sections, these substances have clear pronociceptive or antinociceptive effects when given in vivo or in vitro. However accurate, neurochemical characterization led to relatively scarce functional information on their circuitry

[24] C^{MrgD} nociceptors correspond to the C non-peptidergic rat nociceptors.

in SDH and often (but fortunately not always) these molecules have been simply regarded as mere neurochemical tags. Although very interesting from a conceptual point of view, the aforementioned computational model of the gate theory (Ropero Peláez and Taniguchi, 2016) correctly takes into consideration that all PAFs are excitatory but does not consider that a subset of pPAFs stores together positive and negative high MW modulators of sensory neurotransmission.

Here I propose that gating mechanisms mediated by high MW transmitters/modulators not only occur between the non-nociceptive and the nociceptive PAFs, but also between parallel processing pathways pertaining different subpopulations of pPAFs and between pPAFs and non-pPAFs (Figs. 4-6).

Today, the idea that PAFs are functionally labeled starts to be accepted (Braz *et al.*, 2014): noxious heat, cold, and mechanical stimulation, are transmitted along different subsets of PAFs respectively expressing TRPV1, TRPV1+TRPA1, and *MrgD*. Likewise, specific itch-transmitting PAFs derive from pruriceptors in DRGs expressing *MrgA3* (Han et al., 2013) and/or B-type natriuretic peptide (Bautista *et al.*, 2014). Under this light, new transcriptomics information is also helping in deciphering the functional complexity of DRG neurons (Box 6). Coding of chemical pain in a broader sense may be more complex, as nociceptive C PAFs express an array of ion channels that sense a variety of chemicals of heterogeneous nature. For example TRPV1 not only senses heat but also capsaicin and several other substances (Frias and Merighi, 2016), and TRPA1 not only responds to cold but also to mustard oil and numerous other irritant compounds (Chen and Hackos, 2015).

Computational analysis has confirmed that sensory nociceptive information is also encoded in the temporal discharge patterns of (cutaneous) C PAFs (Cho *et al.*, 2016). As discharge pattern may selectively give way to the release of low and high MW transmitters in pPAFs (see 2.2.4. Neurochemistry of SGR), one may speculate that circuits A (Fig. 4) and B (Fig.5) specifically gate noxious heat/cold and chemical pain. This hypothesis is appealing if one considers that circuit C (Fig. 6) has previously been proposed as a specific pathway to gate mechanical pain (Duan *et al.*, 2014). If correct, a specific label could also exist at the level of, at least, the large vertical/stalked cells (Fig.4-6) as those expressing SP/ENK may integrate noxious heat/cold and chemical stimuli, whereas those expressing SST should intervene in the processing of mechanical stimuli.

Therefore, it would be interesting to experimentally challenge these circuits that put in play so far unconsidered gates in SGR.

5. Spinal gray matter lamination in the third millennium: has the SGR come to age?

Given today's well-established existence of extensive connections of lamina II with other laminae of the gray matter, one could ask whether it still makes sense to hold the SGR as a discrete entity. This is indeed a reasonable question, which poses the alternative of considering at least the SDH as a single functional set that processes the initial sensory information reaching the spinal cord. However, such an alternative view directly challenges the concept of spinal cord lamination.

For an apropos discussion, I must stress that in this paper the term *layer* is used only in reference with previous work depicting the history of SGR discovery (Section 2.3.1. and Box 1). Anywhere else I made reference to the Rexed's laminar subdivision. In spinal cord, the terms *layer* and *lamina* should not be used interchangeably in reason of the well-known differences in embryonic development compared to the most anterior parts of the neural tube. Indeed, it is well accepted that three different strategies are employed to produce distinct classes of neurons during development: dorsal-ventral polarization in the spinal cord, segmentation in most regions of the encephalic trunk, and generation of *cellular layers* in the cerebral and

cerebellar cortices, tectum and retina (McConnell, 1995). Typically, the generation of layered structures in the brain segregates neurons that share distinctive dendritic morphologies, physiological properties, and axonal connections. However, the Rexed's *laminar* subdivision was simply based on cytoarchitectonic features such as shape, size, density and distribution of perikarya, but neglected all the others conceptually cognate to a genuine layer.

Whether Rexed's laminae are indeed different among each other has been the object of some work that used the Sholl's linear method (where dendritic arborization is taken into account) complemented by nonparametric statistics in humans, cats or rats (Milosevic *et al.*, 2005; Ristanovic and Milosevic, 2007; Schoenen, 1982). Specifically, Schoenen carried out a quantitative analysis of laminae I-IV neurons and showed that each lamina had a different dendritic organization. However, Milosevic and co-workers concluded that laminae II-III[25] were statistically different from laminae IV, V and VI but not lamina I. The logical conclusion of their analysis would be that the SDH forms a single, discrete part of the spinal gray matter.

With the rapid progress of molecular biology and mouse genetics in the last decades, it is today indubitable that the organized arrangement of neurons in the mature spinal cord DH derives from a pattern of cell types that is established in the early embryonic neural tube (Lai *et al.*, 2016). Work in the field demonstrated that the interneurons populating *each* of currently recognized DH laminae possessed a different repertoire of transcription factors and displayed different routes of migration. The latter occurred along two temporal windows to reach the SDH or the deeper laminae (Caspary and Anderson, 2003; Matise, 2002). Hence, such additional clues must today be considered is support of the distinctiveness of Rexed's laminae.

In spite of this, an issue of complication in defining the individuality of SGR concerns its functionally heterogeneous input from PAFs and connections with other DH laminae, to the point that, as thoroughly discussed in previous sections, it was possible to further subdivide lamina II in at least two sublaminae. Although one can legitimately embrace the point of view of reconsidering the laminar subdivision of the DH, I believe that still we are far from a coherent view that brings together the array of data stemming from molecular genetics, embryology, histology, physiology, circuitry and pharmacology of the DH. In particular, mouse genetics may be useful for a better definition of laminar boundaries, if indeed necessary (Lai *et al.*, 2016). Under this perspective, I believe useful to here quote a couple of examples. Among the Hox transcriptional factors, loss of Hoxb8 altered the laminar cytoarchitecture of the spinal DH and sensory responses in mice (Holstege *et al.*, 2008). Notably, in Hoxb8 mutants the organization of laminae I and II was altered, in parallel with a deficit in nociceptive and thermoreceptive responses, but deeper laminae were unchanged. Therefore, these results do not reconcile with the idea of SDH as a single entity. Occurrence of neuron positioning errors in the SDH of the *Reeler* mouse offers an additional example that challenges this idea. The *Reeler* is a spontaneous mutant totally devoid of Reelin, a glycoprotein indispensable for correct neuronal positioning within the cortical layered structures of the brain (Katsuyama and Terashima, 2009). Mutants exhibited an aberrant neuronal compaction in laminae I–II and V and reduction of neurons in lateral spinal nucleus, accompanied with thermal hyperalgesia and decreased mechanical sensitivity (Villeda et al., 2006). Specifically, Villeda et al. reported that mutants "had a neuron-sparse region between lamina I and II_o, a feature not normally found in wild-type DHs" and that neuronal positioning errors were accompanied with a "certain bilaminar split of the primary afferent projections to lamina I". Thus, deficiency of Reelin causes a disturbance of the normal dorsal-ventral patterning in the mature spinal cord and thereby gives additional support to the idea that the SDH has indeed a laminar organization.

