**Università di Modena e Reggio Emilia**
**Dipartimento di Scienze Medcihe, Metaboliche e Neuroscienze**
**XXXV ciclo di dottorato in Neuroscienze**

# 5-HT7 Receptors Regulate Excitatory-Inhibitory Balance in Mouse Spinal Cord Dorsal Horn

CANDIDATO
**COMITATO ANTONELLA**

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**INDEX**

# Crab

Un cammino iniziato dalla fine, caratterizzato da molte sofferenze e perdite, sconfitte ed abbandoni. Scelte sbagliate ma consapevoli, falsate dalla percezione di una realtà mistificata. Decisioni dettate da incredibili malintesi e alle quali non si può più rimediare.

Il dolore non è solo danno tissutale. È un meccanismo molto più complesso che coinvolge aree del cervello difficili da analizzare. La componente affettivo con cui si interpretano gli stimoli, si memorizzano i ricordi, si associano le emozioni, conferendo al dolore stesso quell'aspro sapore soggettivo, è un pianeta ancora inesplorato, ma che necessiterebbe di indagini più dettagliate, di finanziamenti più cospicui, di interesse politico-sociale collettivo.

Il dolore è un male subdolo. Ti entra in casa senza chiedere; senza che uno se ne accorga.
Quando lo vedi, è troppo tardi. Allora, si cerca di fare come i granchi, che saltellano, simulando andatura contraria, se minacciati. Ma anche quello è solo malinteso. Il granchio procede solo in avanti come tutti, perché a nessuno è concesso di tornare indietro.

Dedico questo elaborato
-a tutte le persone che ho perso: **a mia madre** e **mio padre**, morti l'anno prima del covid e nell'anno di inizio del mio dottorato; a Valeria Marigo, il mio tutor di sempre, che ha segnato non solo la mia crescita professionale ma anche quella personale;

-a tutte le persone che mi hanno lasciato qualcosa: a Rita, che mi ha insegnato molto più della semplice fisiologia: la tolleranza, la sensibilità e il perdono; a tutti i miei **fratelli** e **sorelle** che, sebbene fisicamente lontani, mi hanno sempre aiutata. Una famiglia unita come quelle di una volta; **al mio sbaglio più grande**, dal quale un giorno spero di allontanarmi, perché l'affetto da solo non deve bastare;

e infine, al mio gatto **Nana**, che è stata con me per tredici anni e che chiude tragicamente questo ciclo, pagando in primis quel maledetto malinteso, che ha drasticamente cambiato tutta la mia vita.

Qualunque sia la direzione che prenderò, io mi auguro solo che ne sia valsa davvero la pena…

**AC**

## ABSTRACT

Serotonergic receptors of the 5-$HT_7$ type (5-$HT_7$Rs) are widely expressed in the central nervous system (CNS), where they modulate several functions, such as pain. Behavioral experiments *in vivo* have shown both anti- and pro-nociceptive actions of 5-$HT_7$Rs, although an analgesic effect seems to be prevalent. In the spinal cord dorsal horn, the mechanisms involved in 5-$HT_7$R-mediated synaptic modulation are still poorly understood, especially those regarding the control of synaptic inhibition. The present study investigated the modulation exerted by 5-$HT_7$Rs on dorsal horn excitatory and inhibitory synaptic circuits, by performing patch-clamp recordings from lamina II neurons in mouse spinal cord slices. Our results show that applying the selective 5-$HT_7$ agonist LP-211 facilitates glutamatergic release by enhancing the frequency of spontaneous postsynaptic currents (sEPSCs) and increasing the peak amplitude of excitatory postsynaptic currents (EPSCs) evoked by dorsal root stimulation. The effects on sEPSCs were still observed in the presence of the 5-$HT_{1A}$ antagonist WAY-100635, while the 5-$HT_7$ antagonist SB-269970 blocked them. LP-211 was also able to increase the release of gamma-aminobutyric acid (GABA) and glycine, as shown by the increase of spontaneous inhibitory currents (sIPSC) frequency and evoked inhibitory postsynaptic currents (IPSC) amplitude. LP-211 was proved to be more effective in potentiating synaptic inhibition as compared to excitation: consistently, 5-$HT_7$R activation significantly enhanced the excitability of tonic firing neurons, mainly corresponding to inhibitory interneurons. Our data bring new insights into the mechanisms of synaptic modulation mediated by 5-$HT_7$Rs in the dorsal horn. Stronger impact on synaptic inhibition supports the hypothesis that these receptors may play an anti-nociceptive role in the spinal cord of naïve animals.

# INTRODUCTION

The concept of pain has been the subject of numerous scientific studies that have attempted to define its different aspects and peculiar characteristics, according to different perspectives dictated by the sciences that investigated it.
The first definition of pain, recommended by the Subcommittee on Taxonomy, adopted by the International Association for the Study of Pain (IASP) Council in 1979 and widely shared and accepted internationally, is formulated as "an unpleasant sensory and emotional experience associated with harm actual or potential tissue, or described in terms of such damage ".
Over the years, however, its definition has been continually revised due to the progress made in the understanding of pain, up to its current remodeling, which taking into account the different components that make up pain, asserts that, although tissue injury is a common antecedent to pain, it can be present even when the damage to the tissue is indistinguishable. Pain is therefore understood as a complex and multidimensional sensory experience, the result of multiple factors (Raja et al., 2020), such as perceptual factors, through nociception and subjective, through the cerebral cortex, which materialize into an emotion . Other components that are part of pain such as affective, motivational, behavioral and sociocultural ones, which intervene between the nociceptive stimulus and the subjective experience of pain and can amplify or inhibit this sensation (Zaki et al., 2016).
Under physiological conditions, pain caused by actual or potential tissue injury has a sudden and limited onset in time. This form of pain is called acute pain and represents a functional response of evolutionary adaptation, essential for survival. In fact, it constitutes the cornerstone of the body's defense against harmful or potentially harmful stimuli, preventing further damage. Its biological significance is underlined by subjects suffering from pathologies due largely to genetic mutations that cause a lack of perception of pain, and which results in a short life full of medical visits and treatments. However, when the pain persists over time and intensity, from acute it becomes chronic and ceases to have its function as a strategy of evolution, turning into a real pathology (Mannion and Woolf, 2000).
Epidemiological data indicate that one in five adults in Europe suffers from moderate to severe pain (Dahlhamer J, et al 2016; Breivik et al., 2013), understood as frequent painful episodes lasting at least three months, but that in some patients extends for over twenty years (Breivik et al., 2006). In countries such as Asia, Africa and America 33 of adults and 56 of the elderly suffer from chronic pain such as joint pain, low back pain and widespread pain (Jackson et al, 2015). In all these patients, pain can be exacerbated by harmless or noxious external stimulation or occurs spontaneously, thus losing its protective function for the body. Not to be overlooked is the psychological aspect that in America has given rise to the so-called phenomenon of deaths from despair from suicide, alcohol and opioid abuse, analyzed in 2015 by the Nobel laureate for economics Angus Deaton. But also in Italy the problem is rampant with a growing percentage of patients suffering from chronic pain and later developing depression (Breivik et al., 2006).
Pain, both acute and chronic, therefore causes great physical and mental suffering to the individual, negatively affecting the quality of life of the patient, family members and society and therefore representing one of the main public health problems in all. the world. In fact, the daily life of affected individuals is compromised due to their ability to participate in common daily and work activities. This also impacts on society, in terms of direct costs on healthcare costs for drugs or other medical services (Hecke et al., 2013; Phillips CJ. Et al, 2009), and in terms of indirect costs, related to lost working days.
At present, pain is mainly managed with drugs that have many defects such as poor efficacy, adverse reactions, dependence, addiction (Turk et al 2011).
Hence the importance of understanding the cellular, molecular and physiological mechanisms that underlie pain transmission.

# Chapter I. PAIN

## Pain transmission pathways

The processing of pain affects a wide variety of structures through the nervous system, the involvement of different mechanisms and a complex regulation.
Its perception consists of 4 phases: **transduction, transmission, modulation, perception**, Pain and disability 1987.
**Transduction** refers to the processes by which tissue-damaging stimuli activate sensory nerve endings. The electrical impulse is then conducted along the sensory fibers in the spinal cord, where the nociceptive information passes largely to the opposite side, decussation, and runs towards the thalamus in the anterolateral system.
**Transmission** refers to the transmission functions by which the message is transported from the site of the tissue injury to the brain regions underlying perception.
**Modulation** is understood as a neural process that acts specifically to reduce activity in the pain transmission system itself.
**Perception** represents the phase in which the impulse reaches the neocortical sites of the localization of pain and the limbic sites responsible for the emotional component of pain. Therefore, through the integration of many sensory messages, the individual becomes aware of pain in a subjective dimension, influenced by different processes including attention, expectation and interpretation. From here we move on to the neuro-humoral, sympathetic and endocrine responses to pain, **Fig. 1**

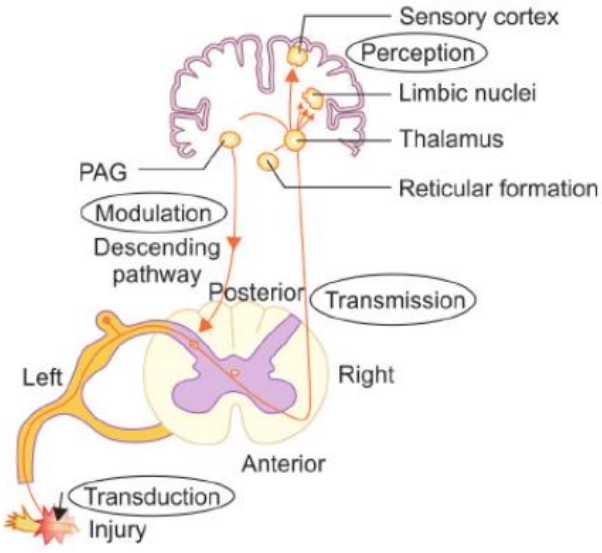



**Fig. 1** After the stimulation of nociceptors, via afferents pathway, an action potential is generated at the site of nociceptor (transduction), which are then further carried by A-delta fibers and C fibers toward the higher center (transmission) and make us feel pain via stimulation of sensory cortex (perception). Complex brainstem circuitry provides both descending inhibitory and excitatory alterations of nociceptive responses at the level of the spinal cord (modulation).

Sensory transduction begins with the activation of the first-order neuron, the peripheral nociceptors, which, stimulated in the peripheral tissues by insults of different nature (mechanical, such as pressure, thermal and chemical, such as serotonin), convert the stimulus into an electrical impulse. This neuron has its cell body in the dorsal root ganglion (DRG) and a long process, the axon, which divides and sends a branch to the periphery, where it makes contact with numerous receptors located in the skin or locomotor, and the other branch into the spinal cord through the dorsal root entry zone (DREZ).

Following its activation, nociceptive action potentials evoked by noxious stimulation are generated, through which signals are conducted along the primary afferent fibers and transmitted to the spinal cord or brain stem (transmission), **Fig. 2**.

The main primary afferent fibers, responsible for the transmission of the pain sensation, are the Aδ fibers and the C fibers.

Aδ fibers are small caliber fibers (1-5μm), myelinated, characterized by a high conduction speed (3-30m /s) and allow to perceive an immediate and well localized painful sensation, due to mechanical and thermal insults. C fibers are large caliber fibers (0.2-1.5μm), polymodal, that is, capable of responding not only to noxious mechanical, thermal and chemical stimuli, resulting for example from the release of inflammatory mediators. They are unmyelinated fibers, with a low conduction speed (0.5-2m/s), and are responsible for the transmission of a dull, diffuse pain that lasts beyond the term of peripheral stimulation of the nociceptors (Raja et al., 1999 Price et al, 1977).

In the spinal dorsal horn, near the DREZ, the nerve endings of nociceptors synaptic with second order neurons (Willis, 1985), activated by neurotransmitters, such as substance P and somatostatin, as well as amino acids such as glutamic or aspartic acid , released by first order sensory neurons.

Ascending fibers originate from the axons of second-order neurons, which decussate and run towards the thalamus in the anterolateral system, **Fig. 2**

The anterior lateral system consists of two main bundles, **neo-spino-thalamic** and **paleo-spino-thalamic.**

The **neo-spinothalamic** bundle is in turn made up of two bundles, anterior thalamic spine, used for gross tactile sensitivity, and lateral thalamic spine, responsible for thermo-pain information. The direct spinothalamic route is phylogenically more recent and more analytical and carries precise information regarding the mobility, intensity and localization of the pain stimulus.

The neo-spinothalamic bundle, exclusively anterolateral, ends in the ventro-postero-lateral and posterior nuclei of the thalamus from which other neurons project towards the primary somato-sensory cortex, an area of the body schema, responsible for our ability to discriminate in a few milliseconds from where a painful stimulus derives and its intensity in order to activate the escape reaction.

The **paleo-spinothalamic** bundle, phylogenically older, homo and counter lateral, comes from neurons in connection with the C fibers and establishes synapses with various nuclei of the reticular formation of the brain stem from which axons depart for the diffuse projection nuclei of the thalamus that project diffusely to the cortex. It is a slow multisynaptic pathway, with poor codification and localization skills and is more connected to the activating and emotional aspects of the painful stimulus, **Fig. 2.**

The whole system receives inhibitory and excitatory synapses from neurons placed in the gelatinous substance of the spinal cord, which finely regulate its activity. From here you can understand the importance of the functional meaning of the entire street.

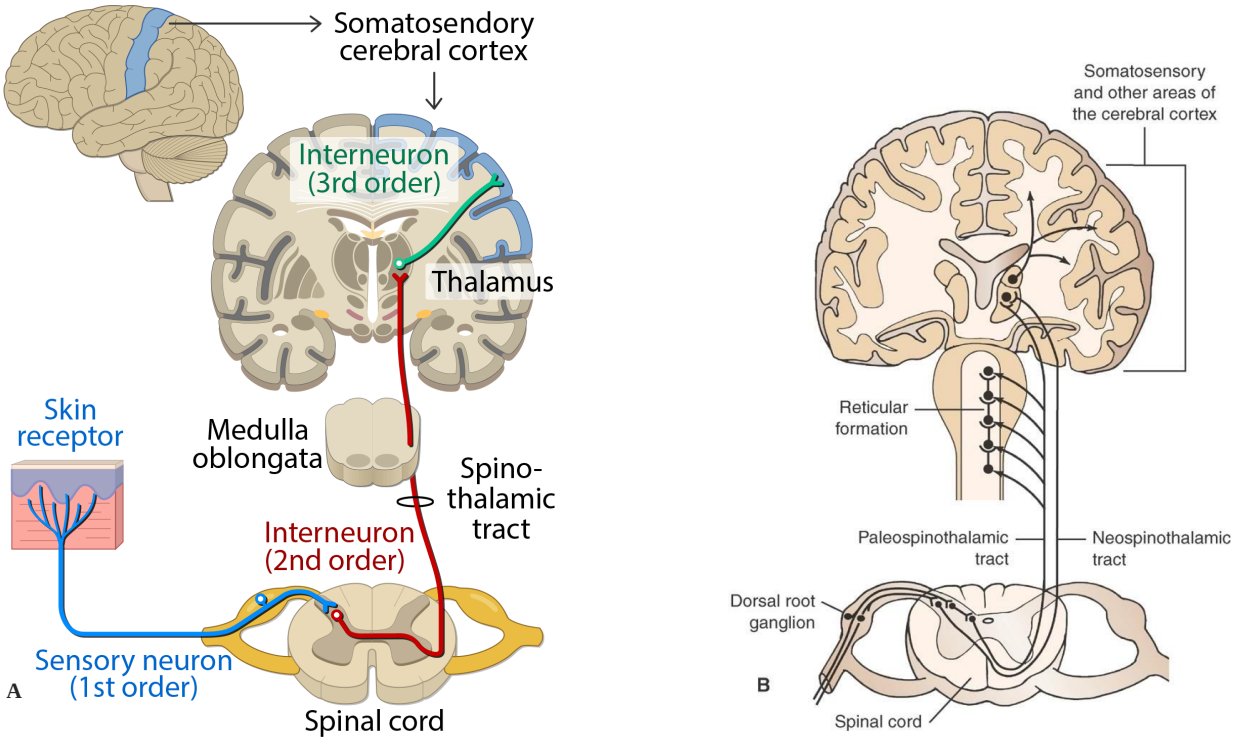
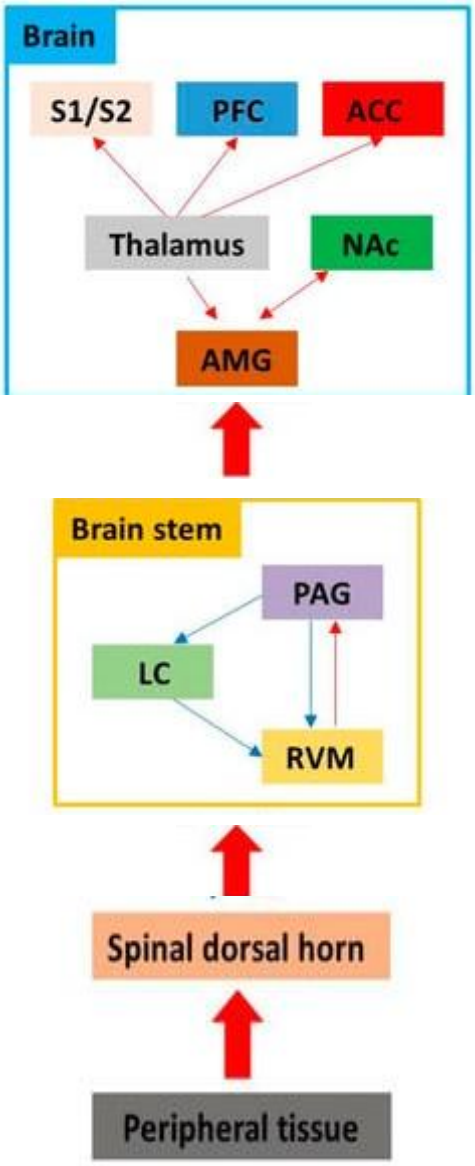



**Fig. 2**
Afferent pathway.
(A) Pain is transmitted at the somatosensory level through three orders of neurons. First-order neurons carry the signal from the peripheral sensory zone to the dorsal horns of the spinal cord. Second-order neurons carry the signal from the dorsal horns of the spinal cord to the thalamic nuclei. Third-order neurons connect the thalamic nuclei to the sensory cortex
(B) The ascending pathways that mediate pain consist of two main different tracts: the neospinothalamic tract and the paleospinothalamic tract. The first-order neurons are located in the dorsal root ganglion (DRG) for all pathways. Each pain tract originates in different spinal cord regions and ascends to terminate in different areas in the CNS.

In fact, studies have been conducted on the properties of the cells of the spino-thalamic tract in different species. In all these species, most spinothalamic neurons respond maximally to noxious stimulation. Furthermore, there is a direct relationship in the cells of the spinothalamic tract between the frequency of activation and the intensity of the stimulus in the noxious range for human subjects (Kenshalo et al., 1980; Willis, 1985). These observations, coupled with decades of careful clinical studies, strongly implicate the spinothalamic tract as a major pathway for pain in humans.




The spino-thalamic tracts then reach the brain stem which includes the Locus coeruleus, LC, the Rostral Ventral Medulla, RVM, part of the medulla oblongata, and the periaquiductal gray matter, PAG, located in the midbrain.
Then, from here, they arrive at the diencephalon where they terminate at the posterolateral ventral nucleus of the thalamus and at the superior centers of the brain, **Fig. 3**

**Fig. 3**
Ascending pathway (red line): A nerve pathway that projects upwards from the spinal cord to the brain carrying sensory information from the body to the brain. Pain signals ascend from the spinal dorsal horn to the rostral ventral medulla (RVM) and periaqueductal grey matter (PAG). Pain signals are then transmitted to the thalamus, where they are sent to higher brain centers, such as the primary and secondary somatosensory cortices (S1/S2), prefrontal cortex (PFC), anterior cortex (ACC), amygdala (AMG), and nucleus accumbens (NAc).

At the thalamic level, the pain pathways have two main termination sites: ventrocaudal and medial. The ventrocaudal thalamus receives nociceptive inputs directly from the protruding spinal neurons. Neurons in the ventrocaudal thalamus project directly to the somatosensory cortex (Willis, 1985). The medial thalamus receives some indirect input from the spinal cord, but in addition it receives important input from the region of the brain stem reticular formation to which nociceptive spinoreticular neurons project. The medial thalamus projects to diffuse areas of the forebrain, including the somatosensory cortex (Jones et Leavitt, 1974). Thus there are two main ascending pathways for pain: a direct lateral spinothalamic pathway and an indirect medial spinoreticulothalamic pathway. The lateral path from the spinal cord to the ventrocaudal thalamus

and cortex is thought to be responsible primarily for sharp, well-localized pain occurring near the body surface. On the contrary, the medial spinoreticulothalamic pathway responds more to the stimuli of deep somatic and visceral structures, **Fig. 4**

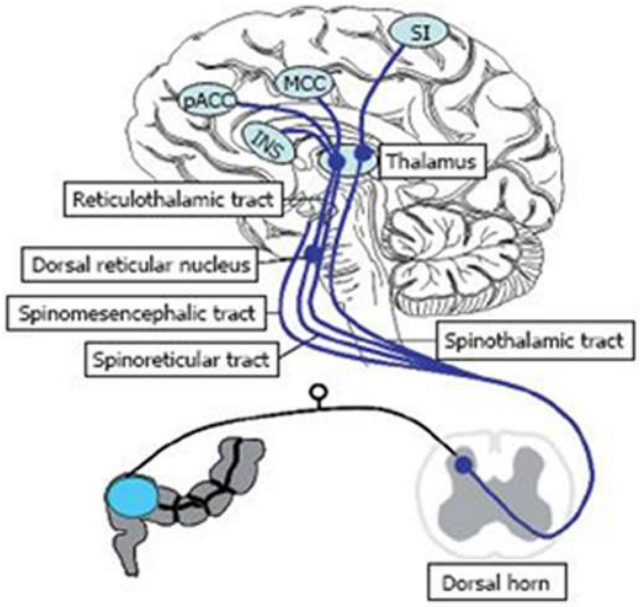



**Fig. 4**
The principal projections from the spinal cord to subcortial and cortical structures (blue lines). The spinothalamic tract terminates in the medial and posterior thalamus. Thalamocortical fibres then project to the primary somatosensory cortex. The spinoreticular tract terminates in the reticular formation to the medial thalamus. The spinomesencephalic tract projects to various regions in the brainstem, including the periaqueductal grey, locus coeruleus, and dorsal reticular nucleus in the medulla. Thalamocortical projections from the medial thalamus project to the cingulate cortex and insula which are involved in processing noxious and somatic information. The brain regions innervated by these pathways that respond to painful stimuli include the thalamus, insula, amygdala and anterior cingulate cortex (ACC). The ACC is comprised of two components, the perigenual ACC (pACC) involved in affect and the mid cingulate cortex (MCC) with behavioural response modification.

## Descending modulation pain

Pain is a high-priority sensory system that serves as a biological warning device. It draws attention, activates automatic defensive motor responses, and motivates organized behaviors to escape or avoid injury. Its perception is the result of the impulses that travel centripetally along the afferent sensory pathways towards the cortical and subcortical nuclei (ascending pathways) and of the impulses that descend from these areas in an inverse centrifugal direction, aimed at modulating the transmission at the spinal level through post synaptic action on the projections of neurons or on interneurons in the dorsal horns of the spinal cord (descending system), **Fig 5**.

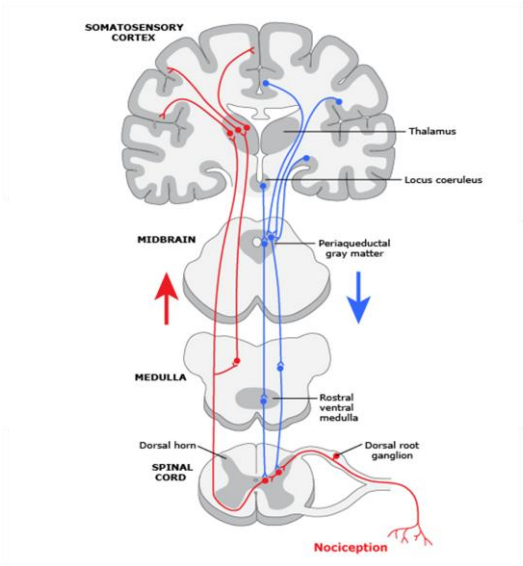
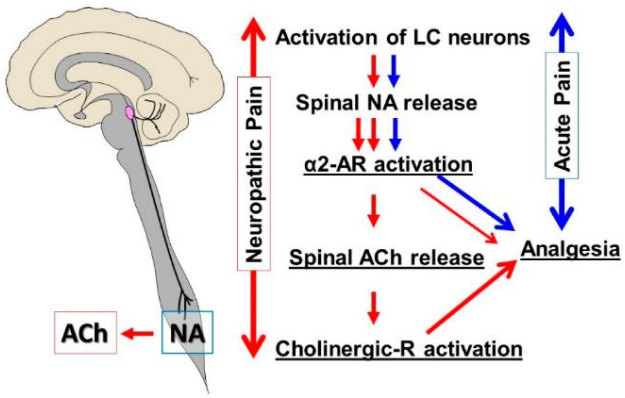



**Fig. 5** Simplified depiction of ascending and descending pain signaling pathways. Ascending pain pathway = red; modulatory descending tracts = blue.
Afferent nociceptive input enters the spinal cord via the DRG. Secondary order projection neurons ascend in the contralateral spinothalamic and spinoreticular tracts that relay the signal to cortical centers.
Descending pathways projecting from the periaqueductal gray (PAG) in the midbrain and the rostral ventromedial medulla (RVM) to the dorsal horn and modulate pain transmission.

In fact, pain may not be the behavioral priority at all times. The pain inhibiting influence allows other biologically significant behaviors to proceed without being prevented by pain.
The analgesic or hypoalgesic effect induced by the descending pathways is essential for regulating other vital processes such as nutrition, urination, mating, pregnancy, childbirth, flight, attack. The function of pain modulation systems is therefore to regulate the transmission of the pain signal with other behavioral and physiological needs, through the integration of sensitive, cognitive, emotional and motivational information with afferent nociceptive information (Gebhart, 2004).
Evidence shows that descending pathways originating from certain supraspinal regions can simultaneously promote and suppress nociceptive transmission via the dorsal horn, referred to as the descending pathway of inhibition (DI) and the descending facilitation pathway (DF). In particular, there is no absolute anatomical separation of the substrates that absorb these processes and the stimulation of a single supraspinal structure can, through divergent actions of different transmitters and different types of receptors, activate both DI and DF simultaneously.
A fundamental center of the descending system is the periaqueductal gray matter (PAG). Recognition of its role in pain modulation comes from the observation that PAG stimulation prior to surgery leads to a decrease in anesthetic requirement (Roizen et al, 1985).
The PAG is a cell-rich region surrounding the cerebral aqueduct in the midbrain and is divided into three sub-regions: ventrolateral, lateral and dorsolateral (Hemington et al, 2015). Its neurons receive direct or indirect inputs from different brain structures, including hypothalamus, amygdala, cortex. So thoughts, emotions and stress can affect their activity. Through both ascending and descending

projections, PAG can reduce or increase pain perception as it propagates nociceptive and analgesic stimuli in a bidirectional way (Benarroch et al, 2008).
Most of its fibers descend on the rostral part of the ventromedial medulla (RVM), which includes the nucleus reticularis gigantocellularis pars alfa and the nucleus raphe magnus (NMR) and which in turn establishes connection with the posterior horns (Fields et al. 1995; Watkins et al. 1980), on which it can exert an inhibitory or facilitating action on the transmission of the nociceptive signal (Basbaum et al. 1984; Heinricher et al. 2009; Mason 2001; Porreca et al. 2002; Ren and Dubner 2002).
The remaining PAG fibers project to the locus coeruleus.
The locus coeruleus, LC, is the largest group of noradrenergic cells in the brain, containing more than 50% of all noradrenergic neurons (Probst et al., 1984, Proudfit, et al, 1991 Singewald et al, 1998) and represents the most important connecting noradrenergic bridge in the inhibition of pain descending from direct projections to the spinal cord (Cao et al., 2020 Pertovaara et al., 2006; Tavares et al., 1997).
While in the absence of pain, the PAG-LC-spinal cord system has limited implications or almost zero activity (Pertovaara et al., 2013), it becomes profoundly effective in descending pain modulation following tissue lesions inducing neuropathic pain.
In the normal physiological state and in acute pain, noradrenaline released by descending noradrenergic axons produces antinociceptive effects in the spinal dorsal horn through the stimulation of α2 adrenergic receptors, which are coupled with inhibitory G protein (Gi / o). which inhibits presynaptic voltage-gated Ca 2+ channels in the dorsal horn, reducing cAMP levels. This in turn causes reduced release of excitatory neurotransmitters in the spinal cord. Furthermore, activation of postsynaptic α2 adrenergic receptors on secondary sensory neurons in the spinal cord causes the opening of internally rectifying K + channels to hyperpolarize cells, thus reducing neuronal excitability (Pan et al., 2008). Through these mechanisms, the activation of the descending noradrenergic inhibitory pathway reduces the transmission of spinal pain, (McGaraughty et al, 2004 de Oliveira et al 2017 Pertovaara et al., 2006 Llorca-Torralba, 2016), promoting the prevention of further injuries and acting as a beneficial and adaptive mechanism in times of stress and / or danger (Millan et al, 1999; Millan et al, 2002). **Fig. 6**

**Fig. 6**
Locus coeruleus (LC) and descending noradrenergic inhibition. In a normal physiologic state (blue pathway), activation of LC neurons results in spinal noradrenaline (NA) release, which stimulates α2-adrenergic receptors (α2-AR) in the spinal cord to produce analgesia. In early-stage neuropathic pain following peripheral nerve injury (red pathway), noradrenergic axons sprout in the spinal cord, and the function of the α2-AR in the spinal cholinergic neurons changes from inhibition (Gi/o-coupling) to facilitation (Gs-coupling). Therefore, activation of LC neurons results not only in an increased release of NA but also the excitation of cholinergic interneurons to induce the release of acetylcholine (ACh) in the spinal cord, which is critical to the antihypersensitivity effect of spinal noradrenaline after nerve injury.




In the early stages of neuropathic pain following tissue injury, in rodents, there is an increase in the brain-derived neurotrophic factor (BDNF) in the dorsal spinal horn, which causes a structural and

functional alteration of the descending noradrenergic pathway. Noradrenergic fibers in the spinal dorsal horn germinate at the dermatomes, surrounding the site of primary sensory input, and allow for an anatomically more extensive release of norepinephrine.
Furthermore, by shifting the balance between Gi and Gs/α2-adrenergic coupling in the cholinergic terminals of the spinal dorsal horn due to modulation in the expression of Gs or Gi proteins or by post-translational modification of these proteins or α2 -adrenergic (Hayashida et al 2008 Hayashida et al 2010, Hayashida et al 2019) the function of the α2-adrenergic receptor in spinal cholinergic neurons changes from inhibition (Gi / o-coupling) to facilitation (Gs-coupling); therefore, the more extensively released norepinephrine at the spinal level excites the cholinergic interneurons to induce the release of acetylcholine, which is critical for the antihypersensitivity effect of spinal norepinephrine after nerve injury (**Fig. 6**), (Hayashida et al 2019 ). The pathway therefore has a predominantly antinociceptive action in acute pain and in the early stages of neuropathic pain. However, recent findings support the hypothesis that the PAG-LC axis may also have an important facilitating role in the transition from acute to chronic pain, as well as during the maintenance of chronic pain after nerve injury (Taylor et al. , 2017, Suarez-Pereira et al 2022), partly explaining the limited therapeutic efficacy of tricyclic antidepressants and norepinephrine reuptake inhibitors such as duloxetine for the treatment of neuropathic pain (Suárez-Pereira et al., 2002).
As neuropathic pain turns into chronic pain, noradrenergic neurons in the LC become less responsive to noxious stimuli, leading to impaired endogenous analgesia. This impairment is mainly due to the reduction of glutamate release by astroglial cells, caused by the activation of histone deacetylase (HDAC) which induces a downregulation of the expression of the astroglial glutamate transporter 1, GLT-1 (Kimura et al., 2015, Pan et al., 2008). Extracellular glutamate is classically removed by two types of astroglial glutamate transporters, mainly GLT-1 and also the glutamate-aspartate transporter (Rothstein et al., 1996, Robensin, 1998, Hayashida et al 2019), Among the various neurochemical inputs in LC, glutamate is the main excitatory neurotransmitter on noradrenergic neurons, acting through the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Singewald et al., 1998). Peripheral nerve injury increases extracellular glutamate down by regulating GLT-1. This inhibits evoked glutamate release by enhanced presynaptic inhibition via presynaptic metabotropic glutamate receptors (mGluRs), (Pan et al., 2008). The increase in the basal concentration of extracellular glutamate results in greater tonic activity of the noradrenergic (NA) neurons and in the reduced release of evoked glutamate which decreases NA neuronal activation mediated by the α-amino-3-hydroxy- acid receptor. 5-methyl-4-isoxazolepropionic (AMPA) important for analgesia induced by noxious stimulation (Kimura et al., 2015, Hayashida et al 2019), (**Fig. 7**)

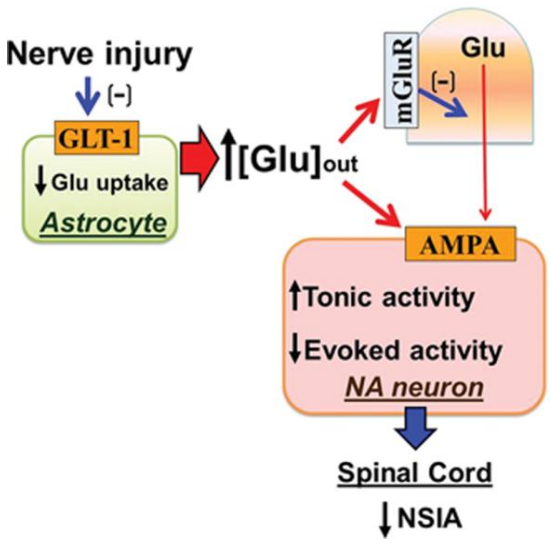



**Fig. 7**
Astroglial glutamate dysregulation is critical to impairment endogenous analgesia. Among the various neurochemical inputs to the LC, glutamate is considered a primary excitatory regulator of noradrenergic neurons, acting through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Glutamate also inhibits its own release from the terminal via group 2 and 3 metabotropic glutamate receptors (mGluRs). In the central nervous system, two types of astroglial glutamate transporters, glutamate transporter-1 (GLT-1) and glutamate-aspartate transporter, regulate extracellular glutamate
In rats with chronic neuropathic hypersensitivity, peripheral nerve injury decreases the expression of GLT-1 via activation of histone deacetylase (HDAC) and increases basal extracellular glutamate concentrations, which reduces noxious stimulation-evoked glutamate release, via activation of presynaptic mGluRs. This reduced glutamate release reduces stimulation-evoked neuronal activity in the LC and noradrenaline release in the spinal cord, thereby impairing noxious stimulation-induced analgesia (NSIA)

The PAG-RVM pathway is considered the main endogenous descending pain modulator. In fact, it has been widely demonstrated that the neurons inside the nucleus raphe magnus and the nucleus reticularis gigantocellularis, included in the RVM, project onto the spinal or medullary dorsal horns. Here they form synaptic connections with primary afferent terminals, second and third order neurons that transmit nociceptive signals to supraspinal sites and interneurons to directly or indirectly increase or decrease nociceptive trafficking, modifying the pain experience (Fields et al., 2005) .

This descending modulatory circuit, which probably represents the last common relay of nociceptive modulation from supraspinal sites (Fields et al., 1976), is relevant to human experience in many contexts, including chronic pain states, and in the action of Pain relieving drugs, including opiates, cannabinoids, NSAIDs and serotonin/norepinephrine reuptake blockers that mimic, in part, the action of opiates and are a primary target for supraspinal opioid analgesia (Calvino et al., 2006 de Oliveira et al., 2006 de Oliveira et al., 2017), as well as being the site of the phenomenon of placebo analgesia (Beecher et al., 1955), probably mediated by the activation of pain inhibitory systems, originating from the cortical and subcortical regions mediated by μ-opioid receptors (Petrovic et al. al., 2002, Zubieta et al., 2005).

Imaging studies have in fact identified areas of the brain, constantly activated by noxious stimuli, defined "pain matrix" in connection with the PAG through RVM that include anterior cingulate cortex, somatosensory cortex 1 and 2, insula, amygdala (Tracey et al., 2009, Binguel et al., 2007, Binguel et al., 2008). These interactions, acting together in the context of nociceptive regulation, provide the basis for the emotional-affective modulation of cognitive functions in pain, guiding tasks such as decision making, pain risk/reward assessment or avoidance of punishment, contributing overall at the origin of the sensory experience (Neugebauer et al., 2009).

The way can therefore work bidirectionally. The inhibitory descending circuit acts as a natural analgesic. Endogenous analgesia is an advantageous condition in highly stressful or dangerous situations in which other behaviors must anticipate responses to pain and recovery behaviors in order to ensure survival.

The descending effort of facilitation, on the other hand, causes hypersensitivity and can be considered an adaptive mechanism of sensitization of the pathways of pain transmission aimed at promoting recovery behaviors during illness or preventing the use of injured body parts and increases alertness in situations where the threat is possible but not imminent.

The biological significance of the PAG-RVM axis is underlined by experiments in which the inactivation of the RVM attenuates or blocks the downward modulation exerted by the PAG, indicating moreover that the influence of the PAG on the spinal cord is mostly indirect and occurs through RVM, the whose main descending projection travels through the dorsolateral funiculus, terminating at all levels of the dorsal horn.

This system influences spinal nociceptive processes important for the local nocifensor reflexes, as well as fundamental ascending messages for affective and sensory discriminatory aspects of pain. However, the PAG-RVM system is not used exclusively for pain processing. Other functions performed are related to physiological and behavioral aspects of defense, body homeostasis and reproduction. However, there is evidence that in each region there are groups of neurons responsible for pain modulation, presumably because these neurons interact with local circuits to implement integrated responses to internal and external changes in the organism.

The pathway mainly uses endogenous opioids at multiple levels to perform its functions.

The mu, delta and kappa opioid receptors are, in fact, widely distributed both in the PAG and in the RVM, but the analgesia produced by the ventrolateral region of the PAG also depends on the functional integrity of the receptors for serotonin, opioids and excitatory amino acids and cholinergics in the RVM. Pretreatments with antagonists of serotonin receptors, μ- and δ-opioid, cholinergic musciarinics and nicotinics and NMDA, in fact, block the analgesic effect of the

pathway, demonstrating that the axis is transmitted through various neuropeptides and neurotransmitters such as serotonin, enkephalins, substance P and above all GABA both for PAG RVM communication and for RVM-spinal cord transmission. Therefore, a functional or structural alteration of the RVM at any point of the pathway results in a modification of the inhibition of pain.
These data suggest a complex heterogeneous network mediated by neurochemical factors that implies the mediation of pain inhibition elicited in the PAG-RVM. The complexity of the entire system is underlined by the double analgesic and hyperalgesic effect induced by opioids. Acute or chronic administration of opioid analgesics over a long period can lead to facilitating output driven by the release of endogenous cholecystokinin CCK, a neural peptide closely related to opioid-induced effects, highlighting how critical is the dynamic balance between inhibition and facilitation of pain exerted. from this region. This balance in relation to different emotional, affective, pharmacological, neurochemical, physical and behavioral states can therefore shift in one direction or the other, engaging very distinct circuits in order to activate deep analgesia (inhibition) or central sensitization and development of secondary hyperalgesia (facilitation) (Chen et al., 2019).
An imbalance between the inhibitory and facilitating system of the nociceptive descending pathway is considered the basis of painful pathological states (Denk et al., 2014, Kehlet et al., 2006, Belfer et al., 2013, Heinricher et al., 2009).
The bidirectionality of the positive and negative pain regulation, exerted in the RVM, reflects the dichotomy in the function of the cells of which the system is composed.
In the RVM nucleus in 1980 Howard Fields identified and classified cells as off, on and neutral in relation to their response to noxious stimulus. In particular with reference to the start of the noxious reflex, the scientist characterized cells in which there was an increase in neuronal activation (cells on), an inhibition of cell activation (cells off) or an absence of response (cells neutral) (Fields et al., 1983, Heinricher et al., 1989). The electrophysiological characteristics of the on cells are consistent with their pronociceptive and hyperalgesic function, while the activation of the off cells represents a necessary and sufficient condition (Fang et al., 1989, Cheng et al., 1986) for the antinociceptive action and the descending analgesic effect. As for the role of neutral, the debate is still open.
In detail, the excitation of OFF-cells is regulated by opioid receptors, μ-opioids, which at the presynaptic level inhibit the release of GABA. GABA is a neurotransmitter responsible for the inactivation of OFF cells. The μ-opioid receptors also suppress, but directly, the activation of ON cells (Heinricher et al., 1992), **Fig. 8**.
However, although the facilitating effect is blocked and in itself is not sufficient to produce a powerful hyperalgesia response in normal state, it is hypothesized that these nuclei contribute to the analgesic action of drugs

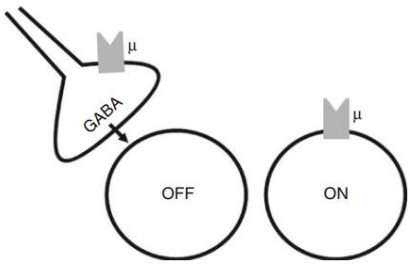



**Fig. 8**
μ-Opioid agonists have both direct and indirect actions in the RVM. ON-cells are inhibited, directly. OFF-cells are disinhibited. The OFF-cell reflex-related pause is mediated by a GABAergic input from outside of the RVM. This GABA input is inhibited by μ-receptor agonists, allowing the OFF-cells to become continuously active

The ON and OFF cells therefore seem to exercise a regulation of "mass action" of the function of the dorsal horn, which leads to the generation of a variable nociceptive threshold to the variation of the balance between the two populations which from the functional point of view alternate passing from an active phase to an inhibited one that lasts from a few seconds to many minutes.
A change in the balance of ON and OFF cell populations such that ON cells predominate for long periods is probably at the basis of the pro-nociceptive influence that RVM develops during chronic inflammatory states and nerve injuries.
The PAG-RVM pathway constitutes the main locus of serotonergic cells that project to most brain regions, including the spinal cord and higher centers (Martin et al., 2006). Among the great variety of chemical messengers that act in nerve cell signaling, 5-HT is at the center of great interest for its implication in almost all physiological functions (nutrition, reward, thermoregulation, cardiovascular regulation, locomotion, pain, reproduction , sleep-wake cycle, memory, cognition, aggression, responses to stress factors, emotion and mood) and in various human pathologies.

# Chapter II. Serotonergic receptors

**Serotonin**

Serotonin (5-HT) is a monoamine neurotransmitter, synthesized mainly in the gastrointestinal tract, although a small percentage is also produced from neurons (Lesurtel et al., 2008, Bertrand et al., 2010).
Its precursor is tryptophan, taken through the diet and subsequently hydroxylated and decarboxylated sequentially, respectively by the enzymes tryptophan hydroxylase 2 (TPH2) and aromatic decarboxylase (AADC), (Cortes-Altamirano et al., 2018, Matthes et al., 2010). Once produced, it is stored in the presynaptic vesicles of neurons, which excitedly release it through their nerve terminals into the extracellular fluids of the different target sites mainly but not exclusively by means of extrasynaptic volume transmission (Hentall et al., 2006, Umbriaco et al., 1995, Agnati et al., 2010, Descarries et al., 1975), (**Fig. 9**).
Its reuptake occurs through SERT, an ATP-dependent 5-HT-specific transporter (Apparsundaram et al., 2008, Steiner et al., 2009). Following reuptake, serotonin can be degraded thanks to the action of the MAO enzyme, associated with the mitochondrial membrane. Alternatively, 5-HT is packaged in vesicles by an (H +)-dependent vector called vesicular monoamine transporter 2 (VMAT2) which is also present in other monoaminergic neurons. Factors leading to packaging rather than degradation of 5-HT within 5-HT neurons remain to be elucidated.
Most intriguing is the recent report on vesicular filling synergy in serotonergic neurons, a mechanism previously found in some cholinergic neurons. Therefore, it was observed that half of the neocortical and hippocampal subsets of SERT-free 5-HT neuronal elements coexpress VMAT2 and the vesicular glutamate transporter VGLUT3 on the same vesicles. It has also been shown that the uptake of vesicular glutamate by VGLUT3 allows vesicular filling of 5-HT by VMAT2, favoring the release of 5-HT from the tonically active terminals involved in volume transmission. Serotonergic and terminal fibers that co-express VGLUT3 and VMAT2 but lacking reuptake by SERT could represent sites of powerful regulatory mechanisms in 5-HT neurotransmission
There is some evidence that 5-HT synthesis, calcium-dependent exocytosis release, selective reuptake by an energy-dependent membrane transporter, metabolism and vesicle reuptake operate in all neuronal elements of neurons. 5-HT (i.e., soma, dendrites, axons and terminals), which participate together in 5-HT homeostasis (Descarries et al., 1975), Hoffman et al., 1998. The wide distribution of axons and terminals 5- HT throughout the neuraxis (**Fig. 10**), frequent non-synaptic neurotransmission such as the abundance of 5-HT receptors help explain the complex relationships between 5-HT and other neurotransmitters and neurohormonal systems.

Retrograde labeling studies show how serotoniergic projections departing from the nucleus raphe and paragigantocellularis of the RVM travel to specific areas of the dorsal horn for each fiber within the spinal cord, leading to the reasonable hypothesis that the rostral region provides a pain regulatory center. dependent and suggesting that the region from which the output originates is instrumental in the descending modulation of pain towards the spinal relay.
Although only 20% of RVM neurons are found to be serotonergic, recent molecular studies of 5-HT gene ablation demonstrate that serotonergic projections descending from RVM are important for positive modulation, facilitation, of pain in inflammatory or neuropathic states, but not necessary for inhibition of opioid-mediated acute pain. Furthermore, intrathecal injections of 5-HT agonists and antagonists after electrical stimulation evoke attenuated antinociceptive and analgesic responses, respectively, confirming that within the spinal cord, the modulating pathways of descending pain can exert an inhibitory influence (inhibition descending) and positive (descending facilitation) on the processing of nociceptive information of the dorsal horn.

**Fig. 9**

The pathways of tryptophan metabolism. In the serotonergic synaptic varicosity, tryptophan (Trp) enters the cell through an amino acid transporter, where it is primarily converted to 5-hydroxytryptophan (5-HTP) through the actions of the rate-limiting tryptophan hydroxylase 2 (TPH2). It is then converted to serotonin (5-hydroxytryptamine, 5-HT) by aromatic L-amino acid decarboxylase (AADC). Alternatively, tryptophan can enter the kynurenine (Kyn) pathway through metabolism by the ubiquitous indoleamine-2,3-dioxygenase enzyme (IDO) or the hepatic tryptophan-2,3-dioxygenase enzyme (not depicted). The vesicular monoamine transporter 2 (VMAT2) transports monoamines, including 5-HT, from the cellular cytosol into vesicles. Upon receiving a stimulus, 5-HT vesicles fuse with the plasma membrane, releasing 5-HT into the synaptic cleft, where it can activate 5-HT receptors on the target cell. 5-HT is then inactivated by reverse transport back into the presynaptic neuron through the actions of the serotonin reuptake transporter (SERT). There, it can either be recycled into vesicles or converted into the metabolite 5-hydroxyindoleacetic acid (5-HIAA) by the actions of monoamine oxidase (MAO).

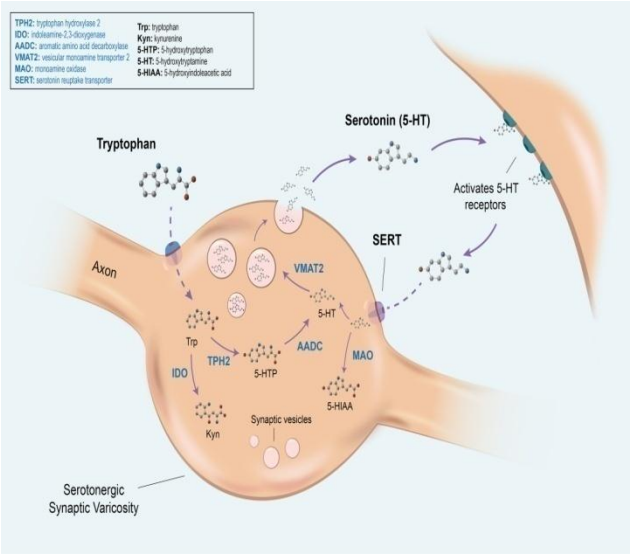
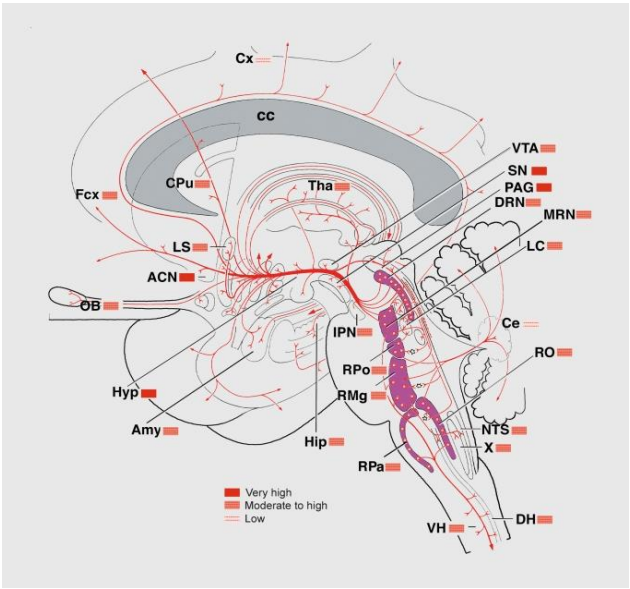



**Fig. 10**

The pathways of tryptophan metabolism. In the serotonergic synaptic varicosity, tryptophan (Trp) enters the cell through an amino acid transporter, where it is primarily converted to 5-hydroxytryptophan (5-HTP) through the actions of the rate-limiting tryptophan hydroxylase 2 (TPH2). It is then converted to serotonin (5-hydroxytryptamine, 5-HT) by aromatic L-amino acid decarboxylase (AADC). Alternatively, tryptophan can enter the kynurenine (Kyn) pathway through metabolism by the ubiquitous indoleamine-2,3-dioxygenase enzyme (IDO) or the hepatic tryptophan-2,3-dioxygenase enzyme (not depicted). The vesicular monoamine transporter 2 (VMAT2) transports monoamines, including 5-HT, from the cellular cytosol into vesicles. Upon receiving a stimulus, 5-HT vesicles fuse with the plasma membrane, releasing 5-HT into the synaptic cleft, where it can activate 5-HT receptors on the target cell. 5-HT is then inactivated by reverse transport back into the presynaptic neuron through the actions of the serotonin reuptake transporter (SERT). There, it can either be recycled into vesicles or converted into the metabolite 5-hydroxyindoleacetic acid (5-HIAA) by the actions of monoamine oxidase (MAO).



In general, most studies have shown that the dual nature of the response depends on multiple factors such as the pharmacodynamics of the drug (serotonin receptor antagonist or agonist), the diversity of 5-HT receptor subtypes that mediate its effect, the duration and nature of the stimulus (Cortes-Altamirano et al., 2018), from the anatomical region in which the action takes place (Gautier et al., 2017).
To these elements is added the complexity of the anatomy of the spinal horn, where the 5-HT receptors are found in the dorsal root ganglion and on the central terminals of the primary afferent fibers (Doly et al., 2005, Pierce et al., 1996) as well as on the GABAergic interneurons in the dorsal horn of the spinal cord (Doly et al., 2005), which while underlining the important role that serotonin plays in the processing of pain (Brenchat et al., 2009). It is difficult to interpret the role of serotonin in pain modulation, so the precise spinal mechanisms involved remain unclear.