[25] These authors have unfortunately considered laminae II and III together

Another example can be considered in relation to the better known and most widely investigated modalities of development of the cerebral cortex. In cortex, it is well known that, after onset of neurogenesis, the time when neurons are generated determines their laminar phenotype (Geschwind and Rakic, 2013). Remarkably, the birthdate of GABAergic neurons in the lumbar spinal cord of GAD67-GFP knock-in mice was demonstrated to vary in different laminae of SDH and to follow a specific temporal sequence (Huang *et al.*, 2013).

In my opinion, these examples are enough to sustain the usefulness of still considering the SGR as a discrete region, at least until Rexed's classification may be substituted with a more appropriate model.

6. Concluding remarks

About two-hundred years ago, Rolando described the SGR simply observing its peculiar aspect in what we would nowadays considered almost useless gross anatomical preparations. Today's picture of the SGR has disclosed a fascinating complexity. However, it is marvelous that the bygone anatomists that followed the initial wick lit by Rolando immediately recognized the importance of the SGR in sensory processing. Subsequent investigations led to establish its role in integrating and gating the nociceptive stimuli collected at periphery, before they were conveyed to the brain. Now we know that the SGR is also directly involved in the processing of itch, a very important sensory modality with relevant clinical implications.

In this review I tried to provide an up-to-date and comprehensive survey on the histology, physiology and neurochemistry of the *normal* SGR. I have also proposed that the cross-talk between different types of PAFs triggered by their peptidergic subpopulations is important in gating specific pain modalities, and suggested that the excitatory large vertical/stalked cells may be functionally heterogeneous. If these propositions will stimulate new experiments, then I will have fully achieved my objectives.

I did not touch, if not by listing them analytically, the constellation of receptors and receptor subtypes of the excitatory and inhibitory neurotransmitters that obviously have a primary role in fast synaptic transmission within the DH, as these issues have been recently and authoritatively reviewed (Ingram *et al.*, 2009; Zeilhofer *et al.*, 2012).

It is well known that nociceptive pathways undergo a remarkable plasticity in conditions of pathological pain leading to central sensitization, hyperalgesia and allodynia (Basbaum *et al.*, 2009), but can anomaly detection work if one doesn't know normality?

Table and Figure legends

Table 1: A summary of the main stages in the accumulation of knowledge on lamina II after the first morphological description by Luigi Rolando

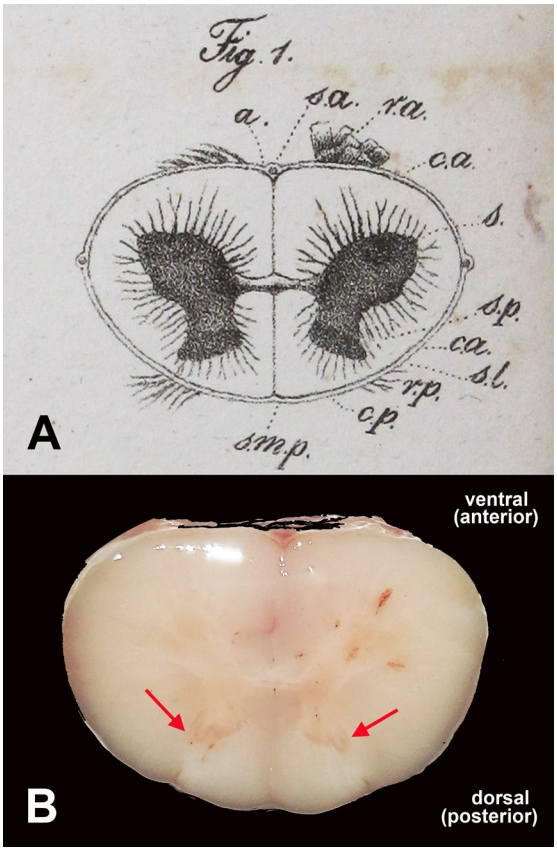

Figure 1: Transverse section of the ox spinal cord. **A:** Rolando's original drawing of the cervical spinal cord (Table III, Fig. 1) Abbreviations: c. a. = anterior columns; c. p. = posterior columns; s. = anterior gray matter; s. p. = posterior gray matter and substantia gelatinosa; s. a. = anterior median sulcus; s. m. p. = posterior median sulcus; a. = anterior spinal artery; r. a. = anterior roots; r. p. = posterior roots; s. l. = lateral posterior sulci. Note that Rolando divided the white matter in an anterior and posterior column only. B = fresh section of the ox cervical spinal cord. The SGR can be seen as a darker undulating brown layer of the gray matter (red arrows).

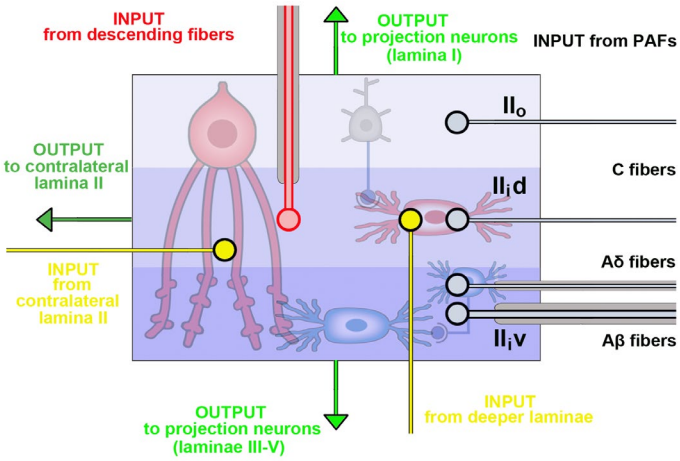

Figure 2: Simplified scheme of the SGR circuitry in rodents. The SGR is divided into three sublaminae that receive an ordered input from functionally different subsets of PAFs. The SGR circuitry (shadowed and depicted only for pictorial reasons) integrates input and output signals. Output signals reach the contralateral SGR or other laminae of DH.

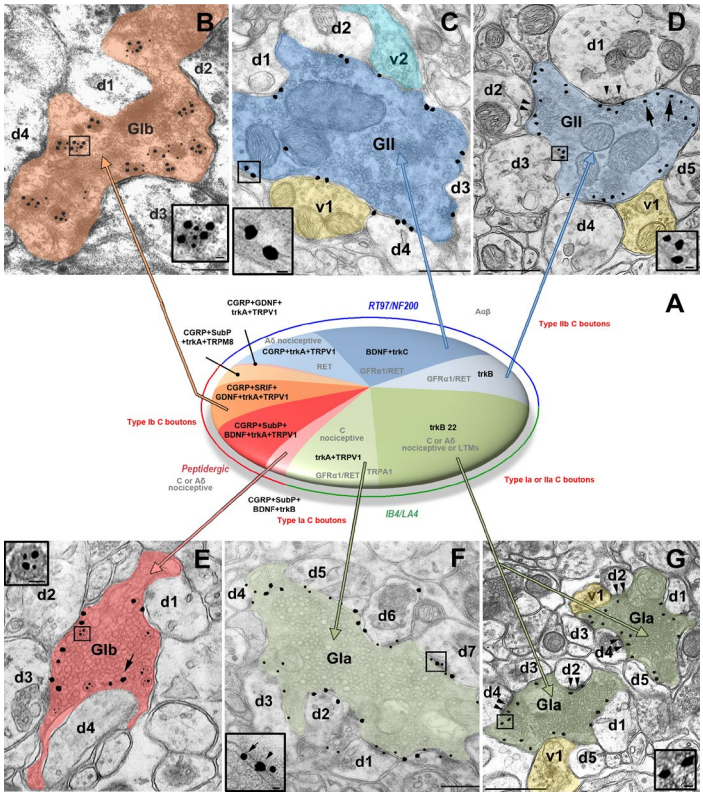

Figure 3: Simplified pie chart of the neurochemistry of the DRG neurons and examples of their central glomerular projections discussed in section 3.1. Input to SGR. **A**: Wedge sizes have been elaborated and partly extrapolated from observations that were, for the most, referred to rodent studies (Averill *et al.*, 1995; Djouhri, 2016; Fang *et al.*, 2006; Josephson *et al.*, 2001; Kestell *et al.*, 2015; Kobayashi *et al.*, 2005; Michael *et al.*, 1997; Molliver *et al.*, 1997; Molliver and Snider, 1997; Price and Flores, 2007; Salio *et al.*, 2005, 2014; Salio and Ferrini, 2016). Whenever possible, differences between rat and mouse (e.g. in the levels of

expression of TRPV1 – see main text) have been taken into consideration in calculating the figures for each class of neurochemically defined DRG neurons in the chart. Note that there are significant discrepancies in the results from single species as a consequence of different experimental approaches, e.g. ISH versus ICC. Nonetheless, data fitted into a coherent frame. DRG neurons were divided into three main categories: the small-sized dark peptidergic neurons, the small-sized dark non-peptidergic neurons, and the medium/large-sized neurons according to Averill et al. (2005). Peptidergic small neurons (shadows of red, orange or pink) express CGRP in combination with other peptides, primarily SP or SST. About ¾ of these neurons also express TRPV1 and give rise to small unmyelinated nociceptive C fibers (Kobayashi *et al.*, 2005). Functionally, in rat these neurons are mechanically insensitive and respond to heat, but in mouse some TRPV1 negative DRG neurons also respond to heat (Woodbury *et al.*, 2004). The TRPV1-negative peptidergic neurons expressing CGRP, SP and trkA (light orange) are likely to also be TRPM8+. They give rise to poorly myelinated Aδ fibers that transduce innocuous cold (Kobayashi *et al.*, 2005). Note that among trkA+ peptidergic DRG neurons there are two quantitatively equivalent subpopulations expressing CGRP, SST and GDNF (orange) or CGRP, SP and BDNF (red). A third, less represented, subpopulation expresses CGRP, SP and BDNF as well as trkB (pink).

Non-peptidergic small neurons (green) can be labeled with IB4 and/or LA4 antibodies. These neurons give rise to C nociceptive (TRPV1+ or TRPV1-) and Aδ fibers that may be nociceptive or non-nociceptive (Funakoshi *et al.*, 2006; Price and Flores, 2007). A subpopulation of these neurons expresses the GFRα1/RET receptor complex for GDNF. The darker green wedge includes the non-peptidergic C polymodal nociceptive neurons expressing - at least in mouse - *MrgD* (Liu *et al.*, 2008; Wang and Zylka, 2009). Three other populations of neurons are not represented because of the relative lack of quantitative data and/or the existence of variations at different segmental levels, e.g. reflecting the presence of glabrous skin in certain dermatomes: the LTMs originating the trkB-positive Aδ D-hair afferents (Shin *et al.*, 2003; Stucky *et al.*, 2002) that do not bind IB4; the C-LTMs chemokine-like secreted protein TAFA4 (Delfini *et al.*, 2013) and the *MrgA3*/*MrgC11* population of putative pruritoceptive afferents (Liu et al., 2008, 2009).

Non-peptidergic medium-to-large size neurons can be subdivided in three subpopulations according to their pattern of expression of trkA, B or C. Note that the DRG neurons expressing trkA also express TRPV1 and CGRP and may be Aδ nociceptors. An additional marker of nociceptive Aδ fibers may be the acid sensing ion channel 3, a putative mechano-sensing channel (McIlwrath *et al.*, 2007).

B: C bouton in a type Ib glomerulus after double immunogold labeling for GDNF (small gold particles) and CGRP (large gold particles); **C:** C bouton in a type II glomerulus after immunogold labeling for GFRα1 (silver intensified gold particles); **D:** C bouton in a type II glomerulus after immunogold labeling for trkB (silver intensified gold particles, some indicated by arrows). Arrow heads indicate postsynaptic densities; **E:** C bouton in a type Ib glomerulus after triple immunogold labeling for BDNF (small gold particles), SP (large gold particles) and trkB (silver intensified gold particles); **F:** C bouton in a type Ia glomerulus after double immunogold labeling for GFRα1 (small gold particles) and IB4 (silver intensified gold particles); **G:** C boutons in type Ia glomeruli after immunogold labeling for trkB. Some synaptic densities are indicated by arrow heads.

C boutons in B-G are pseudo colored with the same colors employed in A for parent neurons. V1 profiles are pseudo colored in yellow. The V2 profile in C is pseudo colored in

azure. Plain dendrites (d) belonging to the same glomerulus are numbered progressively. Panels B-E modified from Salio et al. 2005, 2014.

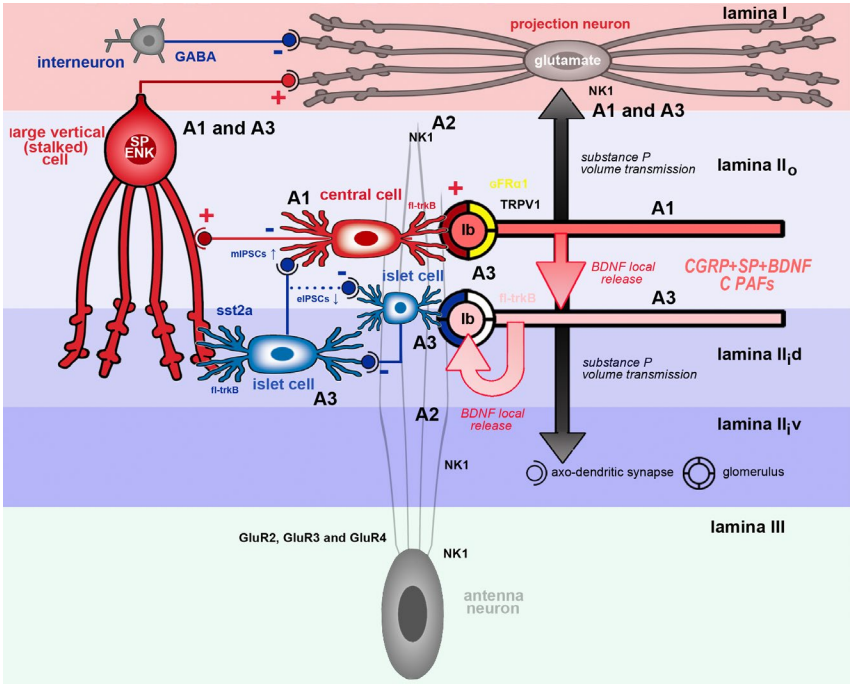

Figure 4: Simplified neuronal circuits involved in excitation driven by pPAFs in SDH. Excitatory and inhibitory SGR neurons are red and blue, respectively. PAFs are color-coded according to Fig. 3.