**Serotonergic receptors**

Since 1987, more than fifteen 5-HT receptors have been identified by various cloning strategies, grouped into seven families in both humans and other mammalian species, although interspecies differences in their neuroanatomic distribution or profiles have been noted. pharmacological. Each receptor is encoded by distinct genes and arises from further alternative splicing mechanisms. In situ hybridization studies locate receptors throughout the nervous system such as the substantia nigra, hippocampal formation, hypothalamus, amygdala, striatum and frontal cortex although their relative density shows a large difference in pattern of expression depending on the area considered.
To date, seven 5-HT receptor families (5-HT1 to 7) have been identified, comprising 15 distinct subtypes (Hannon and Hoyer, 2008). With the exception of the ionotropic 5-HT3 receptor, all 5-HT receptors are G protein-coupled receptors, which can modulate ligand-dependent and voltage-dependent channels (Hannon and Hoyer, 2008). Although the exact types of receptors involved in spinal cord pain modulation are not fully understood, studies have shown the presence of at least four types of receptors (5-HT 1, 5-HT 2, 5-HT 3, and 5-HT 7) and several subtypes that may have an influence on these pain pathways (Hoyer et al., 1994, Gustafson et al., 1996).
Pharmacological studies show that the activity and function of the 5-HT target receptors depend on a variety of factors including

**1. availability of 5-HT in the extracellular space**.

The availability of 5-HT in the extracellular space is regulated at multiple levels, some of which are closely related such as receptor desensitization, reuptake and degradation.
In the prolonged presence of agonists or endogenous 5-HT, such as many G-protein-coupled proteins, GPCRs, 5-HT receptors exhibit rapid intracellular desensitization, sequestration (or internalization), and recycle of the receptor to the membrane. These mechanisms involve the activation of proteins such as SERT, MAO, protein kinase C/G and p38, which modulate the expression of SERT, reducing or accelerating the uptake of serotonin.
Furthermore, the 5-HT1A somatodendritic autoreceptors in the raphe nuclei and the 5-HT1B / 1D autoreceptors in the 5-HT terminal areas represent an additional powerful feedback mechanism, which reduces both the activation of 5-HT neurons and the release of the neurotransmitter. (Bockaert et al., 2008, Millan et al., 2010), **Fig. 11**.

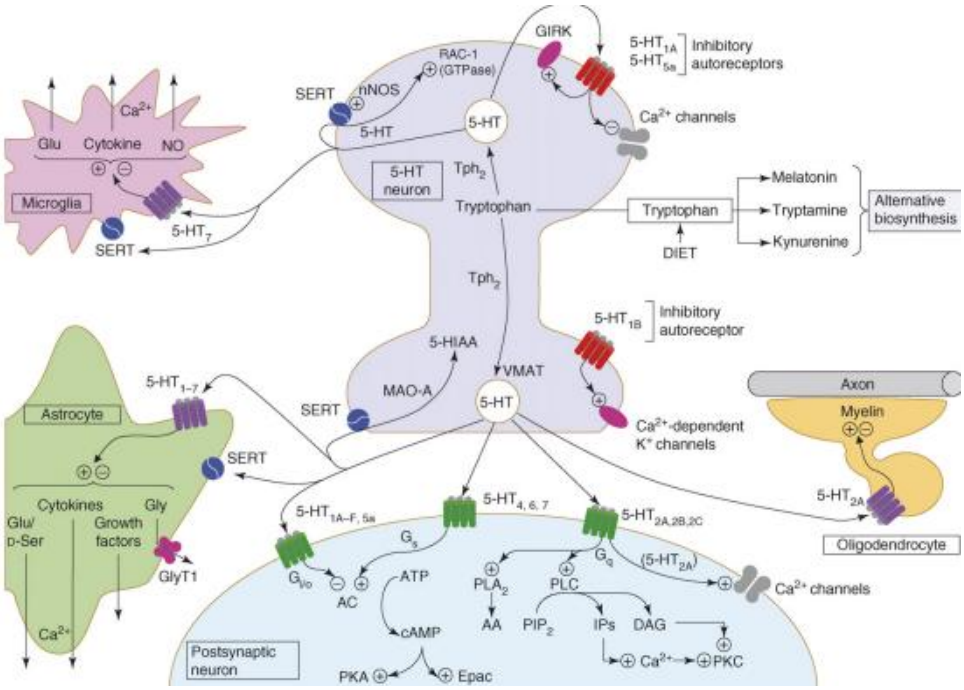



**Fig. 11**
An integrated view of signaling at serotonergic neurons. 5-HT is derived from tryptophan by an action of tryptophan hydroxylase 2 (Tph2), and it is deactivated by monoamine oxidase (MAO) A after release and reuptake via 5-HT transporters (SERT). Generation of 5-HT from tryptophan is an alternative to its conversion into melatonin (in the pineal gland), tryptamine (in neurons) and kynurenine (in astrocytes), which likewise function as neuromodulators in the brain. 5-HT receptors are localized both pre- and post-synaptically to serotonergic neurons, but all subtypes are not necessarily co-localized at the same postsynaptic location. 5-HT1A and 5-HT1B inhibitory autoreceptors are localized on cell bodies and terminals, respectively, and 5-HT5a autoreceptors might also be present on the former. Characterization of non-neuronal 5-HT receptors is far from complete. (Note that ligand-gated ion-channel 5-HT3 receptors, not considered herein, are present postsynaptically both on neurons and on non-neuronal cells.) The major modes of transduction are shown at the postsynaptic level and many receptors converge on specific signaling pathways. Moreover, individual subtypes recruit multiple cascades, for example 5-HT2A receptors couple to Ca2+ channels, PLC and PLA2. In addition, the GTPase exchange factor Epac is a recently identified downstream target of cAMP. Ion currents are an important mode for autoreceptor-feedback inhibition of serotonergic transmission. Serotonergic neurons also reveal two new modes of signaling: direct activation of neuronal nitric oxide (NO) synthase (nNOS) by SERTs and activation of a small G protein–GTPase, Rac-1, by 5-HT itself. Coupling patterns in non-neuronal cells show similarities and differences to neurons, but await further characterization. Abbreviations: 5-HIAA, 5-hydroxyindole amino acid; DAG, diacylglycerol; Gly, glycine; GlyT1, glycine transporter; IP, inositol phosphate; PIP2, phosphoinositol bisphosphate; D-Ser, D-serine; VMAT, vesicular monoamine transporter.

It is also reported that neurnoral nitrix oxide synthase, nNOS, binds to the C-terminal end of SERT, Garthwaite, 2007. This interaction inhibits the transport of SERT to the membrane by competitive binding of the synthetase with Sec23-Sec24, two cytoplasmic proteins that heterodimerize for the export of RE proteins to the Golgi, indirectly modifying the signaling exerted by 5-HT and its receptors, **Fig.12.**

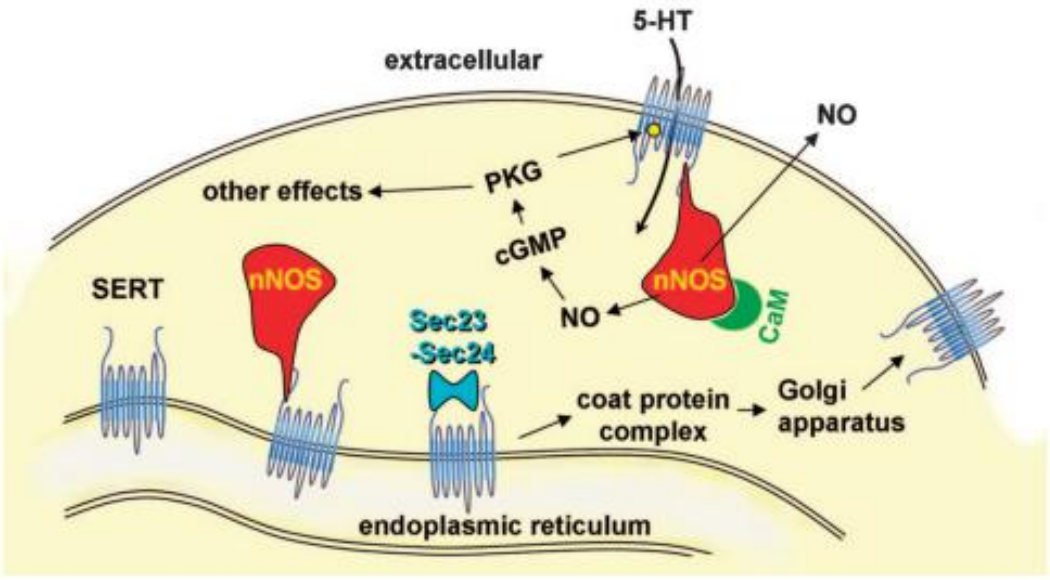



**Fig. 12**
SERT–nNOS interactions. Binding of nNOS to the SERT carboxyl terminus decreases SERT trafficking to the plasma membrane and thereby inhibits 5-HT uptake. Reciprocally, 5-HT uptake induces NO production from nNOS physically linked to SERT, through a calmodulin (CaM)-dependent mechanism. NO formed in association with 5-HT transport might, through cGMP and cGMP-dependent protein kinase (PKG), phosphorylate SERT, increasing its activity.

**2. interaction with other partners**.
Proteomics techniques revealed that 5-HT receptors interact with numerous intracellular proteins in addition to G proteins. These partners control the distribution of 5-HT receptors in specific cell domains, their trafficking in and out of the plasma membrane, Fig, as well as signaling. transduction, Turner et al., 2005. 5-HT receptor associated proteins include ubiquitous GPCR signaling modulators, b-arrestins Marion et al., 2004, serine/threonine protein kinase, protein phosphatase, PDZ Xia proteins et al., 2003, and others that are specific to the individual 5-HT subtypes, Bockaert et al., 2010, with different and distinct mechanisms for each receptor, but which on the whole profoundly modify their functional activity, thus representing all new presumed targets for treatment. of mood and addiction disorders, Millan et al., 2008.

**3. crosstalk with other receptors.**
The biological strategy of this interaction is infinite and acts at all levels of integration, from the input (e.g. endogenous agonist) to the output (e.g. ERK phosphorylation), through G proteins themselves (recruited from different classes of 5-HT receptors). Some exchanges are facilitators (eg 5-HT4 and 5-HT7 receptors converging via Gs to AC), while others are antagonists (eg 5-HT1A inhibition of AC versus 5-HT4 stimulation). Exchanges are both functional and physical.
An example of physical interaction is represented by dopamine which by binding to the 5-HT2A receptor subtype regulates its internalization, Bhattacharyya et al., 2006 or by the activation of protein-kinases such as PKC and PKA that phosphorylate the receptors by modifying their expression, localization and interaction with other protein partners, Hensler et al., 2005, Tobin et al., 2008. Furthermore, a common protein partner between two 5-HT receptors can represent a further modality of crosstalk. Like all GPCRs, 5-HT transactivates protein kinase receptors, PKR. GPCRs can crosstalk with an RTK in different ways, through molecules such as ROS and protein

kinases. Indeed, it has been widely demonstrated that the pathways activated by GPCR and RTK are not mutually exclusive to each other and that they often act synergistically by presenting numerous points of interaction in the signaling cascade (Waters et al., 2004, Wong et al., 2002). The involvement of common molecules in the transduction process determines an integration of the different stimuli through a complex cross-communication defining a more intricate control system of the mechanisms that regulate processes such as cell proliferation and neurogenesis induced by 5-HT (Millan et al ., 2006, Cowen et al., 2007).

**4. homologous and heterologous dimerization.**

Dimerization also represents a receptor regulation mechanism. 5-HT1A, 5-HT1B, 5-HT1D, 5-HT4 and 5-HT2C receptors all form dimers, Berthouze et al., 2007, Lee et al., 2000. Contrary to other protein G, binding is not necessary to the formation of the dimer which occurs in a constitutive manner before the insertion of the receptor into the membrane. the dimer can occur with the same receptor, homodimerization, or with other receptors not necessarily related at the functional level, such as the 5-HT2A/mGLU2 heterodimer, metabotropic glutamate 2, or as for the 5-HT1B/1D subtypes that interact with other GPCRs, Lee et al., 2000.

The proteins assemble via the 4-5 transmembrane domains in the endoplasmic reticulum, Herrick-Davis, K. et al., To form functional complexes that integrate the neurotransmission of serotonin, with a potent inhibitory effect on hallucinogenic drugs, and of glutamate, resulting in neurotransmitter reduction from thalamo-cortical afferents, Zhang et al., 2007, Gonzalez-Maeso, J. et al., 2008.

In this structure, in fact, the heterodimer triggers unique cellular responses when it binds to hallucinogenic drugs, through the enhancement of the coupling of the Gi / o protein to the 5-HT2C receptor subtype, thus modulating the sensory gating functions of the cortex and abolishing by means of mGluR2 the specific signaling of the hallucinogen The fundamental functional role of the dimer is underlined by the altered cortical processes of schizophrenia, Gonzalez-Maeso, et al., 2008.

The figure below, **Fig.13**, shows a schematic summary of the regulatory systems of serotonin and its receptors discussed so far, taking as an example the 5-HT2C receptor that best summarizes what is explained in points 2 to 4.

Subtype 2C can form dimers with other GPCRs or with itself, recruiting, following the binding with the ligand, two different phosplipases, phospholipase C, in case of hetero dimers, PLCb, and phospholipase A2, PLA2, in case of homodimers. PLA2 activates the pathway regulated by phospholipase Cg, while PLCb leads to the recruitment of protein kinase C, which phosphorylates many cellular proteins including ERK1/2. The two pathways together lead to a specific gene expression characteristic of the pathway and responsible for the functional and activity differences of the receptor, factors further regulated by the interaction of the dimer with partner proteins such as nNOS and PDZ, also important for its insertion into the membrane.

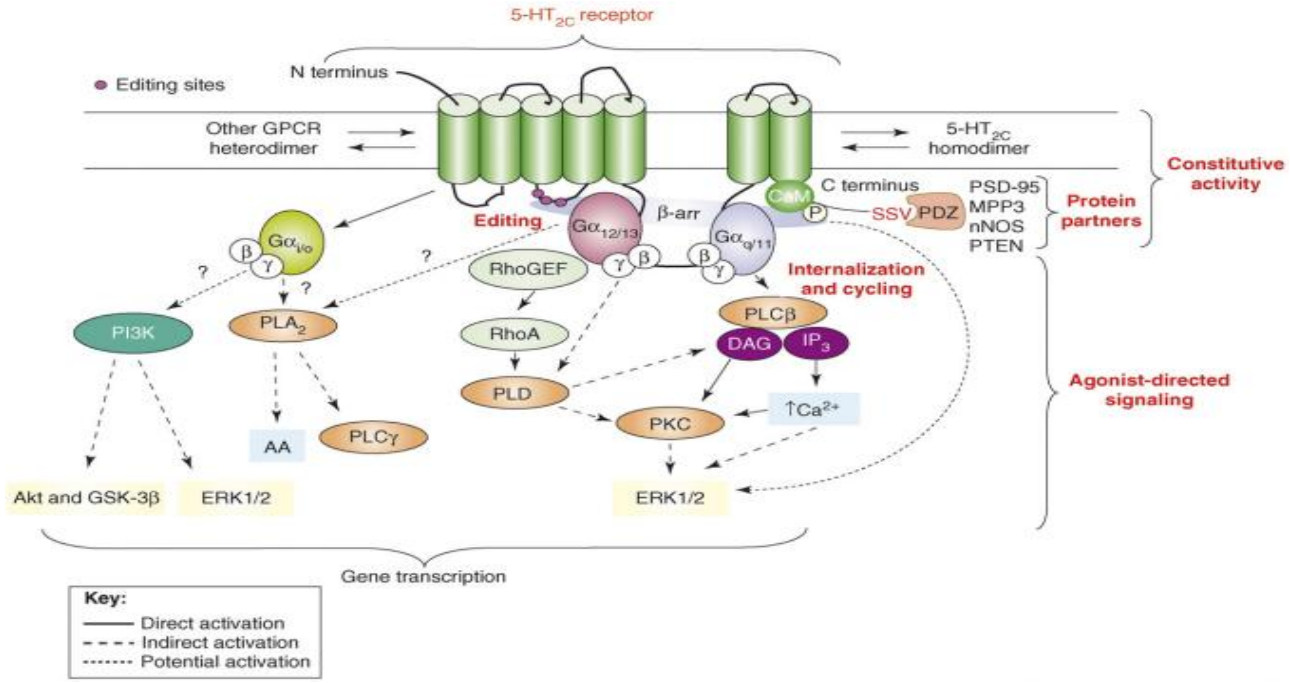



**Fig. 13**

Signaling at 5-HT2C receptors.

5-HT2C receptors illustrate much of the complexity of serotonin signaling. They prototypically recruit phospholipase (PL)Cb via the a subunit of Gq/11. In addition, they possess a further important axis of transduction via PLA2, possibly triggered by G12/ 13, that also indirectly activates PLD. PLA2 itself recruits PLCg and cyclooxygenase 2 resulting in the generation of arachidonic acid (AA). By contrast, stimulation of PLC and PLD ultimately converges onto PKC, then results in the phosphorylation of extracellular regulated kinase (ERK)1/2. A further route to ERK activation is provided by Gai/o, which induces the phosphoinositide 3 kinase (PI3K)–Akt–glycogen synthase kinase (GSK)-3b cascade. Like ERK, this cascade controls gene transcription and it is also implicated in apoptosis and many other cellular functions. In addition to directing internalization by 5-HT2C receptors, b-arrestin can activate ERK1/2. The preference of certain agonists for one transduction pathway over another is called ligand-directed signaling. Constitutive activity refers to spontaneous coupling in the absence of agonists and it is greatest for isoforms unaffected by adenosine-to-inosine mRNA editing. Although 5-HT2C receptors link up with themselves to form homodimers, they probably assemble into heterodimers with other classes of GPCRs. Moreover, 5-HT2C receptors associate with protein partners controlling membrane insertion, constitutive activity and signaling: four proteins, but others are known

Abbreviations: CaM, calmodulin; DAG, diacylglycerol; il-3, intracellular loop 3; IP3, inositol triphosphate; RhoA, Ras homolog A; RhoGEF, Ras homolog guanine nucleotide exchange factor; SSV, Ser-Ser-Val. Akt is also known as protein kinase B.

On the periphery, endogenous serotonin is released as a result of tissue damage mainly by mast cells, platelets and epithelial cells, Sommer 2004. In the formalin test, a model of inflammatory pain, it has been shown that acute nociceptive behaviors such as secondary mechanical allodynia and hyperalgesia are dependent on the release of 5-HT from mast cells (Parada et al., 2001; Godinez-Chaparro et al., 2011).

5-HT binds, activating them, to the receptors present on the terminals of the primary nociceptive afferents and on the DRGs. In fact, the 5-HT1B, 5-HT1D, 5-HT2A, 5-HT2B, 5-HT3, 5-HT4, and 5-HT7 subtypes have been identified on the DRGs (Wu et al., 2001; Nicholson et al., 2003), in which it is reported that the lesion induces upregulation of 5-HT2A, 5-HT3, 5-HT4, and 5-HT7 receptors (Wu et al., 2001; Cardenas et al., 2004; Liu et al. , 2005).

Numerous studies have shown that the administration of exogenous 5-HT increases the excitability of myelinated and non-myelinated afferents, and of DRGs (Michaelis et al., 1997; 1998; Cardenas et al., 1999; Moalem et al., 2005; Lang et al., 2006).

Furthermore, intra-implant injection of 5-HT causes leg edema (Sufka et al., 1992; Hong and Abbott, 1994; Carstens, 1997), while in healthy subjects, intradermal injection of 5-HT at a low concentration produces pain. burning (Lischetzki et al., 2001) and hyperalgesia after injection into the masseter muscle of the jaw (Ernberg et al., 2000), while the peripheral injection of the antagonists 5-HT2A, 5-HT3 and 5-HT7 inhibit the nociceptive effects induced by formalin (Rocha-Gonzalez et al., 2005; Nakajima et al., 2009; Godinez-Chaparro et al., 2011).
All these data clearly suggest that peripheral 5-HT2A, 5-HT3 and 5-HT7 receptors significantly contribute to peripheral nociceptive transmission during inflammation.
Even in neuropathic pain models, a mechanical injury caused by sciatic nerve ligation shows increased levels of serotonin, (Anden and Olsson, 1967; Vogel et al., 2003), while the same injury in HTT null mice records reduced serotonin levels. in injured nerves as well as the absence of harmful behaviors, such as thermal hyperalgesia, causing peripheral sensitization (Vogel et al., 2003). Similarly, injection of the 5-HT2A and 5-HT3 antagonists into the affected hind leg relieves CCI-induced mechanical hyperalgesia (Theodosio et al., 1999), demonstrating on the one hand that even in mechanical pain the serotonergic pathway is fundamental in the pain processing, but also that the release of 5-HT in injured tissues, such as inflamed ones, causes sensitization of the nerve fibers, which contributes to peripheral sensitization through direct or indirect mechanisms.

From primary afferent fibers, nociceptive information is transmitted to ascending spinal neurons in the dorsal horn, DH, (Marlier et al., 1991; Pompeiano et al., 1992; 1994; Hoyer et al., 1994; Fonseca et al., 2001 ; Wu et al., 2001; Wang et al., 2003; Doly et al., 2005; Liu et al., 2005), which represents the site where serotonergic receptors are most widely expressed. The main receptors studied in the dorsal horn so far are 5-HT1A, 5-HT2A, 5-HT3, 5-HT7.

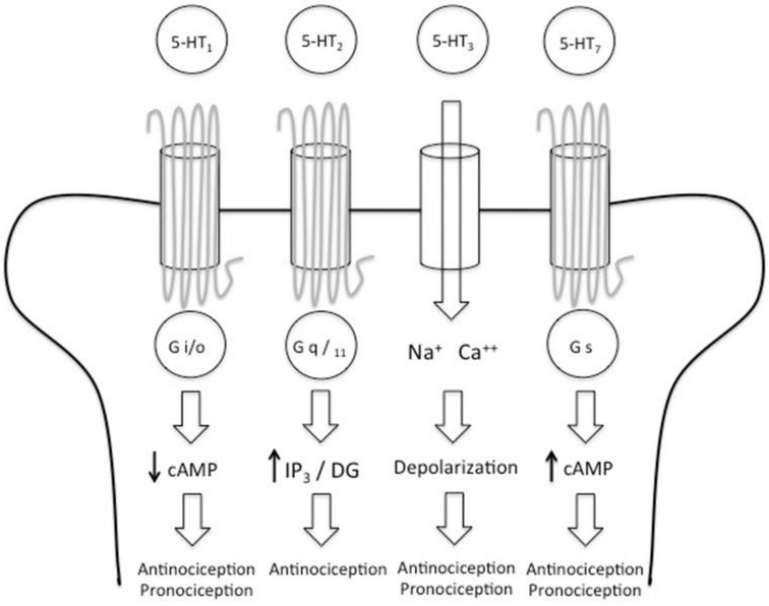



**Fig. 13**
The 5-HT receptors are divided into 7 families (5-HT1, 5-HT2, 5-HT3, 5-HT4, 5-HT5, 5-HT6 and 5- HT7) that comprise 15 receptor subtypes. The receptors involved in the nociceptive pathway are 5-HT1, 5- HT2, 5-HT3 and 5-HT7. 5-HT1 is coupled to the G i/o protein and it reduces levels of cAMP generating anti and pronociceptive effects. 5-HT2 is coupled to the G q/11 protein and its activation leads to an increase of IP3 and DG levels, generating an antinociceptive effect. 5-HT3 is the only one that is not coupled to a G protein; instead this receptor is a ligand-gated cation channel, when activated 5-HT3 depolarizes the neuronal membrane and causes antinociception but also 5-HT3 can maintain the painful stimulus. 5.HT7 is coupled to G s protein, its activation generates an increase in the cAMP levels causing pro and antinociceptive effects.

## 5-HT$_{1A}$ receptor

The 5-HT1A receptor is the most characterized of the 5-HT1 receptors (Pedigo et al., 1981; Middlemiss & Fozard, 1983), largely due to the wide availability of many specific pharmacological tools in addition to the fact that its gene, and related cloning (Kobilka et al., 1987), was identified (Fargin et al., 1988) more than a decade ago. Furthermore, it represents a research target for many neurological and neuropsychiatric disorders, in particular in depression (Chilmonczyk et al., 2015) and pain syndromes, (Salat et al., 2007, Avila-Rojas et al., 2015).

Subdivided further into 6 subtypes, A-F (Zemlan et al., 1988, Peroutka et al., 1988, Cortes-Altamirano et al., 2018), is a Gi/o protein coupled receptor sensitive to pertussis toxin, through which directly or indirectly inhibited or activated enzymes, channels, kinases and the production of second messengers is stimulated or inhibited, **table 1**

In healthy adult rodents, evidence of the antinociceptive effect of 5-HT1a has been provided by both behavioral and electrophysiological studies (Lin et al., 1996, Gjerstad et al., 1996, You et al., 2005). Inhibitory modulation of nociception in a healthy adult rodent is mediated through the 5-HT1a receptor, possibly due to a reduced release of glutamate from the primary afferent terminals (**Fig.14**). There is, however, an electrophysiological study showing that spinal 5-HT1a activation does not affect the C-fiber evoked spinal field potentials, Aira et al., 2010.

Cai et al. showed that optogenetic activation of serotonergic neurons in RVM produces persistent sensitization to mechanical and thermal stimuli and suggested that serotonergic neurons in RVM have a predominant facilitating role on spinal nociception. Indeed, pronociceptive behaviors in healthy rodents are induced by activation of the spinal and systemic 5-HT1a receptor, Wu et al., 1994, Bonnefont et al., 2005, Ardid et al., 2001. This facilitating action of the inhibitory receptor 5-HT1a probably involves the GABA ININ pathway (**Fig.14**), since 5-HT1a receptors are expressed on GABAergic interneurons. Wang et al., 2009. Bonnefont et al. provided evidence of this by demonstrating that the facilitating effect of spinal 5-HT1a receptors could be inhibited by the GABA receptor antagonist bicuculline A. Bonnefont et al., 2005.