Circuits marked with **A** originate from C type (CGRP+) pPAFs expressing SP and BDNF. These circuits explain EPSCs and IPSCs that can be recorded from SGR neurons challenged with SP or BDNF. They are consistent with the overall pronociceptive effects of these two pain modulators. Circuit **A1** is made of a direct trisynaptic excitatory pathway (C fiber → central cell → large vertical cell → excitatory NK1+ lamina I neuron). It is also possible that the glutamatergic large vertical cells themselves release SP acting on the excitatory NK1+ projection neurons in lamina I via volume transmission.

Antenna neurons in lamina III express NK1 receptors at dendrites entering the SGR. Receptors at these dendrites may be activated by locally-released SP (**A2**) also in the absence of synaptic specializations. Under basal conditions, C type pPAFs may also release BDNF. The effects of BDNF occur along both pathways (**A1** and **A2**) after activation of *postsynaptic* fl-trkB receptors.

When excitatory neurotransmission is reinforced (**A3**), BDNF can also act onto *presynaptic* fl-trkB receptors expressed by a different subset PAF C boutons (pink). As these boutons also contain BDNF, fl-trkB receptors are *autoreceptors* for these fibers. The neuronal chain in A3 is C pPAF → 1st islet cell → 2nd islet cell → central cell → large vertical cell → lamina I excitatory projection neuron. As the first islet cell inhibits the second islet cell that, in sequence, is inhibitory to the central cell, the circuit is excitatory onto the large vertical cell. This type of configuration explains why BDNF was shown to produce a depression of the evoked IPSCs (at presumable axo-dendritic synapses between the islet cells) as well as an increase of mIPSCs at synapses between the islet cell and the central cell.

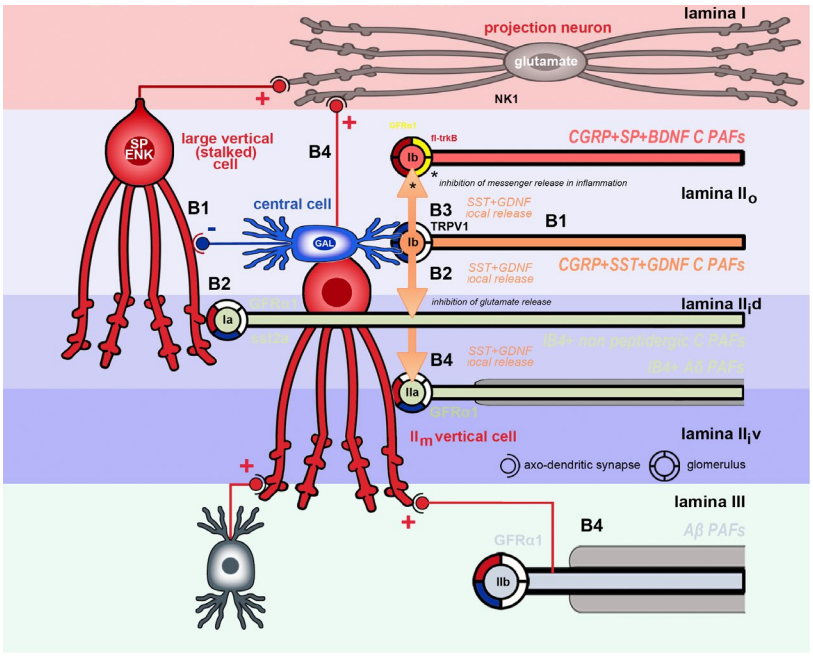

Figure 5: Simplified neuronal circuits involved in inhibition driven by pPAFs in SDH. Excitatory and inhibitory SGR neurons are red and blue, respectively. PAFs are color-coded according to Fig. 3.

Inhibitory circuits originate from C pPAFs (CGRP+) expressing SST and GDNF (orange) and provide tonic inhibition to the outflow of information from SGR. In **B1**, the C PAF, with the interposition of a central cell, inhibits the excitatory large vertical cell connected to the lamina I projection neuron. In **B2**, local release of SST and GDNF from the C pPAF presynaptically inhibits a C type non-pPAF (IB4+ - light green) expressing sst2a and GFRα1 receptors. The fiber forms the C bouton of a type Ia glomerulus connected with the large vertical cell. The **B2** circuit may be important in hyperalgesic priming, as tonic inhibition of non-pPAFs controls the transition from acute to chronic mechanical pain. In **B3**, locally released GDNF acts at type the Ib glomeruli made by SP+/BDNF+ pPAFs (red) that display GFRα1+ peripheral dendrites. In **B4**, both the Aδ non-pPAFs and the Aβ PAFs are under tonic inhibitory control of GDNF, as the C boutons of type II glomeruli also express GFRα1. The functional significance of these interactions remains to be established in full, as no data are available regarding GDNF volume transmission. Interactions with Aβ PAFs may also modulate the flow of information through II_m vertical cells, as a parallel path converging to lamina I projection neurons (see main text).