Signaling characteristics of human 5-HT receptors

| Receptor | Common signaling linkages | Other signaling linkages |
|---|---|---|
| 5-HT$_{1A}$ | Inhibits AC | Activates PLC |
| | Activates $K^+$ channels | Activates NOS |
| | Stimulates ERK | Activates NAD(P)H oxidase |
| | Inhibits $Ca^{2+}$ conductances | Activates NHE-1 |
| 5-HT$_{1B}$ | Inhibits AC | Activates PLC |
| | Stimulates ERK | Activates NOS |
| | | Activates AC2 |
| | | Activates $K^+$ channels |
| 5-HT$_{1D}$ | Inhibits AC | Inhibits $Ca^{2+}$ conductances |
| | | Activates $K^+$ channels |
| 5-HT$_{1E}$ | Inhibits AC | |
| 5-HT$_{1F}$ | Inhibits AC | Activates PLC |
| 5-HT$_{2A}$ | Activates PLC | Activates NHE-1 |
| | Activates PKC | Activates AC |
| | Stimulates ERK | Inhibits AC |
| | Activates $PLA_2$ | Activates Jak2/STAT3 |
| | | Activates $Ca^{2+}$ channels |
| 5-HT$_{2B}$ | Activates PLC | Activates cell cycle |
| | Activates ERK | Activates iNOS |
| | Activates $PLA_2$ | Activates cNOS |
| 5-HT$_{2C}$ | Activates PLC | Activates $Na^+/Ca^{2+}$ exchanger |
| | Activates PKC | PDZ motif signals? |
| | Activates $PLA_2$ | |
| 5-HT$_4$ | Activates AC | Regulates various channels |
| | Activates PKA | |
| 5-ht$_{5a}$ | Unknown | Unknown |
| 5-ht$_{5B}$ | Unknown | Unknown |
| 5-HT$_6$ | Activates AC | |
| | Activates PKA | |
| 5-HT$_7$ | Activates AC | Activates ERK |
| | Activates PKA | |

**Table 1**
The table shows the common signaling linkages associated with a specific 5HT receptor subtype, as well as other non-common signaling linkages.

5-HT1A receptor mRNA expression in the dorsal horn is significantly enhanced after bee venom-induced (Wang et al., 2003) or carrageenan-induced (Zhang et al., 2002) inflammation, correlating with pain spontaneous and heat hyperalgesia.
Furthermore, depletion of 5-HT1A receptors by molecular biology techniques reduces painful behaviors induced by intra-implant injection of bee venom (Wang et al., 2003).
There are studies that have shown, in fact, that spinal administration of 5-HT1A receptor agonists induces analgesia (Xu et al., 1994; Shannon and Lutz, 2000; Bardin et al., 2001; Nadeson and Goodchild, 2002; Bardin and Colpaert, 2004), suggesting a role of the 1A receptor in chronic inflammatory and neuropathic pain.
Many of these studies were carried out following the use of F-13 640, a highly effective selective agonist of the 5-HT1A receptor (Vacher et al., 1998; Wurch et al., 2003). Data show that administration of this agonist produces analgesia in rodent models of acute and chronic pain (Colpaert et al., 2006a, 2006b), as well as the expression of the c-Fos protein is suppressed in the dorsal horn of the spinal cord (Buritova et al., 2006a, 2006b).

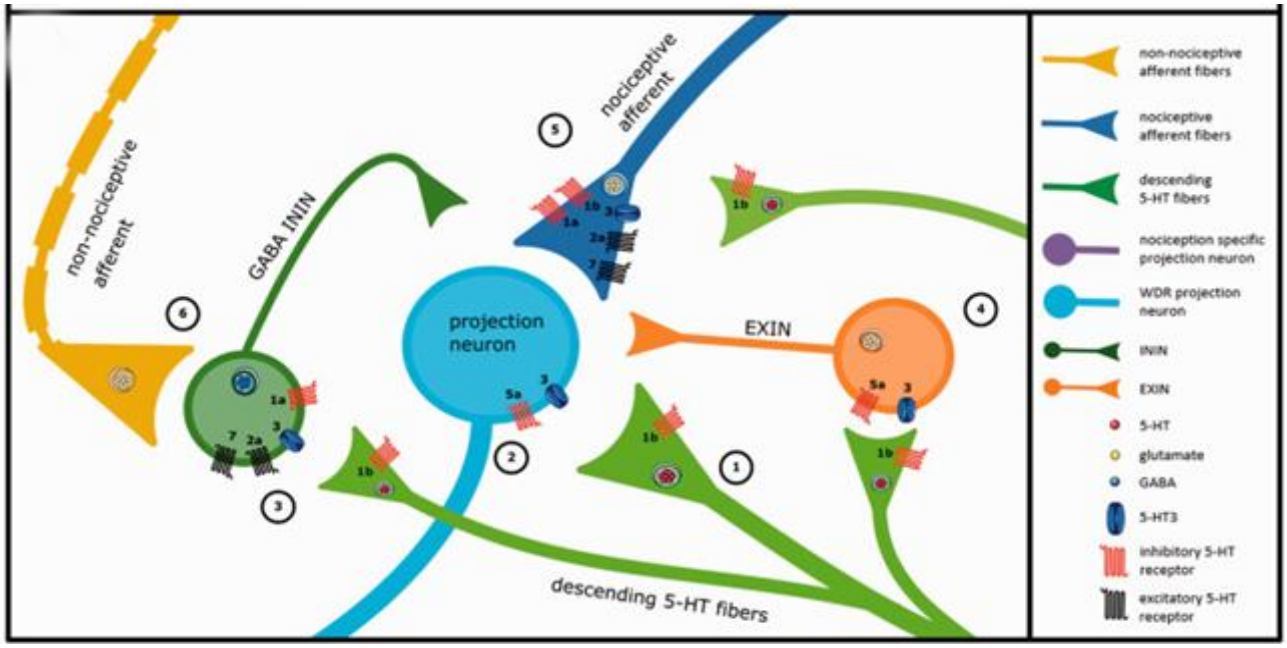



**Fig. 14**
Descending serotonergic fibers and 5-HT receptors in the dorsal horn nociceptive network of the adult rodents. Dorsal horn nociceptive network: Nociceptive afferent fibers (thinly myelinated Aδ-fiber, unmyelinated C-fiber) terminate in the superficial layers (lamina I-II) of the DH where they either synapse on interneurons (lamina I-III) or NK1 receptor expressing projection neurons (lamina I). Neurotransmitters utilized by inhibitory interneurons (ININs) are γ-aminobutyric acid (GABA), glycine, or both. Excitatory interneurons (EXIN) utilize glutamate. The interneurons synapse on projection neurons either in lamina I (nociceptive specific) or in lamina III-V (wide dynamic range (WDR) neurons). WDR neurons have dendrites extending to the superficial lamina and thus synapses form nociceptive fibers, non-nociceptive fibers and interneurons. Non-noxious stimuli are transmitted by touch-responsive, myelinated Aβ fibers that terminate within lamina II-V and synapse onto the WDR and interneurons. 5 Descending serotonergic neurons terminate most abundantly in the superficial laminae (I/II) but they also innervate deeper laminae (IV-VI).
Figure contains numbered pathways of 5-HT mediated nociceptive modulation:
1) Autoreceptor pathway; direct modulation of serotonin release through 5-HT1b autoreceptors on descending serotonergic terminals.
2) Projection neuron pathway; direct modulation through postsynaptic 5-HT3 and 5-HT5a expression on spinal projection neurons) GABA ININ pathway; indirect modulation of projection neurons through 5-HT1a, 5-HT2a, 5-HT3 and 5-HT7 expressed on GABAergic ININs) EXIN pathway; indirect modulation of projection neuron through 5-HT3 and 5-HT5a expression on EXINs) Nociceptive afferent pathway; direct modulation of neurotransmitter release through expression of 5-HT1a, 5-HT1b, 5-HT2a, 5-HT3 and 5-HT7 on nociceptive afferent terminals) Non-nociceptive afferents pathway; modulation via activation of GABAergic ININs by non-nociceptive afferents (Aβ fibers) according to the principle of the Gate-Control Theory.

Regarding the role of 1A receptors in chronic neuropathic pain, many studies suggest an inhibitory effect of subtype 1a on nociceptive neurotransmission in different pain models, following the administration of different drugs that mediate pain-relieving effects following activation of 5-$HT_{1A}$, such as curcumin, ac. ferulic and cannabidiol (Colpaert et al., 2002, Aira et al., 2010, Avila-Rojas et al., 2015).

In a model of intraoperative and postoperative pain induced by orthopedic surgery in rats, pretreatment with F-13 640 reduces the need for isoflurane, anesthetic, in the preoperative phase, while, administered after surgery, F-13 640 suppresses the behavior of the postoperative pain (e.g. leg flexion and elevation) in a lasting way (Kiss et al., 2005). These inhibitory effects of F-13 640 are reversed by WAY-100 635, a selective antagonist of the 5-$HT_{1A}$ receptor, confirming an antinociceptive effect mediated by the 5-$HT_{1A}$ receptor (You et al., 2005). In chronic neuropathic pain models, such as chronic constricting injury, ischemic spinal cord injury, or infraorbital nerve ligation, acute or continuous infusion of F-13 640 strongly inhibits hyperalgesia and allodynia (Brenchat et al., 2010; Colpaert et al., 2002, 2004; Deseure et al., 2002, 2004, 2003; Wu et al., 2003). In addition, chronic exposure to 5-HT agonists or reuptake inhibitors desensitize presynaptic 5-$HT_{1A}$ autoreceptors in raphe nuclei without altering the response properties of postsynaptic 5-$HT_{1A}$ receptors in projection areas, such as the dorsal horn of the spinal cord (Hamon and Bourgoin, 1999). Consequently, the 5-HT1A autoreceptor-mediated inhibitory feedback control of 5-HT neurotransmission fades, which allows for maximal 5-$HT_{1A}$ signaling to postsynaptic receptors (Lanfumey and Hamon, 2004).

Thus, the pronociceptive effects of 5-$HT_{1A}$ receptor agonists appear to be predominantly mediated by supra-spinal 5-HT1A presynaptic autoreceptors, while their antinociceptive effects probably derived from activation of postsynaptic 5-$HT_{1A}$ receptors in the spinal cord (Colpaert et al. ., 2006a, 2006b), **Fig.15**

However, few other studies suggest that the spinal 5-$HT_{1A}$ receptor is not involved in inhibition (Wei et al., 2006) or loses this inhibitory action (Liu et al., 2010). These contradictory results could be related to the nature of the noxious stimulus applied, to the pathophysiological condition (Bardin et al., 1997; Colpaert et al., 2002) as well as to the drug administration site, which causes a different permeability, distribution and affinity to the receptors. located in the different anatomical sites Wei and Pertovaara.

Finally, subgroups 1B and 1D show the same antinociceptive effect as group 1A in neuropathic pain, while for the 1F, 1E, 1C receptors they have not been detected at the moment.

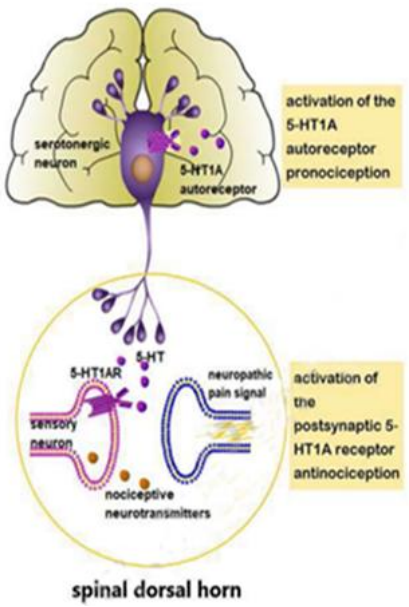



**Fig. 15**
Possible mechanisms of 5-HT1 receptor in neuropathic pain.
5-HT1 is coupled to the G protein and generates both anti- and pronociceptive effects according to the place it located. The activation of 5-HT1A auto-receptors in supraspinal level inhibit the synthesis and release of 5-HT. The lower 5-HT availability to 5-HT1A receptors in spinal level facilitated pain transmission. The activation of postsynaptic 5-HT1A receptors in spinal level can elicit antinociceptive effect..

**5-$HT_{2A}$ receptor**

5- HT2 is a family of receptors associated with the Gα q protein, which after receptor stimulation with the agonist, activates phospholipase C (PLC), which subsequently promotes the release of diacylglycerol (DAG) and inositol triphosphate (IP3), which in turn they induce the activity of protein kinase C (PKC) and the release of Ca 2+. It includes groups 2A, 2B and 2C.

The various subclasses are similar from the structural point of view and for the transduction and pharmacological pathways activated, even if the mechanisms with which they could work, could be different.

The main subgroup is the 2A receptor which mediates neuronal excitation.

5-$HT_{2A}$ receptors are highly expressed in DRGs in humans and rats (Pierce et al., 1996, 1997; Fonseca et al., 2001). Their localization is limited to predominantly myelinated primary afferent fibers (Ad) and to a subpopulation of unmyelinated afferent fibers (C) (Okamoto et al., 2002; Thibault et al., 2008; Van Steenwinckel et al., 2008). The presence of mRNA encoding for 5-HT2A receptors has also been reported on nociceptive neurons synthesizing CGRP or substance P, as well as on nociceptive neurons expressing the capsaicin transient potential type 1 vanilloid receptor in DRG (Okamoto et al., 2002 ; Van Steenwinckel et al., 2009).

All studies conducted to date confirm the important role of peripheral 5-$HT_{2A}$ receptors in the development of inflammation-induced hyperalgesia, particularly in the inhibition of sensitization of primary sensory neurons associated with inflammatory pain (Nishiyama, 2005). Indeed, inflammation is accompanied by robust receptor upregulation. 5-HT released by platelets and mast cells activates primary afferents and increases the excitability of small-diameter neurons in the DRG, promoting the release of CGRP by stimulation of 5-$HT_{2A}$ receptors (Wei et al., 2005; Wang et al., 2010; Huang et al., 2011). Furthermore, systemic or local administration of 5-HT2A receptor antagonists reduces pain behavior after formalin injection into the hind leg (Obata et al., 2001; Nishiyama, 2005; Nakajima et al., 2009, Kayser et al. 2007).

Sasaki et al. in their study examined the effect of an antagonist having an exclusive and selective periphe

ral action of the 5-$HT_{2A}$ receptor, sarpogrelate, on thermal injury-induced hyperalgesia and allodynia in rats. The authors concluded that the antagonist blocks 5-$HT_{2A}$ receptors at the terminals of primary afferent fibers in the periphery to inhibit primary thermal hyperalgesia and secondary mechanical allodynia.

In clinical trials, oral administration of sarpogrelate has been reported to relieve pain symptoms in patients with lumbar disc herniation (Kanayama et al., 2005) or bone atrophy (Otake et al., 1998). Using positron emission tomography, Kupers et al. (2009) demonstrated high levels of 5-$HT_{2A}$ receptor binding in the prefrontal cortex and cortex, areas associated with pain evoked by high tonic thermal stimulation, suggesting that the use of selective 5-$HT_{2A}$ receptor antagonists, at least after administration peripheral, may not only have beneficial effects for the treatment of inflammatory but also for neuropathic pain.

Most spinal cord studies have reported low levels of 5-$HT_{2A}$ receptor mRNA (Zhang et al., 2001a; Doly et al., 2004). In the rat spinal cord, 5-$HT_{2A}$ receptors, localized by immunocytochemistry through the use of an antibody directed against an N-terminal sequence of the receptor, are more pronouncedly expressed in motor neurons, sympathetic preganglionic cells and dorsal horn. where the distribution is mainly limited to laminae I – IV (Thibault et al., 2008; Van Steenwinckel et al., 2008; Xie et al., 2008) on GABAergci interneurons, concluding that the localization of 5-$HT_{2A}$ receptors is mainly postsynaptic .

Current studies have shown that, in a healthy adult mouse, spinal 5-$HT_{2A}$ receptors are not involved in the inhibition of neuronal responses to DH, (Aira et a., 2010), nor in nociceptive behavior, (Nitanda et al., 2005). However, the involvement of spinal 5-$HT_{2A}$ receptors in nociceptive inhibition cannot be completely ignored since activation of spinal 5-$HT_{2A}$ receptors inhibits C-fiber-

evoked WDR responses (Liu et al., 2007), an inhibitory effect probably exercised indirectly through the pathway of the excitatory interneurons (**Fig.14**).

Regarding the role of spinal 5-$HT_{2A}$ receptors, particularly in neuropathic pain, several and divergent researches have been reported which however suggest a function for the receptors in pain modulation.
Nitada et al. studied the possible involvement of the 5-$HT_{2A}$ receptor in the pathogenesis of neuropathic pain using the chronic constriction injury of the sciatic nerve in rats and the antagonists ketaserin, the antagonist, used in different pain models, and sarpogrelate; their results indicated that 5-$HT_{2A}$ receptor antagonists reduce hyperalgesia. Other authors subsequently confirmed the inhibitory role of the antagonists, also exploring the probable activated molecular mechanisms. Wang et al demonstrated that in a fifth lumber nerve ligation model, subcutaneous and topical administration of ketaserine inhibits the expression of the neuropeptide Y and calcitonin gene-related peptides CGRP, which increase after nerve injury, and relieve pain. as a consequence of the blockade of the 2A receptor group. Furthermore, systemic activation of 5-$HT_{2A}$ has been shown to cause pronociceptive effects (Wang et al., 2009, Lopez et al., 2018).
Van Steenwinckel et al also confirmed the pro nociceptive role of subtype 2A.
The authors used a model of neuropathy in rodents induced by a nucleoside analog, reverse transcriptase inhibitor, used in HIV/AIDS therapy. After intrathecal injection of a-phenyl-1- (2-phenylethyl)-4 piperidinemethanol, 5-$HT_{2A}$ receptor antagonist, the mice showed a dose-dependent reduction in mechanical hyperalgesia and allodynia, while the knockout mice for the 5-HT2A receptor do not develop neuropathy induced by dideoxycytidine (Van Steenwinckel et al., 2008).
In neuropathy induced by an antineoplastic drug, vincristine, which induces thermal alloidine and mechanical hypersensitivity, (Thibault et al., 2008), an increase in the immunoreactivity of the 5-$HT_{2A}$ receptor is demonstrated in the superficial layers of the horn. This increase is accompanied by an increase in c-Fos expression throughout the spinal cord. Furthermore, administration of receptor antagonists, such as MDL 11.939, reduces antineoplastic drug-induced mechanical hypersensitivity in a dose-dependent manner, suggesting a pronociceptive role of the 5-$HT_{2A}$ receptor in spinal nociceptive processing, (Thibault et al., 2008) .
In chronic neuropathic pain, the effects of the facilitating role of spinal 5-$HT_{2A}$ receptors can be explained by increased receptor expression in the spinal DH following injury (Aira et al., 2012, Aira et al., 2010, Patel et al., 2018).
The mechanisms that could explain the hyperalgesia induced by the activation of the 5-$HT_{2A}$ receptor were suggested by experiments conducted by Aira et al in a model of Spinal Nerve Ligation.
The authors provided evidence for a novel feedforward activation mechanism between 5-$HT_{2A}$ and metabotropic receptors (mGluRs) in dorsal spinal horn neurons. They demonstrated that 5-$HT_{2A}$ receptors facilitate pain transmission by upregulation of metabotropic receptors (mGluRs) via protein kinase C (PKC) (**Fig.16**).
All these data taken together suggest that the receptor is responsible for the production of neuronal activity at the level of the spinal cord in different pain models.
At the same time the use of 5-$HT_{2A}$ receptor antagonists could help relieve various types of neuropathic and inflammatory pain.

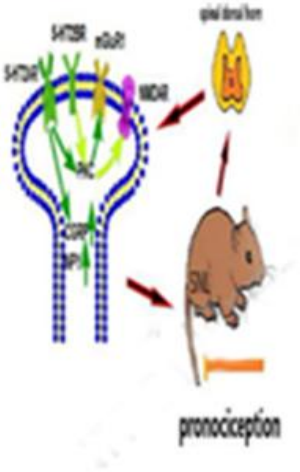



**Fig. 16**
Possible mechanisms of 5-HT2 receptor in neuropathic pain. 5-HT2 receptor is coupled to G protein. Spinal nerve ligation (SNL) leads to an up-regulation of 5-HT2A receptors in spinal dorsal horn neurons. Its activation leads to an increase of calcitonin gene-related peptides (CGRP) and neuropeptide Y (NPY) levels and an up-regulation of metabotropicreceptor1 (mGluR1) through protein kinase C (PKC) generating a pronociceptive effect. The facilitation of neuropathic pain mediated by 5-HT2B receptor is major mediated by the activation of the PKCc/NMDAR pathways

### 5-HT$_3$ receptor

Of the 5-HT receptor family, subtype 3 is the only type of ligand-gated cation channel, (**Fig. 17**).

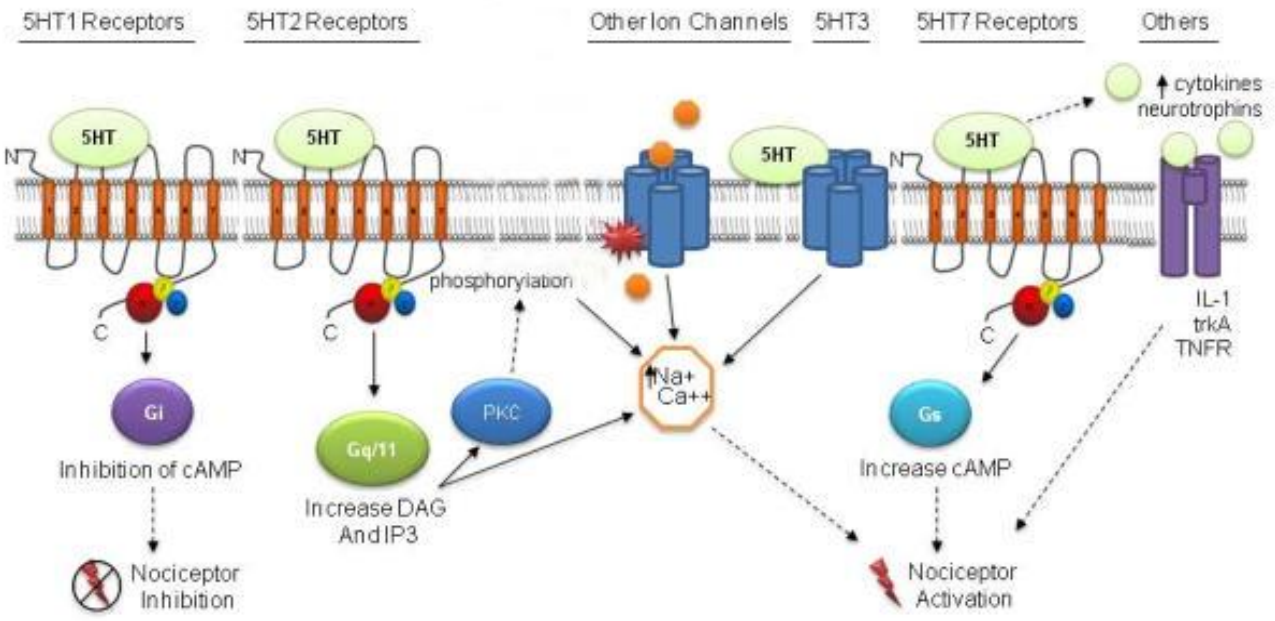



**Fig. 17**
The figure shows the subtype receptors 5HT$_1$, 5HT$_2$, 5HT$_3$ and 5HT$_7$ and the downstream activation pathways

In the periphery, 5-HT$_3$ receptors are located in DRGs of rats and humans (Laporte et al., 1992; Kia et al., 1995; Pierce et al., 1996, 1997) as well as at the terminals of primary myelin afferent fibers (Ad) and non-myelinated (C) within the spinal cord (Zeitz et al., 2002; Maxwell et al., 2003; Conte et al., 2005).
In the spinal cord, 5-HT3 receptors are found on the superficial laminae of the dorsal horn (Laporte et al., 1992; Kia et al., 1995; Millan, 1997), on the terminals of excitatory interneurons and on some neurokinin 1 projection neurons (NK1) and in laminae I/III (Maxwell et al., 2003; Conte et al., 2005). They are also found on inhibitory GABAergic interneurons (Huang et al., 2008).