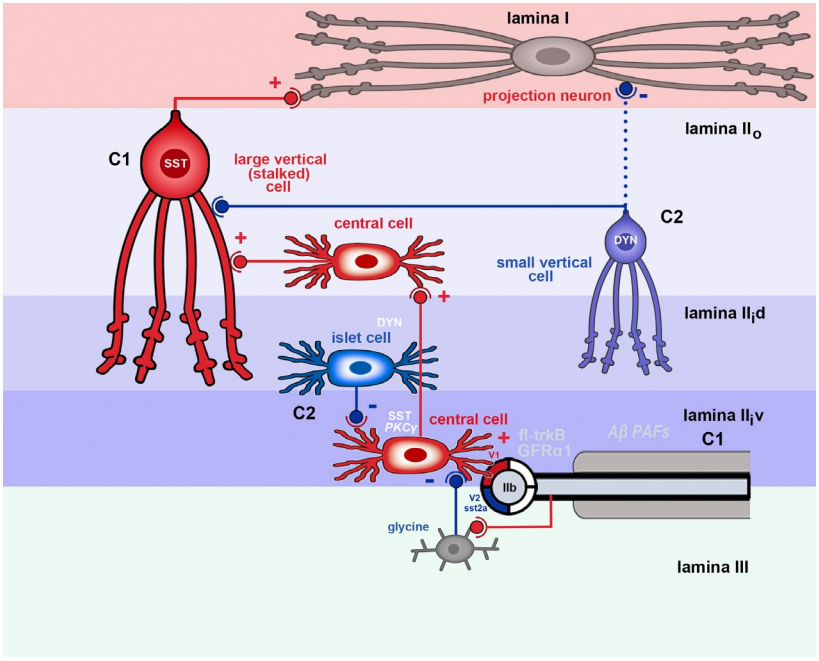

Figure 6: Mechanoreceptive circuits in SDH. Excitatory and inhibitory SGR neurons are red and blue, respectively. PAFs are color-coded according to Fig. 3.
Aβ mechanoreceptive PAFs at the border of laminae II/III and/or in lamina III originate an important circuit that gates mechanical pain and involves excitatory and inhibitory neurons, respectively expressing SST and DYN. In **C1**, a SST+/PKCγ+ central cell, with the interposition of a second central cell, excites the SST+ large vertical cell connected to the lamina I projection neuron. In lamina III, collaterals of Aβ PAFs may regulate the flow of information along **C1** by activating a glycinergic inhibitory interneuron that hyperpolarizes the first central cell of the pathway. In **C2**, DYN+ neurons inhibit the SST+ vertical cell directly (DYN+ small vertical cell) or indirectly (DYN+ islet cell). It has also been hypothesized that the small vertical cells monosynaptically inhibit lamina I projection neurons (dashed line). For simplicity the inhibitory descending input to SGR is not represented. These circuits may be very important in triggering mechanical allodynia.

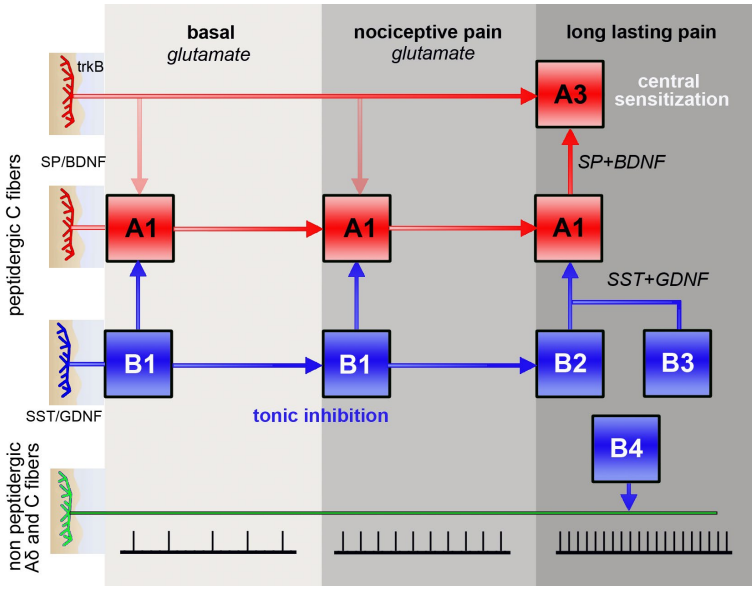

Figure 7: Scheme of the cross-talk of peptidergic C fibers to gate the outflow of nociceptive information from SGR in relation to the differential release of coexisting fast- and slow-acting neurotransmitters. Excitatory and inhibitory circuits described in Fig. 4-5 are in red and blue respectively. According to the pattern of firing, PAFs release glutamate without or with coexisting high MW neuropeptides and trophic factors (see Box 4).

BOX 1: The history of the substantia gelatinosa

The first description of the substantia gelatinosa dates back to 1824 when Luigi Rolando published his "Ricerche anatomiche sulla struttura del midollo spinale"[26] (Rolando, 1824). In describing the posterior gray matter in fresh spinal cord sections from humans and several other mammals, Rolando wrote (page 60): "Then what has most surprised me was seeing that *no one has made mention of the difference that passes between the gray matter in the anterior part* (of the spinal cord -TN) *and that corresponding to the posterior columns*, which, at times, surrounds and, at times, almost entirely forms the posterior horns, as can be seen from an examination of the cross sections of the cord here represented in the attached figures. And then he adds "It is quite true that *only in quadrupeds I was able to see this new grayish substance…* However, *even in the human spinal cord…I could find out the traces of this new jelly-like* (gelatinosa in Italian and Latin -TN) *substance. It even takes a different color that, in general, is less reddish and darker*". The second use of the word "gelatinosa" occurs at page 87 in one of the "corollaries" (number 10) that close the work. There Rolando wrote "*that the primordial cavity of the spinal cord insensibly fills from the accumulation of two different grayish substances, an anterior one, and the other posterior, darker and most gelatinous*". A very precise description of what we still nowadays call the SGR is afterwards found in the legend of Table II/Figure VII where Rolando, in describing a transverse section of the ox cervical spinal cord, wrote "*the gray matter is found in larger quantities. At the ends of the two posterior horns a layer of a different gray matter, more gelatinous and gloomier was discovered, of which no anatomist did ever make mention*". He also described the substantia gelatinosa in the legends of Table III/Figure I (Fig. 1A) and II.

Rolando made his observations on fresh material or on spinal cord sections initially soaked in saline or alcoholic solution and then left to macerate for up to twenty days in water. Obviously, these methods did not allow going beyond the study of certain gross anatomical features (Fig. 1B).

The introduction of the practice of tissue fixation was an important step to achieve further knowledge on the organization of the gray matter in the nervous system. Table 1 summarizes the most important steps in the understanding of the SGR architecture. Readers are invited to read Lenz *et al.* (2010) for an excellent historical account about the progress in knowledge on SGR, and, more in general, spinal cord nociceptive pathways.

[26] Anatomical investigations on the structure of the spinal cord – TN. Rolando's paper was of course written in the Italian of that time. My English translation tries to maintain his original constructions of phrases and terms.

TABLE 1			
Type of preparation	**Major findings**	**Species**	**References**
Chromic acid fixation	• SGR is made of small sized cells • First description of larger fusiform cells at SGR periphery* • SGR is crossed by spared bundles of fibers	ox, calf man fowl, tortoise	Clarke, 1859; Stilling, 1842**
Carmine* **	• SGR is made of neurons	man several mammals	Deiters, 1865****; Kölliker, 1867; Gierke, 1885, 1886; Lissauer, 1886; Virchow, 1887; Waldeyer, 1888
	• SGR is mainly made of glia	man several mammals	Gerlach, 1872; Betchterew, 1886
Myelogenetic techniques	• Myelinated fibers cross the SGR	man	Flechsig, 1876); Kahler, 1888
	• SGR contains small neurons	man	Edinger, 1889
Golgi staining	• Description of different morphological types of SGR neurons • Distinction between marginal cells (giant, fusiform) of SGR and cells of SGR (proper) • Collaterals of PAFs enter the SGR	man several mammals birds, fish, reptiles	Cajal, 1890a; 1890b; 1890c; Cajal, 1891; 1893; 1895; Kölliker, 1890, 1891; Lenhossék, 1895; Retzius, 1891; Van Gehuchten, 1901
Pyridine silver method (unmyelinated fibers)	• Branches of small unmyelinated fibers in dorsal root enter the Lissauer's tract and terminated in the SGR	man	Kölliker, 1890
	• Large marginal cells as a part of a magnocellular group • SGR is called nucleus sensibilis proprius with small cells	man	Jacobsohn, 1908
	• SGR is called posterior sensory zone (nucleus sensibilis proprius) with small cells	man	Massazza, 1922; 1923; 1924
Nissl stain	• SGR is described as a discrete layer	man	Bok, 1928
Myelin stain	• SGR is subdivided in three sublayers*****	man	Bok, 1928
	• SGR is described as a discrete layer	man	Strong and Elwyn, 1943
Nissl cytoarchitectonic stain	• SGR is one of the then laminae in which the spinal cord gray matter can be divided	cat man monkey	Rexed, 1952, 1954 Schoenen, 1982; Schoenen and Faull, 2004; Sengul et al., 2013 Shriver et al., 1968; Sengul et al., 2013