The presence of 5-$HT_3$ receptors has also been reported in brain structures such as the amygdala, hippocampus and cortex (Miquel et al., 2002).
Both electrophysiological and behavioral studies have reported a critical role in the downward facilitatory modulation of neuropathic and chronic pain.
It is widely demonstrated that peripheral 5-HT3 receptors induce pronociceptive effects (Grubb et al., 1988; Guilbaud et al., 1989; Giordano and Dyche, 1989; Lang et al., 2006). Peripheral injection of 5-HT or carrageenan evokes acute pain in rats that is attenuated by relatively selective 5-$HT_3$ receptor antagonists (Richardson et al., 1985; Eschalier et al., 1989; Sufka et al., 1992). Administration of m-chlorophenylbiguanide (m-CPBG), a 5-$HT_3$ receptor agonist, increases the excitability of C-fiber axons in the rat sural nerve (Lang et al., 2006). Similarly, a clinical study demonstrated that administration of granisetron, a 5-$HT_3$ receptor antagonist, abolished the hyperalgesia induced by 5-HT injection into the masseter muscle of the jaw of healthy individuals (Ernberg et al., 2000).
Studies on the contribution of spinal 5-$HT_3$ receptor mechanisms to the pain process have yielded contradictory and unclear results. Although 5-$HT_3$ is originally excitatory, the presence of this receptor on inhibitory GABAergic interneurons causes its activation to also result in a net inhibitory effect on spinal nociceptive neurotransmission. Indeed, electrophysiological studies report inhibitory effects of 5-$HT_3$ receptors (Peng et al., 1996, 2001, Liu et al., 2006).
In a healthy adult mouse, in detail, spinal 5-$HT_3$ agonists produce antinociceptive behaviors (Alhaider et al., 1991, Paul et al., 2001), while spinal 5-$HT_3$ antagonists or 5-HT3 receptor knock-down -HT3 increase sensitivity to nociceptive stimuli and reduce the inhibitory effects of exogenous 5-HT (Scott et al., 2006, Alhaider et al., 1996)
Alhaider et al., 1991, demonstrated that this inhibitory modulation is mediated by γ-aminobutyric acid (GABA).
Thus, 5-HT3 receptor activation involves the inhibitory GABAergic interneuron (ININ) pathway (Fig.14). In addition to a large amount of evidence showing inhibitory effects of 5-$HT_3$ receptor activation (via GABAergic interneurons), activation of this receptor has also been shown to result in pronociceptive modes of action (Bee et al., 2008 , Patel et al., 2018).
This facilitative effect of 5-HT3 receptors can be explained by its expression on primary afferent terminals, excitatory interneurons (via EXIN) and/or projection neurons (Conte et al., 2005, Maxwell et al., 2003, Kidd et al., 1993, Huang et al., 2008).
Several recent studies have demonstrated that 5-$HT_3$ receptor-mediated facilitation of spinal activity is prevalent in central pain and chronic pain models.
Indeed, it has been demonstrated that nociceptive responses to thermal mechanisms and stimuli are similar to those of wild mice, but the behavior of the second phase in the formalin test (i.e. the phase involving centrally mediated mechanisms) is significantly modified, presumably for the involvement of 5-$HT_3$ receptors, whose levels in the spinal cord increase in injury and pain conditions (Kayser et al., 2007; Zeitz et al., 2002). This is in line with behavioral studies showing that intrathecally administered 5-$HT_3$ receptor antagonists reduce only the second phase of formalin-induced nociceptive behavior (Okamoto et al., 2004). Furthermore, administration of the antagonist ondansetron in the formalin assay reduced spinal levels of extracellular kinase-mediated phosphorylation signals, providing evidence that 5-$HT_3$ receptor activation contributes to sensitization of spinal cord neurons (Svensson et al., 2006 ). Furthermore, thanks to in vivo electrophysiological studies, Suzuki et al. 2004b reported that blockade of spinal 5-$HT_3$ receptors with ondansetron strongly inhibited mechanical and, to a lesser extent, heat-induced neuronal responses in the deep laminae (V–VI) in rats after NLS at 14 days post injury, compared with controls.
In behavioral pharmacological studies, acute (Oatway et al., 2004) or chronic (Chen et al., 2009) intrathecal administration of ondansetron produced robust long-term reductions in mechanical allodynia in central neuropathic pain due to SCI, while administrations of the selective serotonin receptor subgroup 3 agonist, m-CPBG, increased allodynia. Ondansetron also induces antiallodynia

or analgesic effects in a spinal nerve injury model (Dogrul et al., 2009), in a rat model of cancer-induced bone pain (Donovan-Rodriguez et al., 2006), in a rat of osteoarthritic pain (Rahman et al., 2009) and reversed opioid-induced chronic hyperalgesia (Vera-Portocarrero et al., 2007).
However, a very recent study by Peters et al. (2010) demonstrated that spinal administration of ondansetron or dolasetron, between 14 and 30 days after L5/6 SNL in rats, failed to reduce mechanical or thermal hypersensitivity. Furthermore, using immunohistochemical and biochemical approaches, spinal serotonin content, density of serotonergic terminals, and 5-$HT_3$ receptor distribution were found to be unaffected after SNL. Hence, suprathreshold or more intense stimuli may be required to recruit descending serotonergic facilitator pathways (Peters et al., 2010). However, the functional anatomy and pharmacology of descending facilitatory pathways mediated by the spinal 5-$HT_3$ receptor is still not fully understood.
All of these results suggest that an integrative sensory pathway that participates in descending facilitatory mechanisms and hyperalgesia involves the release of spinal 5-HT and activation of 5-$HT_3$ receptors
However, further studies focusing on the interaction of spinal 5-$HT_3$ receptors with GABA, substance P and glutamate on the initiation and maintenance of descending facilitation leading to behavioral hyperalgesia are needed. But, clinical data appear to confirm the potential analgesic effects of the 5-$HT_3$ antagonist.
All these results suggested that, clearly, periferic 5-HT contributes to peripheral sensitization through receptor-mediated mechanisms 5-$HT_3$.
Conversely, spinal GABA contributed to the anti-hyperalgesia by intrathecal administration of 5-$HT_3$ receptor agonists. As for the different results with the same drugs in the same models, it might because the different dosage of the drugs induced different banding rates of the receptors, which might trigger different subsequent signal cascades (**Fig 18**).
However, further studies focusing on the interaction of spinal 5-$HT_3$ receptors with GABA, substance P and glutamate on the initiation and maintenance of descending facilitation leading to behavioral hyperalgesia are needed. But, clinical data appear to confirm the potential analgesic effects of the 5-$HT_3$ antagonist.

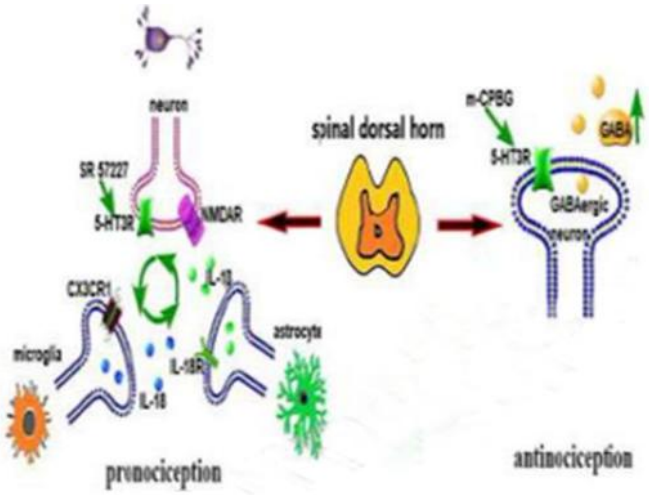



**Fig. 18**
Possible mechanisms of 5-$HT_3$ receptor in neuropathic pain. 5-$HT_3$ receptor is the only one that is a ligand-gated cation channel, and its activation generates pro- and anti-nociceptive effects. The activation of 5-$HT_3$ receptors in spinal level by SR 57227 could induce neuronal hyperexcitability, glial hyperactivity and pain hypersensitivity by promoting the neuron-to-microglia interactions via chemokine fractalkines like IL-18 and IL-1β. While 5-$HT_3$ receptor agonist m-chlorophenylbiguanide (m-CPBG) which reduced hypersensitivity and increased spinal gama-aminobutrylacid (GABA) release in spinal nerve ligation (SNL) rats.

## 5-HT$_7$ receptor

The 5-HT receptor gene 7 is located on human chromosome 10q23.3-q24.3 with an open reading frame containing 1335 base pairs and encoding a protein of 445 amino acids (Bard et al., 1993).

So far, three 5-HT 7 receptor splice variants have been identified in humans, including 5-HT $_{7(a)}$, 5-HT $_{7(b)}$, 5-HT $_{7(d)}$, three in the mouse - 5 - HT $_{7(a)}$, 5-HT $_{7(b}$), 5-HT $_{7(d)}$ and four in rat - 5-HT $_{7(a)}$, 5-HT $_{7(b)}$, 5-HT $_{7(c)}$ , 5-HT $_{7(e)}$ ( Heidmann et al., 1997 ; Liu et al., 2001).

These splice variants differ only in their short sequence of carboxy-terminal amino acids. The receptor isoforms have altered tissue distribution patterns, while no difference in their pharmacological properties and coupling to ACs was observed (Heidmann et al., 1997, 1998; Krobert et al., 2001). The human 5-HT $_{7(d)}$ receptor represents an exception, as this isoform possesses a differential pattern of receptor internalization that may influence receptor-mediated signaling (Guthrie et al., 2005). In this regard, the 5-HT $_{7(d)}$ receptor was constitutively internalized in the absence of agonist suggesting that its carboxy-terminal tail, which is the longest of the known human 5-HT$_7$ receptor isoforms, may contain a motif which interacts with cellular transport machinery that is distinct from 5-HT $_{7(a)}$ and 5-HT $_{7(b)}$ receptors, **Fig. 19**

The functional consequences of the individual splice variants are not clear, although all of them seem to be able to stimulate AC (Jasper et al., 1997; Stam et al., 1997; Heidmann et al., 1998). However, because the different variants may have differing numbers of phosphorylation sites, their regulation by kinases may be different.

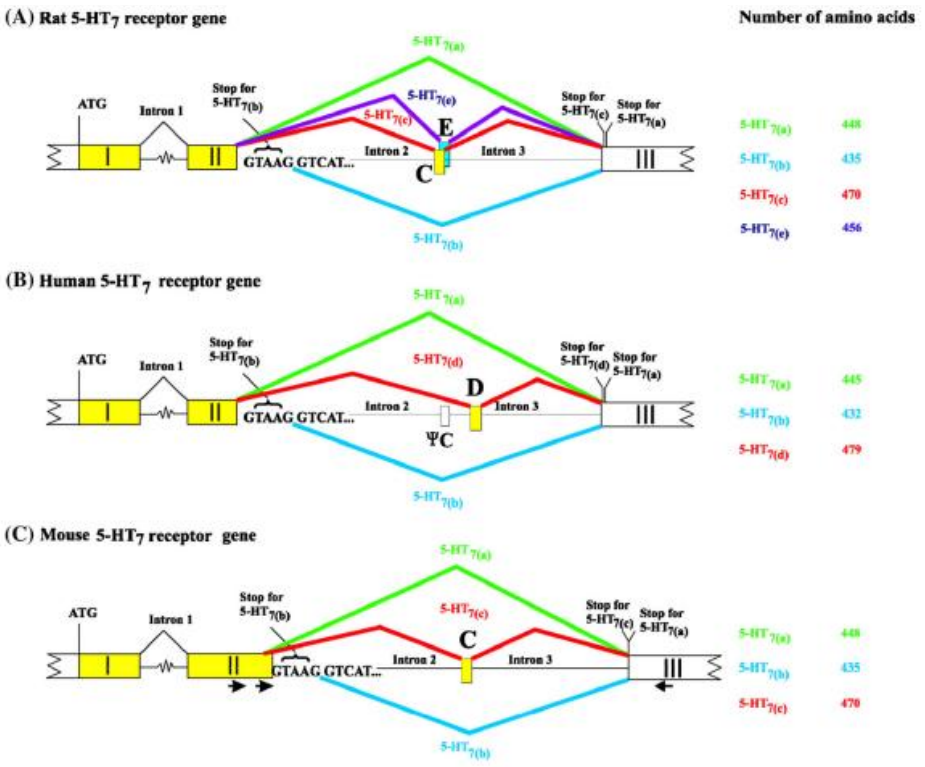



**Fig. 19**

1. Schematic overview of the splicing process leading to different rat (A) human (B) and mouse (C) 5-HT7 receptor mRNA. Exons I, II, III, C, ψC, D and E are indicated by boxes. Those that code for 5-HT7 splice variants are shown in grey. (C) Primers used to clone the different mouse 5-HT7 splice variants are indicated by arrows: OFP = outer forward primer, IFP =inner forward primer and RP = reverse primer. Exon I consists of 549 bp, exon II of 755 bp, exon C of 97 bp and exon III of 43 bp. The introns contain 86902 bp (intron 1), 4832 bp (intron 2) and 3907 bp (intron 3).

The 5-HT receptor 7 is canonically coupled to the stimulatory protein Gs which in turn can activate several AC isoforms (Shen et al., 1993). ACs exhibit unique tissue distribution as well as regulatory properties ( Krupinski et al., 1989 ; Bakalyar and Reed, 1990 ; Premont et al., 1996 ) Coupling between the 5-HT 7 receptor and G protein s results in an increase of AC activity leading to the production of cAMP, which in turn activates protein kinase A (PKA) thereby inducing the phosphorylation of several target proteins (**Fig 20**). This results in the activation of multiple downstream signaling cascades, including ERK and AKT.
Non-canonical signaling pathway of 5-$HT_7$R acts via Gα12 (Guseva et al. 2014). This leads to the activation of Rho, Rac, and cell division control protein 42 (Cdc42) all part of the Rho family of small GTPases, which in neurons promote dendrite sprouting, formation of filopodia, and synaptogenesis (Kobe et al. 2012a; Speranza et al. 2013; Speranza et al. 2015; Marin and Dityatev 2017). Of relevance, Trkb expression (a brain-derived neurotrophic factor (BDNF) receptor) appears to be enhanced by both Gαs and Gα12 (Fig. 19) (Samarajeewa et al. 2014).
These signaling pathways may be of therapeutic relevance for neurodegenerative diseases, although few studies have so far evaluated these effects (Hashemi-Firouzi et al. 2017; Costa et al. 2018; Quintero-Villegas et al. 2019).

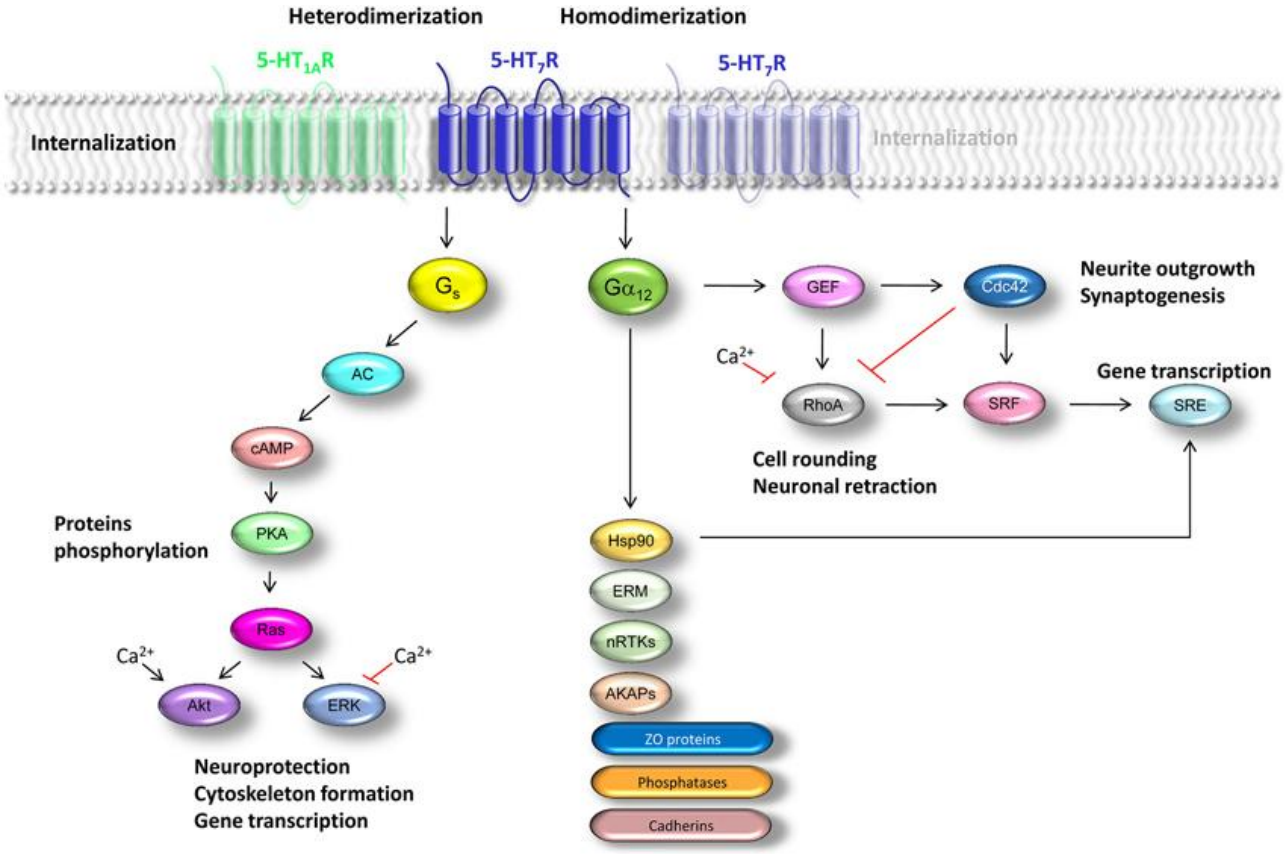



**Fig. 20**
5-$HT_7$ and 5-$HT_{1A}$ receptor signaling pathways and oligo/heterodimer formation. 5-$HT_7$ receptor monomers (in yellow) can form homodimers or homoligomers, with the same signaling pathways and cellular effects. 5-$HT_7$ can also form heterodimers or heteroligomers with 5-$HT_{1A}$ (in teal), resulting in the inhibition of the 5-$HT_{1A}$ signaling pathway, with no net effect downstream of 5-$HT_7$. When activated, 5-$HT_7$ activates Gas (canonical pathway) with a subsequent signaling cascade that results in the activation of ERK (also known as MAPK) and Akt; in contrast, the activation of Ga12 activates mTOR and different Rho family small GTPases. As illustrated, the phosphorylation of Trkb is mediated by both G proteins. *AC* adenylate cyclase, *cAMP* cyclic adenosine monophosphate, *Cdc42* cell division control protein 42 homolog, *ERK* extracellular signal-regulated kinases, *MAPK* mitogen-activated protein kinases, *mTOR* mammalian target of rapamycin, *Trkb* Tropomyosin receptor kinase B

By the combined application of biochemical and biophysical approaches it has recently been shown that 5-$HT_7$ receptors can form heterodimers with 5-$HT_{1A}$ receptors both in vitro and in vivo (Renner et al., 2012), (**Fig 20**).
Functionally, heterodimerization decreases 5-$HT_{1A}$ receptor G protein i coupling and attenuates receptor-mediated activation of G-protein-dependent potassium channels (GIRK), without substantial changes in coupling of the 5-HT receptor 7 to G proteins. Furthermore, heterodimerization greatly facilitates 5-$HT_{1A}$ receptor internalization, whereas the 5-HT7 receptor internalization kinetics decelerates after heterodimerization (Renner et al., 2012). Overall, the dimerization mechanism represents an important receptor regulation mechanism for the functionality of the receptors themselves.
Functional analysis demonstrated that they are associated with a number of physiological responses including serotonin-induced phase shift of the circadian rhythm, locomotor and exploratory activity. But they are mainly associated with limbic brain divisions that receive serotonergic inputs (e.g., the hippocampus, amygdaloid complex, or mammillary nuclei) (Hedlund and Sutcliffe, 2004; Ballaz et al., 2007; Eriksson et al., 2008; Gasbarri et al. al., 2008; Hedlund, 2009). This suggests that 5-$HT_7$ receptors are involved in sleep induction and hypothermia, learning, mood, and neuroendocrine or vegetative behaviors, observations also confirmed in a mouse strain with a 5-$HT_7$ gene discontinued (Hoyer et al., 1994, Hedlund et al., 2003).
Furthermore, a large body of evidence indicates the involvement of the 5-$HT_7$ receptor in several neurological diseases (Hedlund & Sutcliffe, 2004; Thomas & Hagan, 2004).
Indeed, mRNA expression level analysis revealed that the amount of 5-HT 7 gene transcripts in the dorsolateral prefrontal cortex of schizophrenic patients increases, demonstrating that the 5-$HT_7$ receptor may be linked to schizophrenia (East et al. al., 2002; Pouzet et al., 2002; Ikeda et al., 2006). In addition, pharmacological blockade or knock-down of the 5-$HT_7$ receptor has been shown to induce antidepressant-like behavior in animal models (Guscott et al., 2005; Hedlund et al., 2005; Wesołowska et al., 2007). Furthermore, some antidepressants may act directly on the 5-$HT_7$ receptor (Mullins et al., 1999), suggesting this receptor as a novel target for the treatment of depression (Hedlund, 2009; Mnie-Filali et al., 2009).

In relation to the multiple roles of 5-$HT_7$, an increasing number of studies have described the distribution of the 5-$HT_7$ receptor in various animal models using immunohistochemical techniques (Bickmeyer et al., 2002, Muneoka et al., 2003).
These reports have shown that, in the central nervous system, the distribution of proteins is similar to that of mRNA, with the greatest abundance in the spinal cord (Dogrul and Seyrek, 2006), thalamus, hypothalamus, hippocampus, prefrontal cortex, and amygdala where it is expressed in both neurons and glial cells (Hedlund and Sutcliffe, 2004; Thomas and Hagan, 2004; Russo et al., 2005), but significant density was also observed in the raphe nuclei area. In contrast, the expression level of the receptor detected in the putamen and cerebellum is relatively low (Horisawa et al., 2013). **Fig 21**

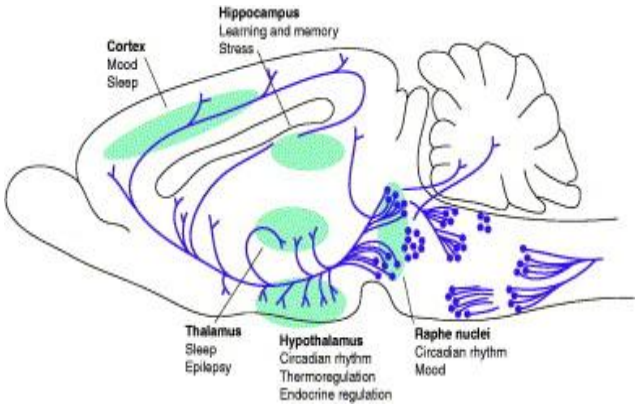



**Fig. 21**
A sagittal view of the rodent brain, showing the 5-HT-producing neurons of the brain stem with their ascending and descending projections (purple). Regions that are relatively rich in 5-HT7 receptor expression (green) and their putative correlation with 5-HT7 receptor-mediated functions are indicated.

Recent studies, show that spinal, peripheral and central 5-$HT_7$ receptors have role in pain information, but the effect of 5-$HT_7$ receptor on the process is known little so far.
Although the 5-$HT_7$ receptor does not seem to play a major role in the modulation of nociceptive transmission, the 5-$HT_7$ receptor produced antinociception in various neuropathic pain models.

In the anterior cingulate cortex (ACC) of CNS, the changes of its neuronal activity are thought to be a reason for the development of neuropathic pain (Baliki MN et al, 2012). Santello and Nevian (2015) adopted a targeted injection into the ACC with a 5-$HT_7$ receptor agonist, and found that specific activation of the 5-$HT_7$ receptors in ACC could increase function of hyperpolarization-activated cyclic-nucleotide-modulated cation (HCN) channel and restore the dendritic integration via adenylate cyclase, then alleviate the neuropathic pain in nerve-injured models (**Fig. 22**).

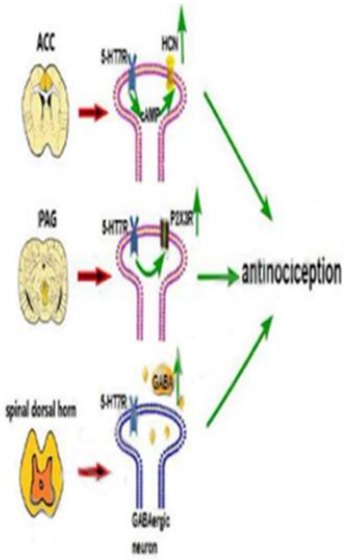



**Fig. 22**
Possible mechanisms of 5-HT7 receptor in neuropathic pain. 5-HT7 is coupled to G protein, activation of the 5-HT7 receptors in anterior cingulate cortex (ACC) could increase the function of hyperpolarization-activated cyclic-nucleotide-modulated cation (HCN) channel and restore the dendritic integration via adenylate cyclase and alleviate the neuropathic pain. The interaction of 5-HT7 receptor and P2X3 receptor in the ventrolateral midbrain periaqueductal gray (PAG) was involved in the antinociception of 5-HT7 receptor. 5-HT7 receptor agonists exerted the analgesic effects partially by the activation of inhibitory GABAergic interneurons in spinal

The appearing of new 5-$HT_7$ receptor agonists which are brain-penetrant helps to make the pathophysiological role of this receptor clearer. In another study of Santello et al. (2012), a new brain penetrant 5-HT7 receptor agonist LP-211 (Adriani W et al., 2012, Hedlund PB et al., 2010, Leopoldo M et al., 2008) was used to modulate neuronal activity and to investigate the possible antinociceptive effect on neuropathic pain. They suggested that LP-211 presented an antiallodynic effect by passing the blood brain barrier and acting in 5-$HT_7$ receptors located in ACC. These results shed light on the promising pain-relieving effect of 5-$HT_7$ receptors in ACC, and provided a novel treatment strategy for neuropathic pain.
5-$HT_7$ receptors are expressed wealthily in the PAG, involving somatic and autonomic responses to nociceptive stimuli, (Martin-Cora et al., 2004).
Li et al., (2014) demonstrated that the 5-$HT_7$ receptor agonist AS-19 provided a dose-dependent anti-hyperalgesia effect when microinjected into the PAG in CCI model, and pretreatment with SB-269970, a selective 5-$HT_7$ receptor antagonist could reverse this effect. They also found that the interaction of 5-$HT_7$ receptor and P2X3 receptor in the PAG was involved in the antinociceptive effect. In the study of Xu, et al., (2013), SB-269970 was used to test the role of 5-$HT_7$ receptor located in ventrolateral orbital cortex, VLO, in the anti-allodynia function of 5-$HT_7$. By microinjecting into VLO, SB-269970 could attenuated the 5-HT-induced inhibition of allodynia. These data showed that the 5-$HT_7$ receptors located in PAG and VOL also participated in the descending inhibition of the process of neuropathic pain.

In the spinal cord, the 5-$HT_7$ receptor is found principally in the superficial laminae of the dorsal horn, postsynaptically in local interneurons, presynaptically in peptidergic fibers (including presumably primary afferents) and in astrocytes (Meuser et al., 2002; Doly et al., 2005), while in the periphery, 5-$HT_7$ receptors have been found in rat and in human DRG (Pierce et al., 1996, 1997; Doly et al., 2005).