		rat mouse	Molander et al., 1984, 1989; Sengul et al., 2013; Sengul, 2015 Sidman et al., 1971; Heise and Kayalioglu, 2009; Sengul et al., 2012
Electrophysiology Immunocytochemistry/ lectin binding	• SGR is subdivided into lamina II_o and lamina II_i	man, monkey, cat, rat, mouse	Light and Perl, 1979a; Rexed, 1952; Rexed, 1954; Schoenen, 1982; Woodbury et al., 2000; Pan and Pan, 2004; Lorenzo et al., 2008; Ribeiro-Da-Silva and De Koninck, 2008

* Marginal or zonal cells (Waldeyer, 1888)

** Stilling was the first to introduce the method of serial sectioning

*** Before the era of hematoxylin and synthesized aniline dyes as histological stains, the method of choice to stain tissue sections employed natural dyes such as carmine and saffron. Carmine is obtained from the bodies (female) of the insect *Dactylopius coccus cacti*. The active coloring agent in carmine is carminic acid (C.I. 75470, Natural red 4). For nuclear staining it is invariably used in conjunction with a mordant such as alum solution

**** Deiters was the first to draw a (spinal cord) neuron using a method of microdissection that remained concealed to other researchers as a consequence of his premature death

***** SG (stratum gelatinosum Rolando), SSE (stratum spongiosum externum substantiae Rolando), SSI (stratum spongiosum internum substantiae Rolando)

Box 2: Lamina I

Lamina I is very important in the processing of noxious, thermal and pruritic stimuli (Braz *et al.*, 2014; Davidson, 2016; Grudt and Perl, 2002; Han *et al.*, 1998; Lima and Coimbra, 1988; Lima, 1998; Prescott and De Koninck, 2002; Wu *et al.*, 2010; Zhang *et al.*, 1996). It contains several types of neurons that, on histological and functional bases, can be divided into three main discrete populations: the fusiform, pyramidal, and multipolar neurons (Todd *et al.*, 2002).

Fusiform neurons are the most numerous. These cells have longitudinal spindle-shaped cell bodies at which extremities emerge two well-developed spiny dendritic trees. Fusiform neurons fire tonically; in cat, they were identified as nociceptive-specific and were activated by mechanic and thermal stimuli (Han *et al.*, 1998).

Pyramidal neurons have prismatic triangular cell bodies, from which stem a few large dendrites that ramify inside the white matter. Altogether, also the dendritic tree has a pyramidal shape. In cat, these neurons displayed phasic firing, were thermoreceptive and responded only to innocuous cooling (Han *et al.*, 1998). However, in rat some pyramidal projection neurons expressing NK1 and receiving a selective innervation from SP-containing PAFs were reported to be nociceptive (Todd *et al.*, 2002).

Multipolar neurons have dendritic arbors of spherical shape. These cells displayed delayed-onset, single spikes at electrophysiological recordings. In cat, multipolar neurons were reported to be polymodal nociceptive cells, and responded to heat, cold and/or pinch (Han *et al.*, 1998).

The list of neurochemicals reported in lamina I includes excitatory (glutamate) and inhibitory (GABA, glycine) transmitter amino acids, biogenic amines (dopamine, 5HT), and several neuropeptides (Sengul, 2015). Neurons also express a wide array of receptors for glutamate, GABA and neuropeptides (Ribeiro-da-Silva, 2015). GABA and sst2a receptors were reported to be expressed in all neuronal types except the pyramidal neurons, whereas SP was detected in multipolar cells. Beside the neurochemicals above, DYN, NK-1, calbindin and NOS were also detected, but appeared not to display a specific localization in a given morphological type of lamina I neurons (Wu *et al.*, 2010).

Although a small fraction (about 5%) of the total neuronal population (Spike *et al.*, 2003), lamina I projection neurons, are very important as they contribute to the transmission of pain messages to the brain. About 80% of these neurons express NK1 and are presumed to be exclusively excitatory. In rodents, the large majority ofNK1+ lamina I projection neurons reach the parabrachial nucleus (Spike *et al.*, 2003), but a few project to the thalamus (Yu *et al.*, 2005). More generally across species, projection neurons reach the thalamus and several areas of the brainstem (for review, see e.g. Table 2 in Wu et al. 2010). Some of those projecting to the thalamus are of the WDR type. Differently from the nociceptive neurons, their functional properties remain unaltered after nerve injury (Lavertu *et al.*, 2014).

Lamina I inhibitory interneurons are for the most GABAergic, but about one third of them also express glycine (Todd and Sullivan, 1990). In the spinal cord dorsal horn of GAD67-EGFP mice, inhibition by GABA and glycine appear to be region-specific and to define two populations of cells: the first is GABA-dominant and prevails in laminae I–II$_o$, the other is

glycine-dominant and occurs at the lamina II_i–III border (Bardoni *et al.*, 2013; Takazawa *et al.*, 2017).

The PAF input to lamina I mainly derive from nociceptors of the Aδ (Light and Perl, 1979b) and CGRP/SP peptidergic C types (Alvarez *et al.*, 1993).

Box 3: Laminae III-V

Laminae III and V are far less well characterized than laminae I and II. They are composed of small interneurons and larger projection neurons, with a relatively low neuronal density.

Lamina III is characterized by the presence of myelinated fibers and contains morphologically heterogeneous neurons (from rounded to spindle-shaped). Also in *lamina IV* neurons have different shapes (round, triangular or multipolar). They tend to be of medium/large size, particularly if compared with those of SGR. *Lamina V* is the most extended of the dorsal horn and its neurons are also of heterogeneous morphology. At least in rodents, it can be divided into a medial and a lateral part. The former consists of small- to medium-sized neurons, while the latter is made of large neurons and well evident bundles of fibers. Dendrites of lamina V neurons may reach laminae II-III, dorsally, and lamina VII, ventrally (Schoenen and Faull, 2004).

Interneurons in laminae III-IV display excitatory or inhibitory phenotypes (Abraira et al., 2017). Neurochemical markers of the former include VGLUT2, SST, neurotensin, preprotachykinin B and PKCγ. A recent quantitative analysis was carried out on the excitatory interneurons in lamina III (Gutierrez-Mecinas *et al.*, 2016). Quantitative data are missing or less well defined for laminae IV-V.

As their main neurotransmitter(s), inhibitory interneurons use GABA and/or glycine. They have been localized with antibodies against the transmitter aminoacids, GAD (Mackie *et al.*, 2003) and GLYT2 (Zeilhofer *et al.*, 2005), or after genetic tagging with FRPs (see main text). In lamina III, 64% colocalization of the two transmitters was found (Polgár *et al.*, 2003; Todd, 1990; Todd and Sullivan, 1990).

The GABAergic interneurons may also express NPY (Iwagaki *et al.*, 2016) or acetylcholine (Mesnage *et al.*, 2011). They have been further classified into two main groups in hamster: the mostly represented local axon cells and the less abundant deep axon cells (Schneider and Lopez, 2002). Lamina IV seems to contain less GABAergic neurons than laminae III and V (Nowak et al., 2011).

Glycinergic neurons (which are known to colocalize GABA) appear to be concentrated in lamina III and much less represented in laminae IV-V (Zeilhofer *et al.*, 2005). Those in lamina III were demonstrated to be connected to PKCγ excitatory interneurons in lamina II_i (see Fig. 6 in main text) and to gate mechanical allodynia by blocking the activation of lamina I projection neurons (Lu *et al.*, 2013).

The input to lamina III-V neurons mainly derives from Aβ and Aδ cutaneous LTMs. However, some neurons in lamina IV also respond to noxious stimuli. Lamina V neurons (WDR, nociceptive or mechanoreceptive) receive an additional polysynaptic input from C fibers. Output from laminae III-V occurs via the spinocervical (vestigial in humans), spinothalamic, spinohypothalamic, spinoparabrachial and spinoreticular tracts (at least in

rat), as well as the postsynaptic dorsal column (PSDC) pathway[27]. Some excitatory STT projection neurons in lamina III (antenna cells) are GluR4-IR and express NK1 (see main text).

[27] Ascending tracts/pathways that originate from laminae III-V neurons transmit stimuli of heterogeneous nature. The widely investigated STTs convey nociceptive (discriminative pain), thermal and gross tactile stimuli. The spinocervicothalamic pathway conveys light touch, but can also be activated by noxious stimuli. The PSDC pathway is implicated in visceral nociception. It has been described in rodents, cat and monkeys; clinical evidence indicates that it is also present in humans, but it is unclear if, at least in certain species, the neurons of origin lie in deeper (VII and X) laminae (Krames and Foreman, 2007). The other ascending tracts projecting to brainstem or hypothalamus integrate autonomic, motor and endogenous analgesic responses to nociceptive stimuli.

Box 4: Neuron communication and neurotransmitter release

Communication among CNS neurons occurs via two main mechanisms: the release and reception of chemical messengers (*neurotransmitters*) and the direct transfer of intracellular signals across the gap junctions (*electrical synapses*). In turn, neurotransmitter-mediated communication goes from the classic *synaptic transmission* at specialized membrane sites (synapses) to the diffuse secretion of neuromodulators (often referred to as *volume neurotransmission*).

Thus, it is possible to distinguish several modalities of neurotransmitter release (Südhof, 2008): *i. "Classical" synaptic neurotransmitters* are released at synapses, as it is the case for glutamate, GABA, glycine, acetylcholine, ATP etc.; *ii. Monoamines* are released by exocytosis of SDCVs, most often in the absence of synaptic specializations; *iii. Neuropeptides* are secreted by exocytosis of LDCVs[28]; *iv. Small permeable mediators*, such as nitric oxide (NO) and endocannabinoids, are liberated by simple diffusion. Only the first mode is responsible for fast point-to-point synaptic transmission, whereas all the others are involved in neuromodulation. Whereas synaptic transmission and volume neurotransmission stay at opposite ends of the spectrum of modalities that neurons can use to communicate with one another, there is a considerable overlap between the two (Südhof, 2008). Another point of complexity is that neurons, as a rule, produce more than a single neurotransmitter. They thus release a cocktail of neuroactive molecules that, in general, contains one fast-acting low molecular weight (MW) principal neurotransmitter and one or more slow acting neuromodulators of larger size (Merighi, 2017). Neuropeptides are small protein molecules contained in neurons. However, their chemical nature and small size are not *per se* sufficient to characterize as a neuropeptide a molecule of this type contained in neurons. Conceptually, the definition of neuropeptides is associated to the notion that these molecules (potentially) act as neuronal messengers, a notion that is based on three seminal observations emerging form the intensive research in the field during the last decades of the twentieth century: 1. The same peptides that are localized in neurons may act as hormone signals in the endocrine system; 2. Neurons can secrete peptides (neurosecretion); and 3. Neurons can be responsive to peptide signals. Altogether, these observations converged to demonstrate that neuropeptides are slow-acting transmitters and, differently from transmitter aminoacids, also exert their effects at extra-synaptic sites (Merighi *et al.*, 2011).

Commonly, neuropeptide (and high-MW neurotransmitters in general) release is triggered by a small increase in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, whereas release of transmitter amino acids (and low-MW neurotransmitters in general) requires a rise of $[Ca^{2+}]_i$ in the proximity of the Ca^{2+} channels at synapses. Therefore, in terminals with both types of neurotransmitter, a focal increase in Ca^{2+} at the synaptic membrane leads to a preferential discharge from agranular SSVs, whereas a more general elevation of Ca^{2+} inside the terminal favors release from the LDCVs.

[28] The terms LDCVs and LGVs can be used interchangeably. In the main text I have used only the term LDCVs to better differentiate this type of vesicles from the amine-containing SDCVs.

Box 5: Main anatomical, physiological and neurochemical features of the SGR neurons

Islet cells

Islet cells form the best characterized discrete population of inhibitory SGR neurons on histological, structural, neurochemical and functional bases. They are GABA-, GAD- and VGAT-IR and form GABAergic synapses (Lu and Perl, 2003; Zheng *et al.*, 2010). The exclusively inhibitory phenotype of the islet cells was confirmed by the lack of fluorescent neurons in transgenic mice expressing EGFP under the control of the VGLUT2 gene (Punnakkal *et al.*, 2014). Their firing pattern is, for the most, tonic. GABAergic islet cells were reported to be presynaptic to transient-firing central cells (Lu and Perl, 2003), whereas they receive a synaptic input from PAFs of the Aδ and C type (Grudt and Perl, 2002; Hantman *et al.*, 2004; Lu and Perl, 2003, 2005; Yasaka *et al.*, 2007) and from central cells (Zheng *et al.*, 2010). The primary afferent input from the C fibers is excitatory, whereas that from the Aδ fibers is inhibitory and indirect, along a polysynaptic chain (Yasaka *et al.*, 2007). The islet cells, in turn, form dendro-axonic synapses onto the PAF C boutons at glomeruli (V1 profiles). Local DH inputs to the islet cells are both excitatory and inhibitory and derive almost entirely from within SGR (Kato *et al.*, 2007). Kato and co-workers (2007) also found that, along the rostro-caudal axis of extension of the islet cell dendritic tree, inhibitory sites tended to be located closer to the cell soma than those excitatory. This, at least in part, corresponded to a differential distribution of potential excitatory and inhibitory postsynaptic sites, as revealed by maps of the direct responses to glutamate or GABA/glycine, respectively.