The first available electrophysiological, immunohistochemical, and behavioral data in the literature suggest a pronociceptive role of peripheral and spinal 5-$HT_7$ receptors in dorsal horn of spinal cord.
Infact, intraplantar or spinal administration of 5-carboxamidotryptamine, a nonselective 5-$HT_7$ receptor agonist, increased formalin-induced nociceptive behavior and these effects were significantly reduced by SB-269 970, a selective 5-$HT_7$ receptor antagonist (Rocha-Gonzalez et al., 2005).
Nevertheless, the most convincing data were obtained by Brenchat et al. (2009).
Brenchat et al. (2008) evaluated the potential role of the 5-$HT_7$ receptor in nociception associated with a sensitizing stimulus in mice; intrinsic efficacy as an activator of human 5-$HT_7$ receptors and the selectivity of 5-$HT_7$ receptor agonists used were also investigated.
They reported that subcutaneous administration of AS-19, MSD-5a, and E-55 888, all 5-$HT_7$ agonists, exerted a clear-cut and dose-dependent inhibition of capsaicin-induced mechanical allodynia in mice, which was blocked by coadministration of two different 5-$HT_7$ receptor antagonists (SB-258 719 and SB-269 970). In contrast, coadministration with the selective 5-$HT_{1A}$ receptor antagonist, WAY-100 635, was unable to reverse the effect of AS-19. The rank order of efficacy of these agonists was correlated with their invitro efficacy (Brenchat et al., 2009). Using the same 5-$HT_7$ receptor ligands, Bourgoin et al. (2008) have reported that acute administration of these compounds induced long-lasting antihyperalgesic effects in rats with CCI
Subsequently, Brenchat et al, (2010), examined also whether 5-$HT_7$ receptors participates in some modulatory control of nerve injury-evoked mechanical hypersensitivity and thermal hyperalgesia in mice. They found a significant increase of 5-$HT_7$ immunoreactivity in laminae I–II and III–V of the dorsal horn on the ipsilateral side of the spinal cord after nerve injury. In add, the authors found that acute or repeated administration of AS-19 or E-57 431, a new highly selective potent 5-$HT_7$ receptor agonist, induced a clear-cut inhibition of mechanical allodynia and thermal hyperalgesia reducing mechanical hypersensitivity in nerve-injured mice, suggesting that 5-$HT_7$ receptors play an antinociceptive role.
The 5-$HT_7$ receptors also play roles in the antinociceptive effects of some medicines. Dogrul et al, 2012 explored the relative contribution of spinal 5-$HT_7$ receptors to the antiallodynic effects of morphine. Their results suggested that the inhibition of the spinal 5-$HT_7$ receptors by SB-269970 could block the antiallodynic effects of morphine, which is consist with that the activation of spinal 5-$HT_7$ receptors participates in the antinociceptive effect induced by morphine.
Nefopam is widely used in European countries as a non-opioid analgesic for postoperative pain, Evans et al., 2008. The pharmacological properties of Nefopam is different from those of opioids and anti-inflammatory drugs, (Conway et al., 1997).
Dam et al., 2014, adopted intrathecal injection of SB-269970 and the pain-relieving effect of Nefopam was attenuated, which suggested that the activation of the spinal 5-$HT_7$ receptor mediates the antinociceptive effect of Nefopam. Recently, many uncommon analgesics have been used in studies of neuropathic pain. For example, Fisetin was reported to be anti-allergic, (Cheong et al., 1998), cancer chemo-preventive, Ravinchandar et al., 2011, and neuroprotective, Patel et al., 2012..
In the study by Zhao et al., 2015, chronic administration of Fisetin in CCI models produced an anti-hyperalgesia effect on heat stimuli. They believed that the anti-hyperalgesia effect of Fisetin might be mediated by the activation of spinal 5-$HT_7$ receptors, which was congruent with recent studies reporting the engagement of 5-$HT_7$ receptors in neuropathic pain, (Brenchat et al., 2012, Viguer et al., 2012). Areca nut is a traditional herbal medicine peng et al., 2015. Previous studies have

showed that Areca nut had antinociceptive activity in some pain animal models, (Bhandare et al., 2010).
A study by Lee et al, 2018, showed that pre-administration with 5-$HT_7$ receptor antagonists could attenuate the antinociceptive effect of Areca nut. Zhao et al., 2014, explored the anti-hyperalgesia effect of Resveratrol in CCI models. Chronic treatment with Resveratrol could reduce the thermal hyperalgesia, and SB-258719 could counteract this effect. These studies demonstrated that the activation of spinal 5-$HT_7$ receptor paly an essential role in the pain-relieving effect of Fisetin, Resveratrol and Areca nut.
Contrariwise, Amaya-Castellanos et al., 2011, suggested that the activation of spinal 5-$HT_7$ receptors exerted a pronociceptive effect in neuropathic rats. In their study, the results showed that systemic or spinal treatment with SB-269970 could reduce tactile allodynia in a dose-dependent fashion in L5/L6 SNL models.
In add, chronic pain inflammation is also accompanied by a strong upregulation of 5-HT7 receptor in the DRG of rats after bee venom-induced (Liu et al., 2005) and Complete Freund's Adjuvant-induced inflammation (Wu et al., 2001).
The respective opposite roles of peripheral and spinal 5-$HT_7$ receptors in the modulation of mechanical hypersensitivity were investigated under two different experimental pain conditions by Brenchat et al. (2012); they demonstrated that activation of 5-$HT_7$ receptors exerts antinociceptive effects at the spinal cord level and pronociceptive effects at the periphery.
A previous study, Brenchat reports also that 5-$HT_7$ receptors co-localize with GABA in neurons of the spinal-cord dorsal horn (2010).
In another study by Viguier et al., (2012), a CCI model was adopted to examine whether GABA participated in the antinociceptive effect induced by the activation of 5-$HT_7$ receptors. After administering the receptor agonists AS-19, MSD-5a and E-55888, the mechanical and thermal hyperalgesia were reduced markedly.
Blocking of the spinal GABAA receptor by bicuculline could prevent the antinociceptive effect of these agonists, which suggested that the activation of GABAA receptor may be a downstream signal pathway of the activation of 5-$HT_7$ receptor in pain-relieving effect (Fig. 20), in line with the conclusion by Brenchat et al., (2010).
These data is really important. It is possible to note infact that these receptors are positively coupled to adenylate cyclase and that their stimulation is excitatory (Hannon and Hoyer, 2008). Therefore, a direct inhibitory effect on primary afferents or nociceptive dorsal horn neurons cannot be possible. Rather, an indirect action through inhibitory enkephalinergic or GABAergic interneurons would explain their effects on nociceptive transmission.
In summary, 5-$HT_7$ receptors seem to be involved in nociceptive inhibition upon injury. However, one study suggests a facilitatory and pronociceptive effect of 5-$HT_7$ receptors in chronic neuropathic pain. In SNL animals antinociceptive effects of spinal and systemic 5-$HT_7$ inhibition and a reduced 5-$HT_7$ protein content in DH tissue ipsilateral to injury was shown. The complexity of the role of 5-$HT_7$ receptors in neuropathic pain can be attribute to differences in receptor expression and/or protein content as increased 5-$HT_7$ expression was involved in inhibitory antinociceptive effects and the facilitatory pronociceptive effect was observed with reduced 5-$HT_7$ protein content, different gender (male versus female), species (rats versus mice), model types (L5/L6 spinal nerve injury versus partial sciatic nerve ligation) and primary administration route (spinal versus systemic). Though most studies showed an antinociceptive effect of the activation of spinal 5-$HT_7$ receptor on neuropathic pain, in order to confirm the effect, more studies are still needed in the future.
All these results support the involvement of the 5-$HT_7$ receptor subtype in pain control and point to a new potential use of 5-$HT_7$ receptor agonists for the treatment of pain with central sensitization. However, further studies focusing on the site and mechanism of action of 5-$HT_7$ agonists as well as on their effects in chronic inflammatory pain models are needed to confirm this interesting approach.

On the basis of the evidence, peripheral 5-HT clearly contributes to peripheral sensitization through direct or indirect mechanisms (**Fig. 23**).

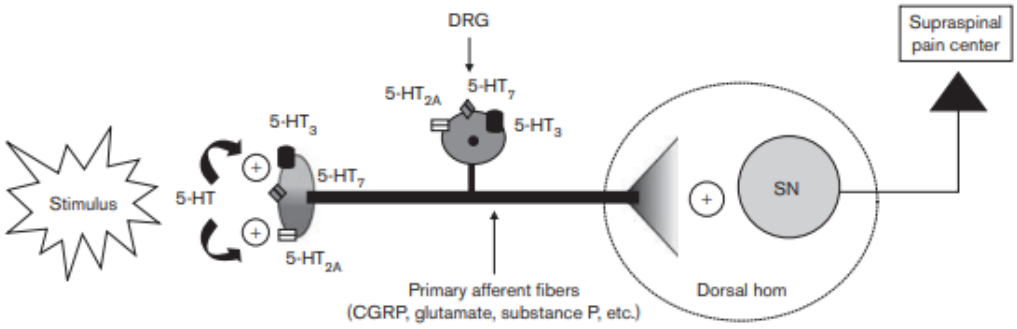



**Fig. 23**
Schematic representation of the involvement of 5-hydroxytryptamine (5-HT) receptors in the generation of nociceptive messages at the periphery. Tissue injury induces release of 5-HT from platelets, mast cells, and endothelial cells, thereby activating excitatory 5-HT2A, 5-HT3, and 5-HT7 receptors on nociceptors. This contributes to activation or sensitization of primary afferent fibers by direct (tetrodotoxin-resistant Na + current, etc.) or indirect (calcitonin gene-related peptide, prostaglandin E2, substance P, etc.) mechanisms, ending with pain sensation through message transfer to supraspinal center through spinothalamic neurons (SN) (see text). DRG, dorsal root ganglia.

In contrast, and depending on acute or chronic pain states, the descending 5-HT pathways can exert both an inhibitory or facilitatory influence, **Fig. 24**

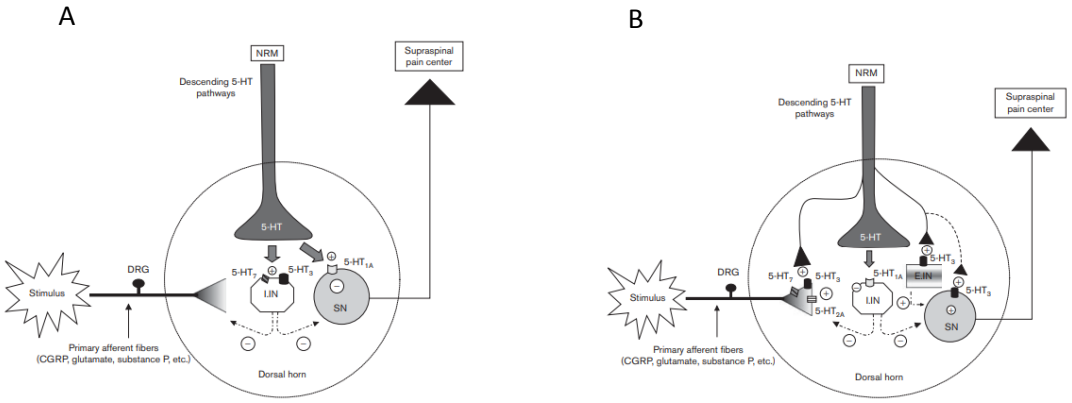



**Fig. 24**
Schematic representation of the inhibitory (A), or facilitatory, (B), influence exerted by 5-hydroxytryptamine (5-HT) descending pathways in the dorsal horn of the spinal cord.
(A). 5-HT, released from descending bulbospinal projections [particularly from the nucleus raphe magnus (NRM)], interacts with postsynaptic 5-HT receptors expressed by inhibitory interneurons (I.IN) and spinothalamic neurons (SN). Through direct stimulation of 5-HT1A receptors on SN, or indirectly by activation of 5-HT3 and 5-HT7 receptors located in I.IN (GABAergic, opioidergic), 5-HT exerts inhibitory (Y) control on pain transmission.
(B). 5-HT, released from descending bulbospinal projections [particularly from the nucleus raphe magnus (NRM)], interacts with 5-HT receptors located presynaptically on the terminals of primary afferents fibers, and postsynaptically with 5-HT receptors expressed by both inhibitory (I.IN) or excitatory interneurons (E.IN) and spinothalamic neurons (SN). Through direct stimulation of 5-HT2A, 5-HT3, and 5-HT7 receptors on the terminals of primary afferents fibers or of 5-HT3 receptors located in SN, 5-HT exerts facilitatory (') influence on pain transmission.
5-HT can also act indirectly on 5-HT1A receptors located in I.IN or on 5-HT3 receptors located in E.IN to facilitate (') pain transmission. CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia.

The balance between facilatation and inhibition of 5HT is important to define the shift from inhibitory to facilitatory mode of action and viceversa. The choise is related to both injury-induced changes in 5-HT content, changes in iupregulated expression of excitatory serotonergic receptors 5-$HT_{2a}$, 5-$HT_{2b}$ and 5-$HT_3$ receptors and and changes in functionality of spinal 5-$HT_{2a/c}$, 5-$HT2_b$, 5-$HT_3$, 5-$HT_4$ and 5-$HT_7$ receptors, **Fig. 25**.

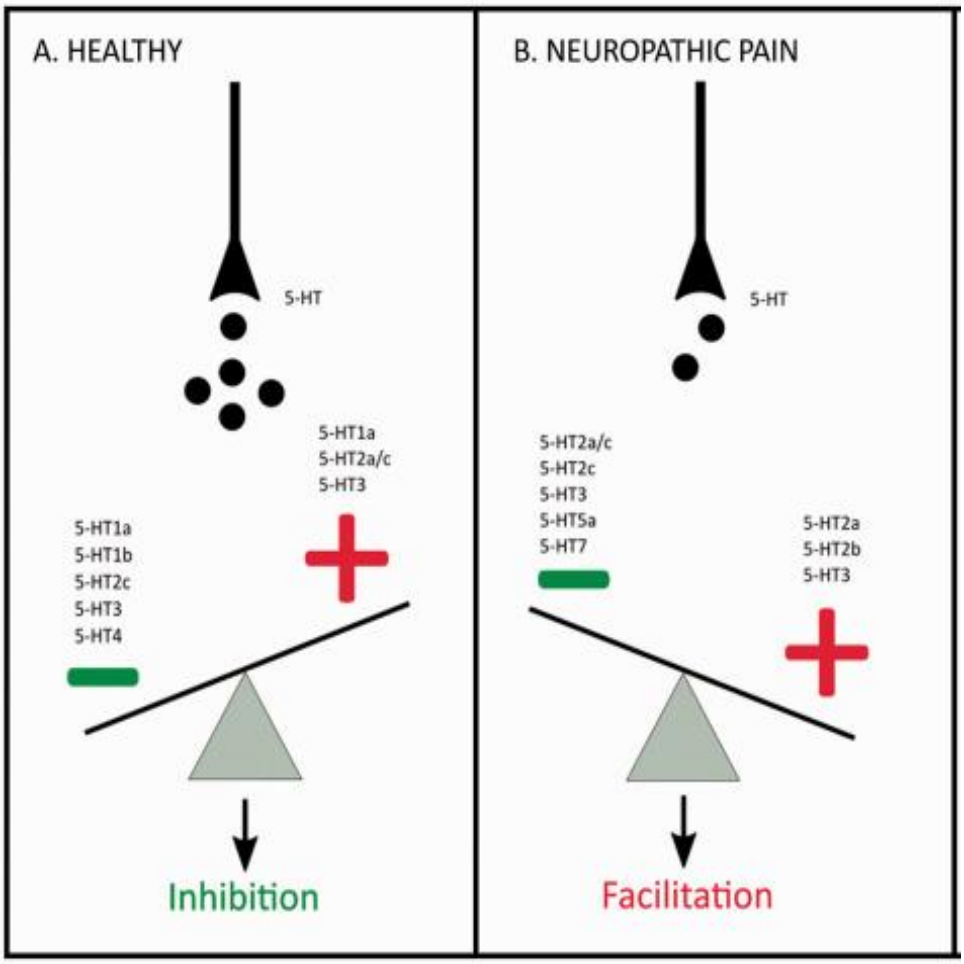



**Fig. 25**
Schematic overview of the effect of descending serotonergic modulation on nociceptive transmission in the DH and the involved receptors in the healthy (a) and neuropathic (b) rodent. Upon injury (b), 5-HT content in the DH is reduced and 5-HT1b and 5-HT4 receptors lose their inhibitory function. The – symbol means inhibitory mediation, the + symbol means facilitatory modulation.

# Chapter III. MATERIALS AND METHODS

## Reagents and Solutions

**Dissecting Krebs solution (high-sucrose Krebs)**

Prepare 500 ml of 10× low-sodium stock solution with the following composition:
950 mM NaCl
25 mM KCl
260 mM $NaHCO_3$
12.5 mM $NaH_2PO_4$ monohydrate
Store up to 1 week at 4°C

Prepare 500 ml dissecting solution by adding 8.55 g sucrose (50 mM), 2.25 g glucose (25 mM) and ∼400 ml distilled water to 50 ml of 10× low-sodium stock solution. Bubble the solution with carboxygen for ∼15 min, to avoid $CaCO_3$ precipitation. Add 3 ml of 1 M $MgCl_2$ stock (to 6 mM) and 0.75 ml of 1 M $CaCl_2$ stock (to 1.5 mM) and dilute to 500 ml. Add 90 mg kynurenic acid (to 1 mM) and sonicate for a few minutes to dissolve. Store in the freezer until ice cold and then use for dissection

*If the solution is saturated with $CO_2$, pH is ∼7.4. The use of high $Mg^{2+}$ and low $Ca^{2+}$, together with kynurenic acid, is intended to decrease synaptic activity This solution is used during spinal cord dissection and cutting, always bubbled with carboxygen.*

**Incubation Krebs solution**

Prepare 500 ml of 10× high-sodium stock solution of the following composition:
1250 mM NaCl
25 mM KCl
250 mM $NaHCO_3$
10 mM $NaH_2PO_4$ monohydrate
Store up to 1 week at 4°C On
Prepare 250 ml incubation solution by adding 1.125 g glucose (25 mM) and ∼200 ml distilled water to 25 ml of 10× high-sodium stock solution. Bubble with carboxygen for ∼15 min. Add 1.5 ml of 1 M $MgCl_2$ stock (to 6 mM) and 0.375 ml of 1 M $CaCl_2$ stock (to 1.5 mM) and dilute to 250 ml. Store no more than 2 days at 4°C.

*If the solution is saturated with $CO_2$, pH is ∼7.4. This solution is used for the incubation of the slices during the day (always bubbled with carboxygen) and to dissolve agarose for embedding.*

**Intracellular solution (potassium-based)**

Prepare 50 ml of solution by adding the following components (in mM) to ∼40 ml distilled water:
120 mM potassium methanesulfonate (Sigma-Aldrich, cat. no. 83000)
10 mM NaCl
10 mM EGTA (Sigma-Aldrich, cat. no. E4378)
10 mM HEPES (Sigma-Aldrich, cat. no H3375)
1 mM $CaCl_2$ (50 µl from 1 M stock)
0.5 mM NaGTP (Sigma-Aldrich, cat. no. G3776)
5 mM ATP-Mg (Sigma-Aldrich, cat. no. A9187)
Adjust pH to 7.2-7.3 with KOH (EGTA will dissolve at pH ∼7)
Dilute to 50 ml and make aliquots of 0.8-1 ml. Store 2-3 months at −20°C.

**Intracellular solution (cesium-based)**
Prepare 50 ml of solution by adding the following components (in mM) to ~40 ml distilled water:
130 mM cesium methanesulfonate,
10 mM sodium methanesulfonate,
10 mM EGTA (Sigma-Aldrich, cat. no. E4378)
1 $CaCl_2$ (50 µl from 1 M stock)
10 mM HEPES (Sigma-Aldrich, cat. no H3375)
2 mM ATP-Mg (Sigma-Aldrich, cat. no. A9187)
Adjust pH to 7.2-7.3 with CsOH (EGTA will dissolve at pH ~7)
Dilute to 50 ml and make aliquots of 0.8-1 ml. Store 2-3 months at −20°C.

*The osmolarity of this solution is ~300-305 mOsm/liter, without adding sucrose. On the day of the experiment, defrost one aliquot, place it in a 1-ml syringe with filter (see Materials), and keep the syringe on ice (to reduce nucleotide degradation). If patch-clamp recording is performed only in voltage-clamp mode (and especially if the cell is held at a depolarized potential), use a cesium-based solution, by substituting potassium methanesulfonate with cesium methanesulfonate (130 mM) and adjust the pH to 7.2 with CsOH. This solution is used to fill the electrodes for patch-clamp recording.*

**Recording Krebs solution**
Prepare 1 L recording solution by adding 4.5 g glucose (25 mM) and ~800 ml distilled water to 100 ml of 10× high-sodium stock solution (same as for the incubation solution).
Bubble with carboxygen for ~15 min.
Add 1 ml of 1 M MgCl2 stock (to 1 mM) and 2 ml of 1 M CaCl2 stock (to 2 mM) and dilute to 1000 ml.
Store for no more than 2 days at 4°C.

*If the solution is saturated with CO2, pH is ~7.4. Osmolarity is ~330 mOsm/l. This solution is used for extracellular and patch-clamp recording.*

**Spinal Cord Slice Preparation**
The Italian Ministry of Health approved all the experiments conducted on postnatal CD1 mice of either sex (P18–P28) following the Guide for the Care and Use of Laboratory Animals and the EU and Italian regulations on animal welfare. Spinal cord slices were obtained following the procedure described previously (Betelli et al., 2015; Bardoni et al., 2019).

The animals were anesthetized with isoflurane and decapitated.
We removed the dorsal portion of the skin corresponding to the vertebral column using surgical scissors. We cut through the rib cage and rapidly removed the lumbosacral segment of the spine, placing it in the dissection chamber, which was filled with ice-cold Krebs dissecting solution bubbled with carboxygen. This step should be as quick as possible because the spinal cord is not oxygenated during this process.
We performed ventral laminectomy, cutting the dorsal and ventral roots and isolating the spinal cord.
At this point, we fixed the isolated spinal cord to the bottom of the dissection chamber with fine pins. With the ventral side up, we removed the meninges (especially the dura), then, cut the ventral roots. With the dorsal side up, we removed the rest of the meninges and separated the dorsal roots from each other. The lumbar spinal cord was isolated and dried by Pasteur pipet. If the spinal cord is not sufficiently dry before the agarose is poured, it will not be embedded properly and the cord could be unstable during the cutting.
After embedded in a low melting point agarose (Thermo Fisher Scientific, Waltham, MA, United States), exposing the dorsal part towards the blade, we cut transverse slices at 400–500 µm

thickness, using the vibratome, (WPI, Sarasota, FL, United States), at minimum speed and high vibration frequency. With a glass Pasteur pipet, we transferred the slices in a becker containing oxygenated incubation Krebs' solution.
The slices mantained at 35°C for 20 min and then used for recording.
Slices are typically viable for 5-6 hours.

## Patch-Clamp Recording and Stimulation

### Background

The *in vitro* preparation of spinal cord slices has been used for electrophysiological and optical recordings for about three decades. The main challenge has been the characterization of synaptic circuits mediating sensory transmission in the dorsal horn. More recently, the possibility of selectively labeling specific neuron populations has provided very important information on the spinal processing of sensory information, permitting to identify several populations of inhibitory interneurons (mainly releasing GABA and/or glycine) and numerous excitatory interneurons.
Action-potential firing patterns seem to differ between inhibitory and excitatory interneurons in superficial dorsal horn: a large proportion of inhibitory interneurons tend to fire tonically in response to depolarizing current steps, whereas the delayed and phasic patterns seem to be more common among excitatory interneurons (Bardoni et al., 2019; Heinke et al., 2004; Punnakkal et al., 2014; Yasaka et al., 2010). Furthermore, inhibitory interneurons tend to be more excitable than excitatory neurons and differ in their expression levels of several postsynaptic receptors (Punnakkal et al., 2014; Yasaka et al., 2010). Although these differences are not absolute, they have proved useful for studying the physiological properties of the two different neuron populations.

Therefore, to distinguish the different neurons in the superficial dorsal horn lamina and to better characterize the response to the agonist drug, LP211, we analyze the neuronal firing properties, determine the firing patterns of the action potential, the rheobase (current required to evoke the minimum number of action potentials) and the action potential threshold (membrane potential at which the rising phase of the action potential changes slope; Fig. 1A)

The most represented firing patterns in dorsal horn lamina II are tonic, delayed, phasic, and single spike. Tonic firing neurons generate action potentials during the whole current step, delayed firing neurons start action potentials after a delay, phasic (or "initial firing") neurons fire only at the beginning of the step, and a single spike generates only one action potential (Fig.1A). The initial delay in the delayed pattern is mainly due to the potassium $I_A$ current. Keeping the membrane potential at negative values (for example, around −80 mV) prevents the inactivation of this current, revealing a larger proportion of delayed firing neurons (Fig.1D). At a membrane potential of −60 mV, many delayed firing neurons fire tonically (Bardoni et al., 2019; Yasaka, Tiong, Hughes, Riddell, & Todd, 2010).
To analyze the properties of the different firing patterns and distinguish between tonic and delayed firing neurons, we have to consider the following properties, in current-clamp mode:
a. Tonic firing neurons in superficial dorsal horn usually have lower rheobases and more negative action potential thresholds than delayed firing neurons, leading to more excitable membranes (Heinke et al., 2004; Punnakal et al., 2014)
b. The ratio between the initial delay and the average interspike interval (determined as the mean of intervals between the action potentials generated at 2× rheobase) is ∼1 for tonic firing neurons and >2 for delayed firing cells (Fig. 1B);
c. Tonic neurons fire a significantly higher number of action potentials than delayed firing neurons for the same amount of injected current (Fig. 1C).

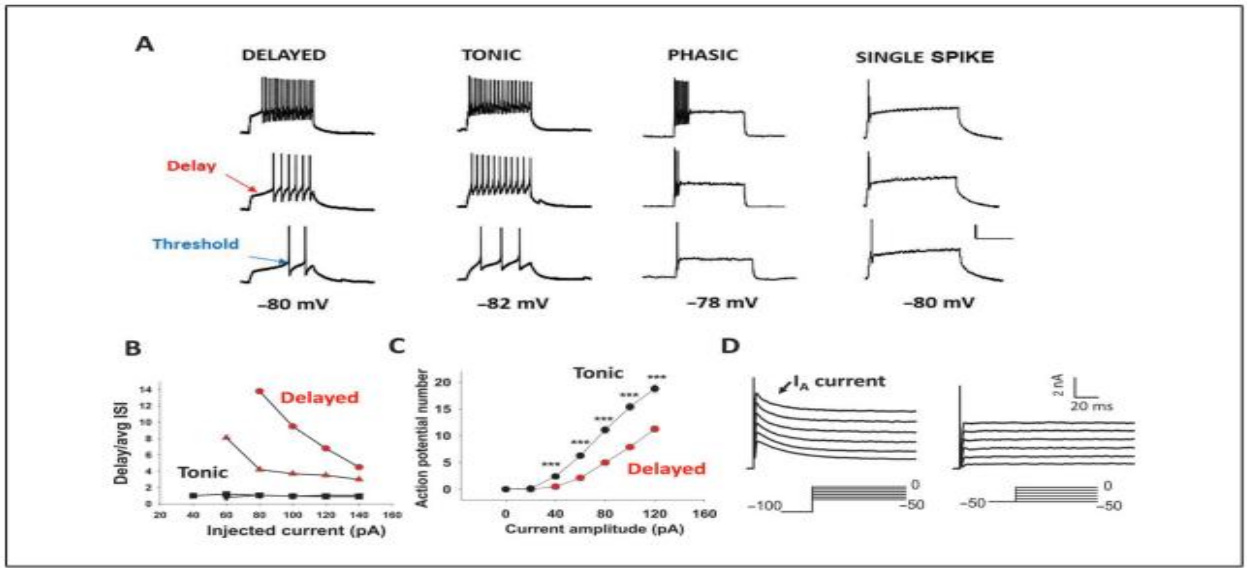



**Figure 1** Analysis of action potential firing patterns observed in lamina II neurons. (A) Examples of firing patterns (delayed, tonic, phasic, and single spike) recorded in different mouse lamina II neurons, in current-clamp mode. The three traces for each firing pattern represent (starting from the lower trace) response to the first current step able to induce action potentials (rheobase) and responses to two current steps above rheobase. The red arrow indicates the delay from the beginning of the current step to the first action potential; the blue arrow indicates the point where the action potential threshold is measured (the point of slope change in the rise phase). Scale bar: 20 mV, 100 ms. (B) Graph representing the ratio between the initial delay of firing and the average interval between spikes, as a function of the injected current. The data were obtained from two delayed (red symbols) and tonic firing (black symbols) lamina II neurons. (C) Average number of action potentials generated at different amplitudes of injected current by tonic and delayed neurons (10 neurons each). Tonic neurons fire a significantly higher number of action potentials at most current steps (unpaired t-test, $p < 0.001$). (D) Delayed firing neurons present the potassium IA current (arrow), which is activated in voltage clamp by holding the neurons at negative potentials (−100 mV, left) but is absent at more positive potentials (−50 mV, right). Panels A, B and D are reproduced with permission from Bardoni et al., 2019, licensed under the Creative Commons Attribution 4.0 International License.