Central cells

Central cells resemble islet cells but are of smaller size. These neurons may be glutamatergic (Lu and Perl, 2003, 2005) or GABAergic (Hantman *et al.*, 2004; Hantman and Perl, 2005; Maxwell *et al.*, 2007; Todd and McKenzie, 1989; Zheng *et al.*, 2010). Central cells display different firing patterns (tonic, transient-I_A, and transient non-I_A). Tonic and transient non-I_A central cells can form glutamatergic synapses onto vertical cells (Lu and Perl, 2003, 2005), whereas the tonic GABAergic central cells make synapses onto vertical and islet cells (Hantman *et al.*, 2004; Zheng *et al.*, 2010). Central cells receive a synaptic input from Aδ and C PAFs (Grudt and Perl, 2002; Hantman *et al.*, 2004; Lu and Perl, 2003; Yasaka *et al.*, 2007). The input from the C fibers may be either excitatory or inhibitory, whereas that from the Aδ fibers is indirect and exclusively inhibitory (Yasaka *et al.*, 2007).

Stalked cell/large vertical cells

The stalked cells correspond to a subpopulation of large vertical cells in rodent's classifications. Most are glutamatergic excitatory interneurons after electrophysiological (Lu and Perl, 2005) and neurochemical (VGLUT2+/GABA-) characterization. At least some large vertical cells contain ENK (Bennett *et al.*, 1982; Cruz and Basbaum, 1985) in combination with SP (Ribeiro-Da-Silva *et al.*, 1991), others contain SST (Todd *et al.*, 2003) - see also main text section 3. Neuronal circuitries in SGR. The firing pattern of the stalked/large vertical cells is of the delayed type. Their primary synaptic target, particularly of those cells having their perikaryon in lamina II_o, are the lamina I spinothalamic nociceptive neurons (Gobel, 1978) that express the NK1 receptor (Polgár *et al.*, 2010) – see also Box 2 and Fig. 4-5. Electrophysiological recordings indicate that the stalked/large vertical cells are either nociceptive-specific or WDR neurons (Bennett et al., 1980). They also receive excitatory and inhibitory inputs from both Aδ (indirectly) and C PAFs (Grudt and Perl, 2002; Hantman *et al.*, 2004; Lu and Perl, 2003; Yasaka *et al.*, 2007). Based on previous TEM observations by Gobel et al. (1980), the PAF input to the vertical cells' dendrites was hypothesized to occur at type Ia glomeruli (Maxwell *et al.*, 2007). A

study on the intralaminar organization of the SDH in rat (Kato et al., 2009) has shown that vertical cells also receive excitatory inputs at their ventrally-oriented dendritic domains with a ventral displacement of the input zone greater than other SGR neurons. Thus, vertical cells receive translaminar excitatory inputs from sources located ventrally to their cell bodies, *i.e.* the lamina II_i or the laminae III/IV – see also Box 3. Input from these deeper laminae was mostly confined to a specific subgroup of vertical cells located near the II_o/II_i border, also referred to as II_m vertical cells (Kato *et al.*, 2009). The vertical cells' dendritic spines were also shown to receive numerous contacts from VGLUT1-IR boutons, at least some of which were likely to belong to A-LTMs (Yasaka *et al.*, 2014) - see Fig. 5.

Small vertical cells

The small vertical cells are subpopulation of inhibitory SGR neurons mainly populating lamina II_o (Grudt and Perl, 2002; Maxwell *et al.*, 2007; Yasaka *et al.*, 2007). They are GABAergic and GAD-IR and display less extensive dendritic trees than their larger glutamatergic counterpart. Another difference with the large vertical cells is that the small vertical cells display a tonic firing pattern instead of delayed firing.

At least some small vertical cells express DYN (Fig. 6) and have been implicated in mechanical nociception (Cruz and Basbaum, 1985; Duan *et al.*, 2014), but it remains unclear whether these were inhibitory, or whether they belonged to the previously identified subset of excitatory DYN cells, which seems more likely – see also main text section 3. Neuronal circuitries in SGR.

Radial cells

Radial cells are excitatory glutamatergic neurons that likely form a discrete population morphologically characterized by compact discoidal dendritic trees and I_A firing (Grudt and Perl, 2002; Yasaka *et al.*, 2007, 2010). Some radial cells have a more diffused dendritic tree and are GABA-IR (Maxwell *et al.*, 2007; Todd and McKenzie, 1989). Radial cells receive excitatory and inhibitory inputs from both Aδ (indirectly) and C afferents (Grudt and Perl, 2002; Hantman *et al.*, 2004; Lu and Perl, 2003; Yasaka *et al.*, 2007).

Box 6: Transcriptomics of DRG neurons

DRGs express a very complex array of proteins with different functions. These proteins may be component of the cytoskeleton (such as those of the neurofilaments that define the large neurons), the neurolemma or the axolemma (functioning as ion channels and receptors), the synaptic vesicles and the high MW neurotransmitters (in the the small peptidergic neurons). Omics techniques, such as next generation RNA-sequencing, have been recently employed to gather novel insights into the biological properties of DRG neurons. Although DRGs are relatively simple in terms of the cell types herein contained, cellular heterogeneity is a significant obstacle to analysis and interpretation of transcriptomics data. To overcome this problem, magnetic purification was used to obtain almost pure neuronal preparations that were subjected to genome-wide RNA-sequencing (Thakur *et al.*, 2014). By this approach the small size C type nociceptors were investigated and demonstrated to express a number of novel markers among which ion channel and transcription factor genes. The same group has more recently used fluorescence activated cell sorting to compare transcriptomic datasets in DRGs and trigeminal ganglion (Lopes *et al.*, 2017). A different strategy of investigation employed large scale single-cell RNA sequencing, unfortunately with somewhat conflicting results (Li *et al.*, 2016; Usoskin *et al.*, 2015). Thus, Usoskin et al. (2015) have been able to define eleven types of DRG neurons: three distinct LTMs, two proprioceptive, and six principal types of thermosensitive, itch sensitive, type C LTM and nociceptive neurons. Li et al. (2016) have, instead, classified small diameter DRG neurons into one type of LTM and five types of mechanoheat nociceptors, whereas large DRG neurons were categorized into four types. Their ten types were also reported to consist of several subtypes. The datasets deriving from these two works have been compared and critically discussed (see Fig. S9 in Li *et al.* 2016).

It is of course very difficult to correlate data on mRNA and protein expression in any type of cell, but this is even more challenging in neurons where *RNA localization*, a regulatory mechanism conserved throughout evolution, may be of paramount importance in relation to axonal or dendritic protein trafficking. By isolating RNA specifically from NGF-dependent DRG mouse neurons, i.e. the peptidergic neurons, it was demonstrated that the axon is enriched in mRNAs encoding secreted proteins, transcription factors and the translation machinery, whereas largely depleted from mRNAs encoding transmembrane proteins (Minis *et al.*, 2014). Such an observation can explain why the mRNA levels of the proteins undergoing activity dependent secretion, i.e. the neuropeptides and the anterogradely transported growth factors such as BDNF and GDNF may be not particularly high in the perikaryon, and thus difficult to detect with the above omics approaches. This, of course applies to those transcripts and proteins which are likely to be present in very low percentages of neurons.

Acknowledgement

I am greatly indebted to Prof. Alberto Granato for critical reading of the manuscript and for very fruitful and enlightening discussions on cortical structures of the brain and their relationship with spinal cord lamination.

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