The patch-clamp recording in whole-cell configuration was performed on lamina II neurons at room temperature. The slices were perfused at 2 ml/min with recording Krebs'solution (see solution previous paragraph). Recordings of excitatory postsynaptic currents (EPSCs) were performed in voltage-clamp at −70 mV by using a potassium-based intracellular solution (see solution previous paragraph). The data were recorded and acquired using a MultiClamp 700A Amplifier and the pClamp 10 software (Molecular Devices, Sunnyvale, CA, United States). The sampling rate was 10 kHz, and the data were filtered at 2 kHz. Series resistance was not compensated, and cells with a resistance higher than 30 MOhm were discarded. Junction potentials were corrected off-line. Inhibitory postsynaptic currents (IPSCs) were recorded in voltage-clamp at 0 mV by using a cesium-based intracellular solution

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Evoked EPSCs were obtained by stimulating the dorsal root attached to the slice with a suction electrode. The applied stimuli (500 μA intensity and 0.1 ms duration) activated both Aδ and C fibers synapsing onto lamina II neurons. Monosynaptic EPSCs were identified from the absence of failures during a train of 20 stimuli at 1 Hz (Daniele and MacDermott, 2009; Bardoni et al., 2019).

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Evoked IPSCs were elicited by focally stimulating the region around the recorded cell with a glass pipette (tip diameter 2–5 μm) at an intensity of 0.1–1 mA and duration of 0.1 ms. Monosynaptic IPSCs were recognized by constant latency and by the absence of failures at 1 Hz.

Single EPSCs or IPSCs were recorded by stimulating at 0.1 Hz. EPSCs or IPSCs recorded with the paired-pulse protocol were evoked by applying 2 stimuli at 400 ms intervals. The time between consecutive pairs of stimuli was 20–30 s.
Recordings in the current clamp were performed by using the potassium-based intracellular solution. Resting potential was determined within the first 5 min of recording: cells with membrane potential more positive than −50 mV were discarded. The neuronal firing pattern was determined by applying at least 10 current steps (amplitude: 10–20 pA; duration: 500 ms). Between the steps, the membrane potential was held at about −80 mV to unmask the delayed firing pattern better, as previously described (Yasaka et al., 2010; Bardoni et al., 2019). Once the firing pattern was established, root stimulation was performed as described above, using the paired-pulse protocol to record paired excitatory postsynaptic potentials (EPSPs). The neuron potential was maintained at −60/−65 mV.

**Drugs**
All the components of Krebs and intracellular solutions were obtained from Sigma-Aldrich (Merck Group, Darmstadt, Germany). LP-211 tartrate was provided by Prof. Marcello Leopoldo at the University of Bari (Italy). Aliquots of 1 μM stock solution in dimethyl sulfoxide (DMSO) were initially prepared and then diluted to 1 μM in recording Krebs' solution on the day of the experiment. NBQX, D-AP5, and tetrodotoxin (TTX) were provided by Abcam (Cambridge, United Kingdom), and WAY-100635 and SB-269970 were obtained from Sigma-Aldrich.

**Data analysis**
Spontaneous EPSCs or IPSCs were analyzed offline using pClamp10 software and Minianalysis (Synaptosoft, United States). The responsivity of individual cells to LP-211 was assessed by performing the Kolmogorov–Smirnov test on cumulative distributions of inter-event intervals

Evoked EPSCs and IPSCs were analyzed using pClamp10 software: sensitivity to LP-211 was established by comparing with an unpaired *t*-test, the peak amplitudes of 5–10 currents in control and in the presence of the 5-$HT_7$ agonist. Paired pulse ratio was determined as the ratio between the second and the first EPSC.

The data are expressed as the mean ± SEM, and differences were considered significant for $p < 0.05$. Comparisons between 2 groups were performed by using an unpaired or paired t-test. Non-parametric tests were applied when the data were not normally distributed. Graphs and statistical analysis were obtained by using GraphPad Prism 9.3 (GraphPad Software, San Diego, CA, United States).

## AIM OF PROJECT

Pain is necessary for our survival as it is a process that helps protect us from danger and it serves as a short-term response to resolve injury. However, pain that persists for longer than 3 months is considered to be a pathological state known as chronic pain.
Serotonin (5-HT, 5-hydroxytryptamine) has been widely related to pain modulation through peripheral and central actions. 5-HT acts on specific receptors that contribute to the maintenance of pain (Suzuki et al., 2004; Bannister and Dickenson, 2016, Bardoni et al., 2019).
Currently, there are seven families of 5-HT receptors and one of the most recently identified subtypes in 1993 is the 5-$HT_7$, 5-$HT_7$-R.
Immocytochemical studies dimostrate that receptor is localized on axon terminal and soma of interneurons of spinal cord, but the controversal role played by 5-$HT_7$-R in nociception has been poorly or not thoroughly investigated, specially the role regarding the control of synaptic inhibition.

The goal of our project is to identify micro-circuitries regulated by 5-$HT_7$ receptors in superficial dorsal horn, involved mainly in pain modulation.

Therefore, we test the new agonist of 5-$HT_7$-R, LP211, never used on spinal cord, to activate in specific-manner 5-$HT_7$-R and to record both spontaneous glutamatergic (**point 1**) and GABAergic (**point 2**) electrical activity by performing patch-clamp recordings from lamina II neurons in mouse spinal cord slices.

Subsequently, we record excitatory (EPSCs) (**point 3**) and inhibitory (**point 4**) postsynaptic currents evoked by dorsal root stimulation.

Because in dorsal horn the interaction between inhibitory and excitatory neurons is key to define the final output of pain, ascending in CNS, it is very important to characterize the contribute of 5-$HT_7$-R on GABA-ergic and glutamatergic neurons

# Chapter IV. RESULTS

**LP-211 increases spontaneous sEPSC in a subpopulation of lamina II neurons**

Given the prominent role that 5-HT plays in the modulation of synaptic transmission and the several lines of evidence about the involvement of 5-$HT_7$ receptors (5-$HT_7$Rs) in pain control at spinal cord level, we sought to evaluate the role of these receptors in modulating synaptic transmission in the superficial dorsal horn.

As such, we have bath-applied the selective agonist LP-211 on mouse spinal cord slices at two different concentrations (0.1 μM and 1 μM) for 3–5 min and we have recorded excitatory or inhibitory postsynaptic currents (EPSCs or IPSCs) from a total of 109 lamina II neurons.

Although Costa et al (2012) demonstrated that LP-211 was active at nanomolar concentrations in hippocampal slices and cultured hippocampal neurons, we did not observe a significant increase in spontaneous excitatory postsynaptic currents (sEPSCs) frequency at 0.1 μM (mean frequency in control: 0.58 ± 0.13 Hz; LP-211: 0.57 ± 0.13 Hz; paired t-test $p = 0.93$, $n = 8$).

Instead, the application of LP-211 at 1 μM to the slice caused a significant increase of glutamate release in a subpopulation of lamina II neurons, recorded in voltage-clamp at −70 mV (Figures 1A–C).

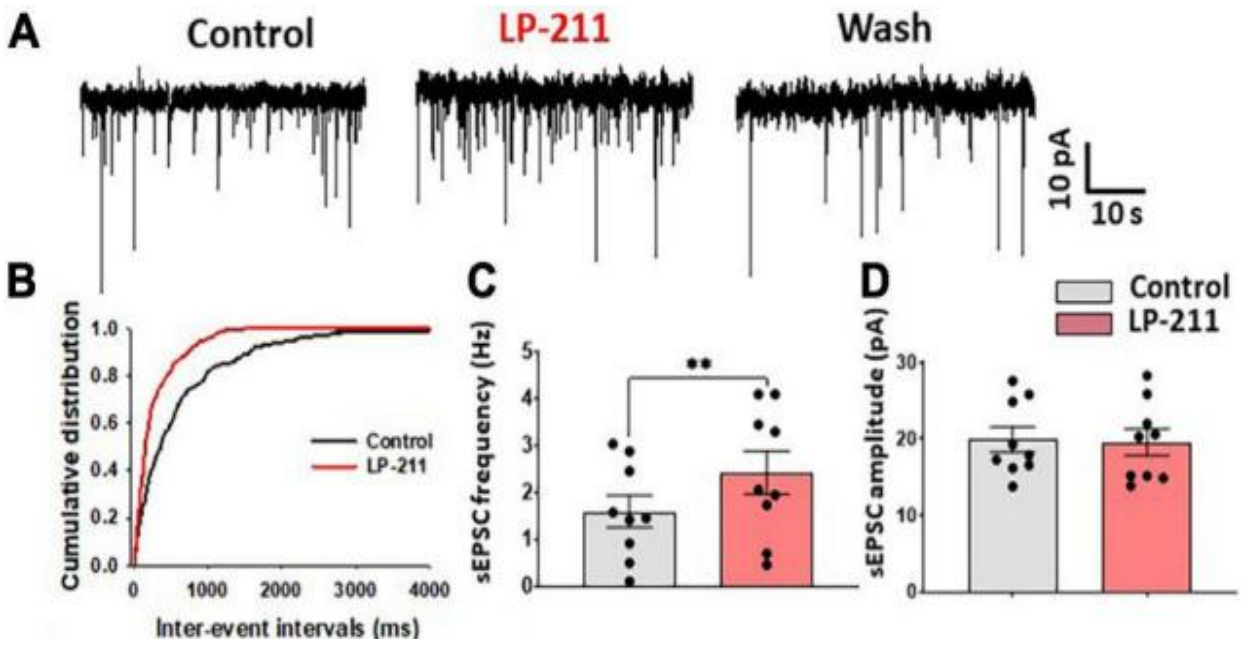


**Figure1** Application of LP-211 increases spontaneous EPSCs in a subpopulation of lamina II neurons. (A) Examples of spontaneous EPSC recordings (sEPSCs), obtained in voltage-clamp at –70 mV. LP-211 (1 μM) caused a significant increase in sEPSC frequency, which was reversible in wash. (B) Cumulative distributions of the inter-event intervals determined from the neuron shown in panel (A). LP-211 caused a shift of the curve to the left, indicating an increase in sEPSC frequency (Kolmogorov–Smirnov test, $p = 0.000$). (C) Summary graph of sEPSC frequencies obtained from the neurons responsive to LP-211 (9 out 14 tested cells; paired *t*-test, $p < 0.01$); (D) LP-211 did not change sEPSC amplitude in the responsive cells (paired *t*-test, $p = 0.69$, $n = 9$). Asterisks reported in the graphs represent statistical significance: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

The analysis was assessed on individual cells based on the significant change in frequency of sEPSCs (Figure 1B).
As showed in Figure 1C, in the presence of LP-211 sEPSC frequency was enhanced in a subpopulation of neurons with a mean percentage increase of 86.9 ± 32.9 (9 cells out of 14). The change was reversible after wash (Figure 1A), while there was no significant alterationofsEPSC amplitude (Figure 1D).
In some neurons, we also observed a slow inward current during LP-211 application (mean amplitude: 8.9 ± 1.1 pA; mean duration: 3.5 ± 0.3 min; 5 out of 11 neurons), indicating the activation of postsynaptic 5-$HT_7$Rs located on the recorded cell.

**Effect of LP-211 and tetrodotoxin on mEPSC amplitude or frequency**

To examine 5-$HT_7$R effect on glutamate release and to understand if the receptor efficacy was dependent on action potential firing, miniature (m)EPSCs were recorded in the presence of 1 μM tetrodotoxin (TTX) to block voltage-dependent sodium channels. Subsequently, 1 μM LP-211 was used to activate 5-$HT_7$Rs.
As shown inFigure 2A-B, 5-$HT_7$R activation by LP-211 significantly increased the frequency of mEPSCs in only 2 out of 9 tested lamina II neurons,whereas mEPSC amplitudes were not affected.
These data suggest that the potentiating effect exerted by 5-$HT_7$Rs partially involves the generation of action potentials in the excitatory networks. Furthermore, the lack of effect on mEPSC amplitude indicates that 5-$HT_7$Rs act at the presynaptic site.

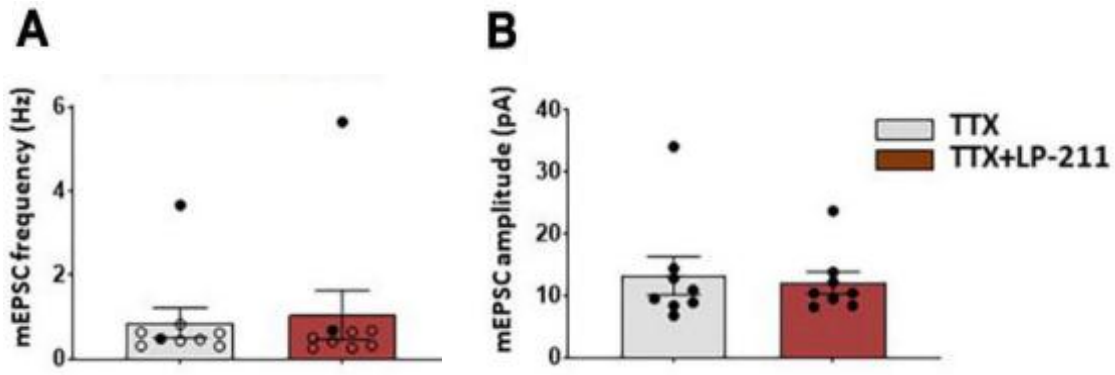


**Figure 2** Application of LP-211 and tetrodotoxin in a subpopulation of lamina II neurons had no significant effect on either mEPSC amplitude or frequency (A) Tetrodotoxin (TTX, 1 μM) reduced the effect of LP-211 on spontaneous glutamate release: in only 2 neurons (black circles) out of 9 a significant increase of mEPSC frequency was observed. The overall effect of LP-211 on mEPSC frequency was not significant (Wilcoxon rank-sum test, $p = 0.91$). (B) No changes in mEPSC amplitude were observed in LP-211 + TTX (Wilcoxon rank-sum test, $p = 0.96$, $n = 9$).

**WAY-100635 did not alter the effect of LP211**

Several studies in brain and spinal cord suggested that some 5-$HT_7$ agonists can also act as mixed 5-$HT_{1A}$R and 5-$HT_7$R agonists (Costa et al., 2012, Mattot et al, 2016).

In order to determine if the alteration of synaptic properties elicited by LP-211 in spinal cord slices were due, in part, to 5-$HT_{1A}$R activation, we recorded sEPSCs in the presence of the 5-$HT_7$R agonist LP-211 (1 μM) and of the selective 5-$HT_{1A}$R antagonist WAY-100635 (WAY, 10 μM) (Ostrowski et al., 2014). Purpose of these experiments was to exclude the involvement of 5-$HT_{1A}$Rs in the observed modulation of glutamate release.

As shown in Figure 3A-B, a significant increase in sEPSC frequency was still observed in the presence of WAY.

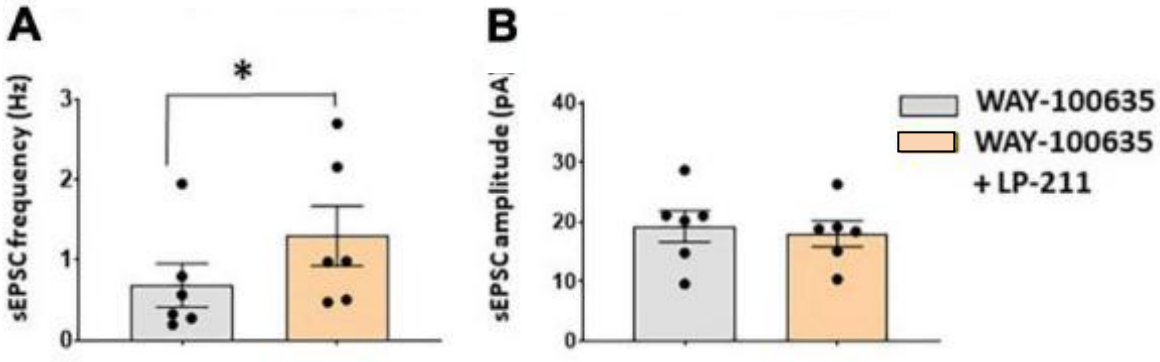


**Figure 3** Application of WAY-100635 on spinal cord slices did not alter the effect of LP-211 in a subpopulation of lamina II neurons (A,B) Co-application of the 5-$HT_{1A}$ antagonist WAY 100-635 (10 μM) did not prevent the increase of sEPSC on 6 out of 10 tested neurons (paired *t*-test on responsive cells, $p < 0.05$, $n = 6$). The sEPSC amplitude was not significantly changed by LP-211 in the presence of WAY (paired *t*-test, $p = 0.39$, $n = 6$).

Infact, as with 5-$HT_7$R activation by LP-211 alone, increased sEPSC frequency was seen in response to the agonist in the presence of WAY. Specifically, sEPSC frequency was enhanced in 60% of cells(6/10), while sEPSC amplitude did not change compared to WAY alone.

Taken together, the block of 5-$HT_{1A}$Rs did not appreciably alter the 5-$HT_7$R-mediated modulation of sEPSCs.

**SB-269970 abolishs the effect of LP211on sEPSCs**

To determine if the effect of LP-211was specific on 5-$HT_7$Rs, we used the selective 5-$HT_7$Rantagonist SB-269970 (SB, 1 μM) and tested whether it was able to block the effect of LP-211 on sEPSCs.

The data in Figure 4 show that, differently from WAY, LP-211 effect on sEPSCs was completely inhibited by applying SB-269970, confirming the selective activation of 5-$HT_7$Rs by the agonist.

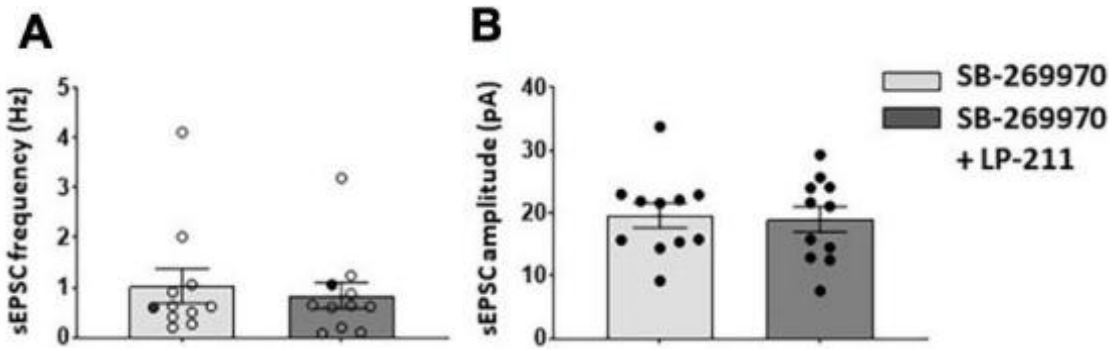


**Figure 4**(A) Co-application of LP-211 with the 5-HT$_7$ antagonist SB-269970 (10 μM) blocked the LP-211 effects on sEPSC frequency[only one responsive cell (black circles) out of 11 tested, Wilcoxon rank-sum test, $p = 0.17$, n = 11]. (B) The sEPSC amplitude was not altered [(B): paired *t*-test, $p = 0.4$, $n = 11$].

**Activation of 5-HT$_7$Rs by LP-211 potentiates spontaneous GABA and glycine release**

Given the variable response of 5-HT$_7$R activation, we postulated that part of these effects may be due to the influence of 5-HT$_7$Rs on inhibitory network activity.

Consistently, Tokarsky et al (2011) demonstrated that the activation of these receptors results in a potentiation of the GABAergic transmission in the hippocampal CA1 area via the activation of 5-HT$_7$Rs located on interneurons, resulting in an enhancement of GABA release.

Therefore, we examined the effect of 5-HT$_7$R activation with LP-211 on miniature and spontaneous inhibitory postsynaptic currents (mIPSCs and sIPSCs), recording in voltage-clamp at 0 mV. Recordings were performed in the presence of the AMPA and NMDA receptor antagonists NBQX (10μM) and D-AP5 (50 μM), respectively, in order to isolate GABA and glycine -mediated IPSCs.

As shown in the representative traces of Figure 5A, 5-HT$_7$R activation by LP-211 was also effective in potentiating sIPSCs mediated by GABA and/or glycine, compared to the control conditions.

In particular, in the presence of the antagonists NBQX and D-AP5, 1 μM LP-211 strongly increased sIPSC frequency in 70.1% of cells evaluated, corresponding to 10 out of 14 lamina II neurons (Figure 5B; mean percentage increase: 236.7 ± 75.2%). On the contrary, sIPSC amplitude was not significantly affected (Figure 5C).

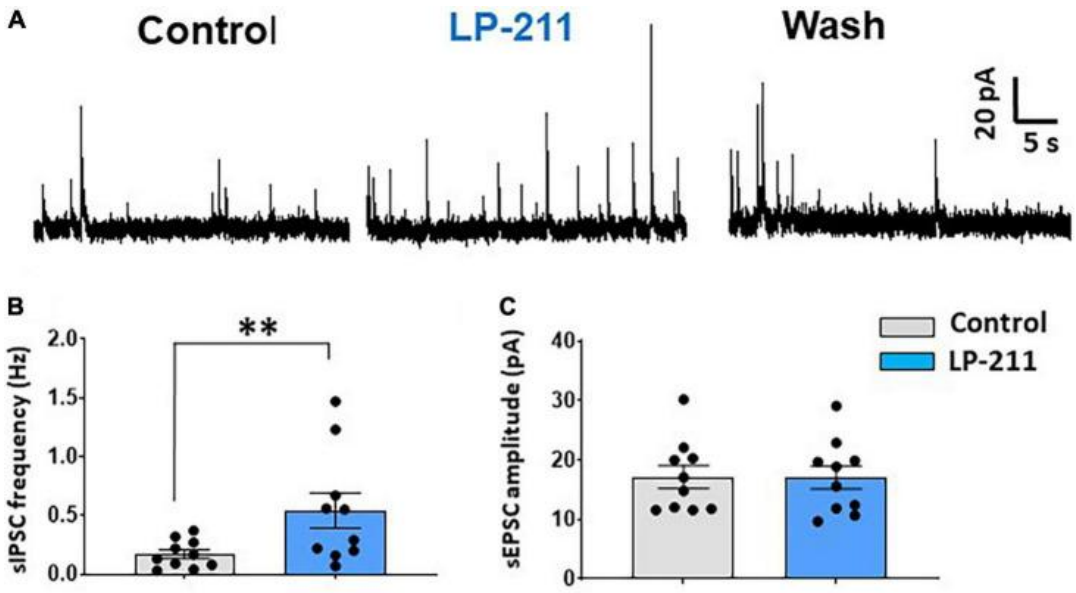


**Figura 5** Activation of 5-HT$_7$Rs by LP-211 potentiates spontaneous GABA and glycine release. (A) Representative traces of spontaneous inhibitory postsynaptic currents (sIPSCs), recorded in voltage-clamp at 0 mV, in the presence of the AMPA and NMDA receptor antagonists NBQX (10 μM) and D-AP5 (50 μM). An increase in sIPSC frequency was evident in the presence of LP-211 (1 μM). (B,C) Summary graphs obtained from the lamina II neurons responsive to LP-211. The agonist caused a significant increase of sIPSC frequency in 10 out of 14 tested cells [(B) paired *t*-test, $p < 0.01$, $n = 10$], without affecting the mean sIPSC amplitude (paired *t*-test, $p = 0.96$, n = 10.

**SB-269970 abolishs the effect of LP211on sIPSCs**

The effect of LP-211 on inhibitory transmission was blocked by 10 μM SB-269970, confirming the involvement of 5-HT$_7$Rs (Figure 6).

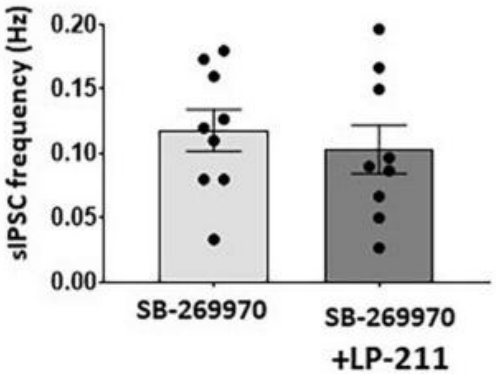


**Figura 6.** Co-application of LP-211 with SB-269970 (10 μM) inhibited the increase of sIPSC frequency, confirming the involvement of 5-HT$_7$Rs (zero responsive cells out of 9 tested, paired *t*-test, $p = 0.21$, $n = 9$).

**Effect of LP211 and tetrodotoxin on mIPSC amplitude or frequency**

To study if 5-$HT_7$Rsact presynaptically to increase inhibitory transmission, mIPSCs in superficial dorsal horn neurons were recorded in the presence of TTX and the effects of LP-211 were determined (Figure7).

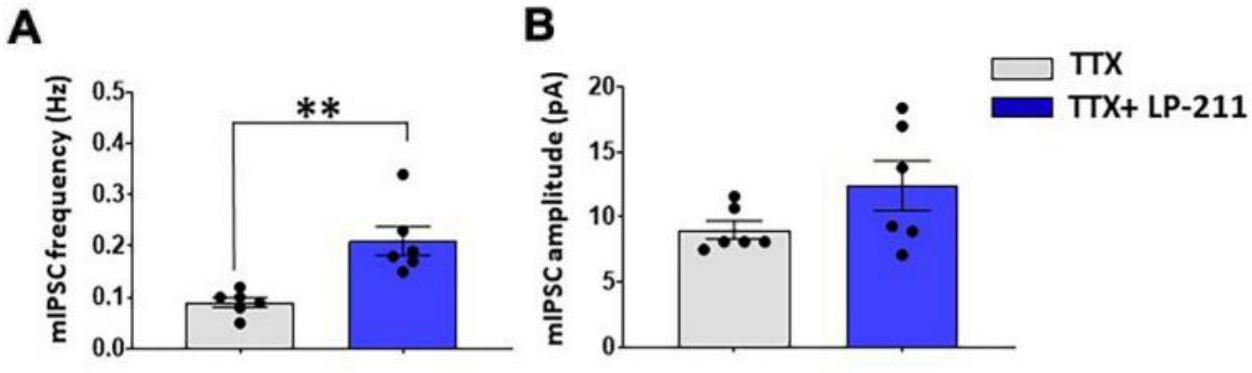


**Figura 7.** Effects of co-application of LP-211 with TTX (1μM) on mIPSCs **(A,B)** In the presence of TTX, LP-211 still produced a significant increase in mIPSC frequency in 6 out of 11 cells tested [**(A)** paired t-test, $p < 0.01$, $n = 6$], while mIPSC amplitude was not altered [**(B)** paired *t*-test, $p = 0.12$, $n = 6$].

The data showed that, after blocking action potentials with TTX, a significant enhancement of mIPSC frequency was still observed in 54.5% of tested neurons, corresponding to 6 out of 11 cells (Figure 7A). Although the overall mean mIPSC amplitude was not significantly changed by LP-211 in the responsive cells (Figure 7B), the increase in frequency was accompanied by an enhancement of amplitude in three neurons. The analysis of the frequency distribution of mIPSC amplitudes showed that this was likely due to the recruitment of additional GABA/glycinergic synaptic terminals, generating larger mIPSCs.

Taken together, 5-$HT_7$R-mediated facilitation of GABA/glycine release was not inhibited after pretreatment with TTX, which indicates that these processes are not dependent on action potential firing.

**LP211 effect on sIPCS vs sEPSC**

Because 5-$HT_7$Rs play a role both in glutamate and GABA/glycine release, we compared the effect of LP-211 agonist on sIPSC and sEPSC frequency (Figure 8).

As shown in the figure below, the percentage frequency increase of sIPSCs resulted significantly higher than that observed for sEPSCs, indicating that 5-$HT_7$Rs exert a more effective potentiation on inhibitory synaptic transmission in spinal cord dorsal horn.

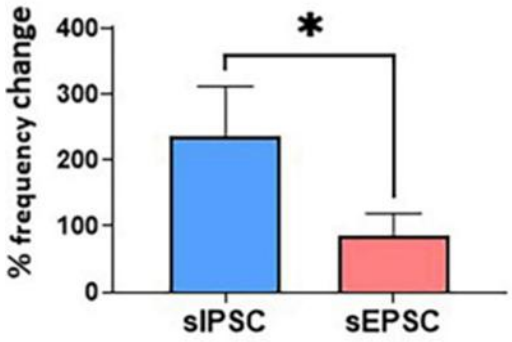


**Figura 8.** LP-211 exerted a stronger effect on sIPSC frequency as compared to sEPSC (Mann–Whitney test, $p < 0.05$, $n = 10$ and 9, for sIPSCs and sEPSCs, respectively).

Since 5-$HT_7$R expression has been detected on DRG neurons and primary afferent fibers, we have then tested whether LP-211 could modulate EPSCs evoked by dorsal root electrical stimulation. A paired-pulse protocol was applied, recording two EPSCs at an interval of 400 ms; the stimulus intensity was 500 μA, able to recruit both Aδ and C fibers.

As shown in Figure 9A–C, a significant increase in the first evoked EPSC was observed in 5 out of 11 lamina II neurons, which was not accompanied by a significant change in the second peak (first peak percentage change: 24.3 ± 3.7%; second peak: 5.3 ± 4.8%, Figure 9B). The paired-pulse ratio (PPR) of the evoked EPSCs was significantly decreased, suggesting a presynaptic site of action of 5-$HT_7$Rs on primary afferent terminals (Figure 9C).

Evoked IPSCs, mediated by GABA and/or glycine, were strongly facilitated by LP-211 (Figures 9D–F). The IPSCs were elicited by focally stimulating lamina II with the paired-pulse protocol and were recorded in the presence of NBQX and D-AP5 to block glutamatergic transmission (Figure 9D). The activation of 5-$HT_7$Rs by 1 μM LP-211 induced an increase of both IPSC peaks in 5 out of 11 tested neurons (first peak percentage change: 71.2 ± 21.6%; second peak: 27.9 ± 6.9%, Figure 9E). The stronger potentiation of the first IPSC, as compared to the second, resulted in a significant decrease in the PPR (Figure 9F). This is consistent with the results on spontaneous IPSCs, suggesting a presynaptic modulation exerted by 5-$HT_7$Rs on inhibitory neurotransmitters. Similar to what was shown above for spontaneous synaptic transmission, a comparison between percentage changes of evoked EPSCs and IPSCs confirmed a stronger potentiation exerted by LP-211 on GABA/glycine mediated transmission (Figure 9G).

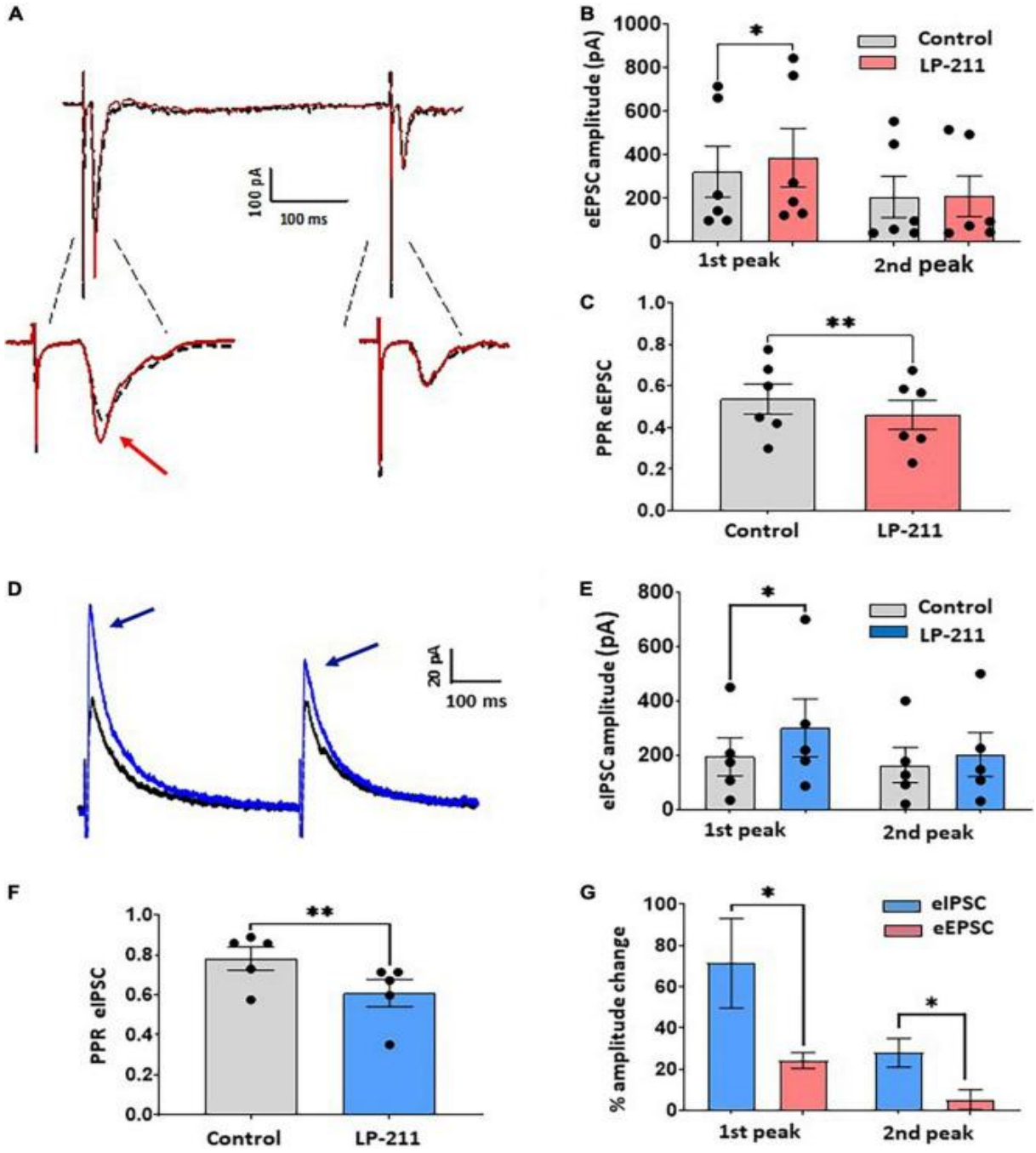


**Figura 9** Application of LP-211 facilitates evoked excitatory postsynaptic currents (EPSCs) and Inhibitory postsynaptic currents (IPSCs), exerting a stronger action on inhibitory transmission. **(A)** Example of EPSC recordings (eEPSCs), evoked by dorsal root stimulation (500 mA, 0.1 ms) and recorded at –70 mV. Two stimuli were applied at 400 ms intervals (paired pulse protocol). LP-211 (1 μM, red trace) caused a moderate potentiation of evoked EPSCs in 5 out of 11 cells tested. **(B)** The first EPSC was significantly increased (Wilcoxon rank-sum test, $p < 0.05$, $n = 6$ EPSCs from 5 cells), while the second peak was not changed (Wilcoxon rank-test, $p = 0.76$, $n = 6$). **(C)** Paired pulse ratio (PPR = second EPSC/first EPSC) was significantly decreased by LP-211, suggesting a presynaptic action (paired *t*-test, $p < 0.01$, $n = 6$). **(D)** Recordings of IPSCs evoked by focal stimulation (paired pulse protocol) and recorded at 0 mV, in the presence of NBQX (10 μM) and D-AP5 (50 μM): in this example, LP-211 caused the increase of both IPSCs (blue trace). **(E)** The first IPSC underwent a stronger potentiation than the second peak, as observed in 5 responsive cells out of 11 (first peak, paired *t*-test, $p < 0.05$; second peak: paired *t*-test, $p = 0.07$, $n = 5$). **(F)** Paired pulse ratio was significantly decreased (paired *t*-test, $p < 0.01$, $n = 5$), confirming a presynaptic effect. **(G)** Evoked inhibitory currents were more affected by 5-$HT_7$R activation compared to excitatory responses, as revealed by the higher percentage increase of both evoked IPSC peaks (Mann–Whitney test, $p < 0.05$, $n = 5$ and 6 for IPSCs and EPSCs, respectively).

Based on these considerations, we finally determined whether LP-211 can modify the excitability of lamina II inhibitory interneurons. As shown by previous studies, excitatory and inhibitory lamina II neurons exhibit different action potential firing patterns in response to current steps. While excitatory neurons show a delayed firing pattern, most inhibitory interneurons tend to fire tonically (Figures 10A,C). Based on this classification, we established the firing pattern of the recorded neuron at the beginning of the experiment. Afterward, we tested the effect of LP-211 on the number of action potentials evoked by dorsal root stimulation (paired-pulse protocol). The LP-211 did not affect the number of action potentials in delayed firing neurons (Figures 10B,E). Still, it effectively increased action potential firing at the first stimulus in tonic neurons (Figures 10D,F). The higher impact of 5-$HT_7$R activation on the excitability of tonic firing neurons is consistent with the stronger effect exerted by LP-211 on inhibitory synaptic transmission.

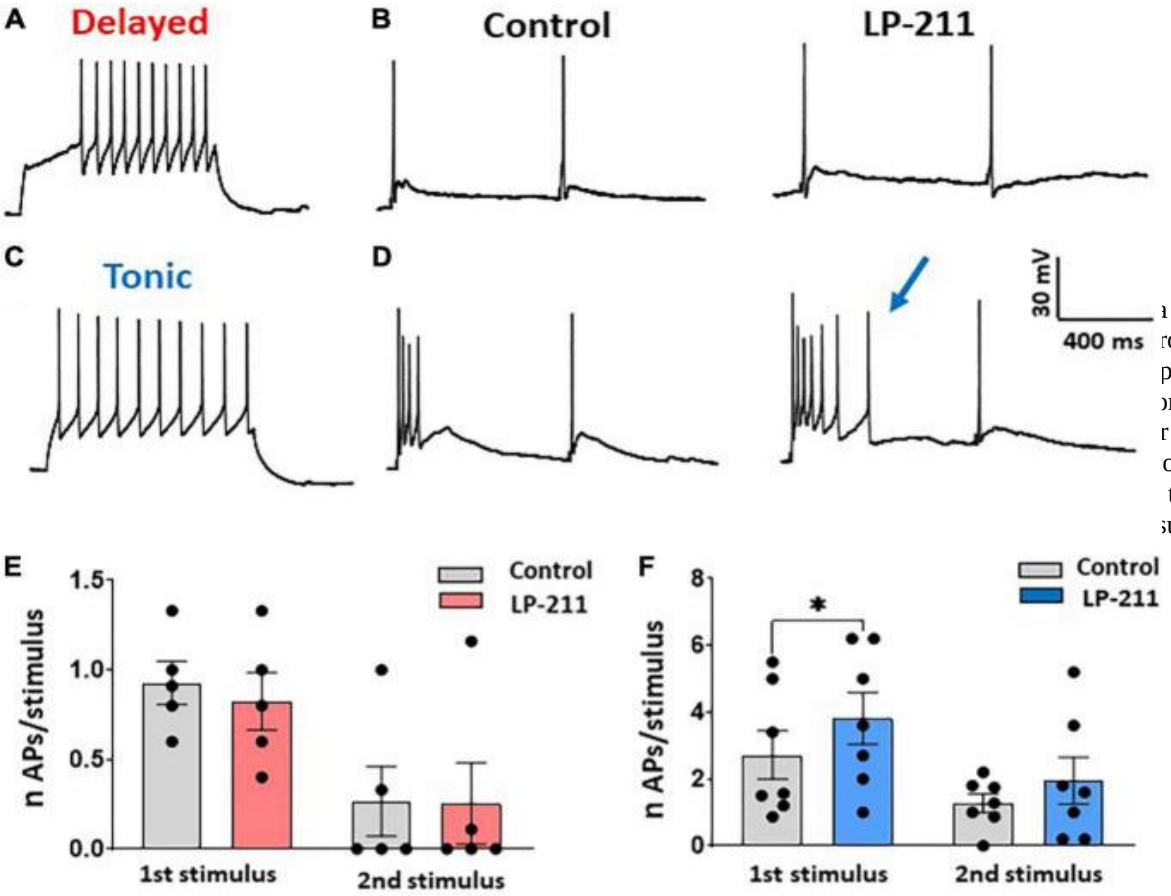


**Figure 10** Activation of 5-$HT_7$Rs differently affects the excitability of delayed and tonic firing lamina II neurons. **(A)** Example of a delayed firing neuron, recorded in current clamp at –80 mV. **(B)** In the same neuron, dorsal root stimulation (2 pulses) evoked action potentials (APs) superimposed to the excitatory postsynaptic potentials (EPSPs). The number of APs was not changed in the presence of LP-211 (1 μM). **(C)** Example of a tonic firingneuron, recorded at a potential of about –80 mV. **(D)** In the same neuron, LP-211 increased the number of APs elicited by root stimulation at the first pulse. **(E)** The meannumber of APs evoked in delayed firing neurons was not changed by LP-211 (first stimulus: paired *t*-test, $p = 0.08$; second: paired *t*-test, $p = 0.85$, $n = 5$). **(F)** In the tonic firing neurons, the number of APs was significantly increased at the first stimulus (first: Wilcoxon rank-sum test, $p < 0.05$; second: paired t-test, $p = 0.2$, $n = 7$).

# Chapter V. DISCUSSION

Chronic pain represents a significant clinical problem affecting up to 20% of the general population (Breivik et al., 2006; Reid et al., 2011) and it is a symptom of a variety of different underlying pathologies including arthritis, nerve injury, depression and cancer.
Unlike some neurobiological disorders which can be attributed to specific brain regions, pain incorporates multiple components of the nervous system, at the peripheral, spinal, and supraspinal levels.
The first components of the pain pathway are the peripheral nociceptors, the majority of them terminating in the dorsal horn of spinal cord.

The dorsal horn (DH) is divided into six layers, referred to as laminae, whose cells have been grouped based on their structure and function, rather than solely on location (Rexed, 1952; Molander et al., 1984).
The neuronal organization and synaptic circuits of the region allow communication between the laminae of the dorsal horn (Dubner and Ren, 1999; Duan et al., 2014; Bourane et al., 2015; Pagani et al., 2019), suggesting the presence of a network through which the integration of the nociceptive process takes place.
In DH, in particular, we identify superficial dorsal horn (SDH, lamina I and II) and deep dorsal horn (lamina III to VI). It is now universally accepted that SDH plays a key rolein carryingnoxious information. The great majority of the neurons in SDH are interneurons. The interneurons can be divided into two broad functional classes: inhibitory cells, which use GABA and/or glycine as their principal transmitter, and excitatory (glutamatergic) interneurons. The balance between excitation and inhibition is known to play a critical role in the transmission of painful messages in the spinal network.

Multiple neurotransmitter systems have been considered to participate in the modulation of the transmission, process and control of pain.The serotonergicsystem has been recognized as one of the main ones.
The direct effects of serotonin on superficial DH neurons are mediated by different receptors, such as 5-$HT_7$, (5-$HT_7$Rs). Immunocytochemical studies found that 5-$HT_7$Rs are localized in the superficial layers of the spinal cord dorsal horn, but the controversial role played by 5-$HT_7$ receptors in nociception has been poorly or not thoroughly investigated, especially regarding the control of synaptic inhibition.
However, morphological and behavioural studies performed so far would suggest an important role of 5-$HT_7$Rsin the control of nociception.

The results from the present study confirm the presence of 5-$HT_7$Rs in the SDH, although their functional role seems to becomplex. Specifically, 5-$HT_7$Rs modulate inhibitory neurotransmission in a more powerful manner than that exerted on excitatory synaptic circuits in dorsal horn, suggesting that the overall effect of 5-$HT_7$R in spinal cord may be anti-nociceptive in acute pain.

We started to analyze the serotonergic modulation of 5-$HT_7$ receptor on glutamatergic synaptic responses.
To activate 5-$HT_7$Rs, we used the compound LP-211, reported by Leopoldo et al. (2008) to be a high affinity 5-$HT_7$ agonist. LP-211 has beenused in several approaches such as in subcutaneous administration of animal models (Adriani et al., 2012, Ruocco et al., 2013, Romano et al., 2014), in neuronal primary cultures (Speranza et al. 2013) and on neuron brain slices (Costa et al., 2012, Lippiello et al., 2016),buthas never been applied in studies on spinal cord neurons.
In recent works, 5-$HT_7$receptor selective activation was induced by LP-211 on hippocampal neurons slicesat the concentration of 10 nM (Costa et al., 2012) or on cerebellar slicesat 1 μM (Lippiello et al.2016).

To activate 5-$HT_7$Rsand identify theirpotential role in modulating glutamate release, we used LP-211 at the concentrations of 0.1 mM and at 1 mM on spinal cord slices.
The application of 1uM LP-211 for 5 minutes induced a rapid increase of sEPSC frequency, while 0.1 μM was ineffective. The necessity of using higher concentrations of agonists in spinal cord slices as compared to other preparations could derive from their particular morphology and from the location of the recorded cells. Indeed, most recordings were obtained from neurons located quite deep into the slice, where synaptic connections are well preserved.

Our data also demostrated that the effect of LP-211 on spontaneous EPSCs was reduced in the presence of TTX, suggesting that 5-$HT_7$Rs expressed on excitatory interneurons could contribute to the modulation of glutamate release through an action potential-dependent mechanism.

Block of 5-$HT_7$R with the antagonist SB-269970 abolished the positive effect of LP-211 in potentiating excitatory transmission. SB-269970 was applied at the concentration of 10 μM that could also inhibit the activity 5-$HT_{5A}$Rs. However, these receptors are negatively coupled to adenylate cyclase *via* Gi/o proteins whose activation opens $K^+$ channels. This mechanism of action is not compatible with the increase of transmitter release and action potential firing observed in our study. Furthermore, SB-269970 administered at similar concentrations has proved to be ineffective on locomotor-like rhythmic activity recorded from *in vitro* spinal cord in 5-$HT_7$R knockout mice (Liu et al., 2009). Interestingly, 5-$HT_2$Rs that are involved in the generation of this activity together with 5-$HT_7$Rs (Pearlstein et al., 2005) were not affected by SB-269970 at these concentrations.

Over the years, several agonists and antagonists for 5-$HT_{1A}$ and 5-$HT_7$ receptors have been developed and applied to investigate the pharmacology of these receptors. It was reported that there is a pharmacological and functional cross-talk between 5-$HT_{1A}$ and 5-$HT_7$ receptors.
Infact, due to the high affinity of5-$HT_7$ agonistsfor 5-$HT_{1A}$ (Naumenko et al., 2014), analysis of 5-$HT_7$ receptor would require parallel application of 5-$HT_{1A}$ receptor antagonists. Several antagonists of 5-$HT_{1A}$ receptors became available in early 1990. One of them, WAY-100135 is the prototypical silent 5-$HT_{1A}$ receptor antagonist, being highly selective and devoid of agonist activity. The compoundhas been widely used as a pharmacological probe to investigate the distribution and function of 5-$HT_{1A}$ receptors.
In our experiments, the inactivation of 5-$HT_{1A}$R with WAY-100135 did not alter the increase of sEPSC frequency induced by LP-211 in SDH.

Taken together, we could assume that, in mouse spinal cord slices, the effect of LP-211 on the activation of 5-$HT_7$Rsand induction of increase of sEPSC frequency is specific. Furthermore, our data suggest that it is possible to use the agonist LP-211 at higher concentrations, even if future studies of molecular biology employing 5-$HT_7$ knockout mice, shRNA, or CRISPR viral delivery will provide a more precise and specific clarification of the role of 5-$HT_7$Rs in modulating nociceptive transmission in the spinal cord dorsal horn.

Immunocytochemical studies of the distribution of the 5-$HT_7$Rin the spinal cord reveals that the receptor is mainly localized in the two superficial laminae, expressed on dorsal horn excitatory peptidergic neurons and GABAergic interneurons.
In the rat, GABA is present in approximately 25% and 30% of neurons in laminae I and II, respectively (Polgar et al., 2003).
Furthermore, the negative modulation of the GABAergic inhibitory pathway is known to contribute to neuropathic pain hypersensitivity (Moore et al., 2002; Lu et al., 2013).

In addition,Viguier et al. (2012) reported that in a model of neuropathic pain caused by sciatic nerve ligation (SNL), acute administration of 5-$HT_7$R agonists markedly reduces mechanical and thermal hyperalgesia, supporting the idea that 5-$HT_7$R-mediated inhibitory control of neuropathic pain is underlain by excitation of GABAergic interneurons within the dorsal horn.
To study the potential role of 5-$HT_7$ receptor in the modulation of inhibitory pathway mediated by GABA interneurons, we analyzed the release of GABA after activation of 5-$HT_7$R, using 1 μM LP-211. We have shown that LP-211 is able to potentiate both spontaneous and evoked inhibitory synaptic transmission by a presynaptic mechanism.

Block of 5-$HT_7$R with SB-269970 abolishes the positive effect of LP-211 in potentiating of spontaneous and evoked GABA/glycinergic IPSCs, confirming the role of 5-$HT_7$Rs expressed on inhibitory interneurons.
Finally, although 5-$HT_7$Rs are able to potentiate both inhibitory and excitatory transmission, the impact on inhibitory interneurons is stronger than that on excitatory interneurons.
5-$HT_7$ receptors differently modulate the activity of GABAergic inhibitory interneurons in distinct brain areas.In the hippocampus, 5-$HT_7$ receptor activation was shown to stimulate the activity of GABAergic interneurons. Application of a 5-$HT_7$ receptor agonist enhanced the frequency of GABA-mediated sIPSCsrecorded from rat CA1 pyramidal neurons, indicating an increased GABA release from interneurons (Tokarski et al., 2011). 5-$HT_7$ receptor activation enhanced GABAergic transmission also in the rat globus pallidus (Chen et al., 2008).
Several cellular mechanisms could be involved in the synaptic facilitation mediated by 5-$HT_7$Rs (Ciranna and Catania, 2014).
As shown in many CNS areas, activation of these receptors can increase neuronal excitability by (i) reducing action potential hyperpolarization (through the inhibition of a $Ca^{2+}$-dependent potassium current; Goaillard and Vincent, 2002); (ii) inhibiting the $I_A$ potassium current (Siwiec et al., 2020); (iii) enhancing the hyperpolarization-activated cation current ($I_h$; Larkman and Kelly, 1997; Cardenas et al., 1999; Chapin and Andrade, 2001; Bickmeyer et al., 2002; Tang and Trussell, 2015); and (iv) potentiating the activity of voltage-dependent T-type calcium channels (Lenglet et al., 2002).
Our study showed that LP-211 is particularly effective in increasing the excitability of tonic firing neurons, which mainly correspond to inhibitory interneurons (Daniele and MacDermott, 2009; Yasaka et al., 2010; Hughes et al., 2012). Interestingly, tonic-firing cells in lamina II have the highest expression level of Ih, which has also been often associated with inhibitory neuron populations (Hantman and Perl, 2005; Hughes et al., 2012; Smith et al., 2015). Together with the expression of T-type $Ca^{2+}$ channels in some neuron populations (Smith et al., 2015), this property would make tonic firing inhibitory interneurons favorable targets for 5-$HT_7$R-mediated modulation. The persistence of the LP-211 effect on mIPSCs in TTX would also suggest the presence of an action potential-independent, direct modulation at the inhibitory neuron axon terminal, similarly to what was observed in the hippocampus (Tokarski et al., 2011).

Based on all our results, we can postulate the following hypothetical dorsal horn circuit.
In Figure 10, we show that functional 5-$HT_7$Rs are expressed by some nociceptive primary afferent terminals (nociceptive fiber Ad/C, in red). They enter within lamina I/II where form synapses with the recorded neuron (indicated in gray).

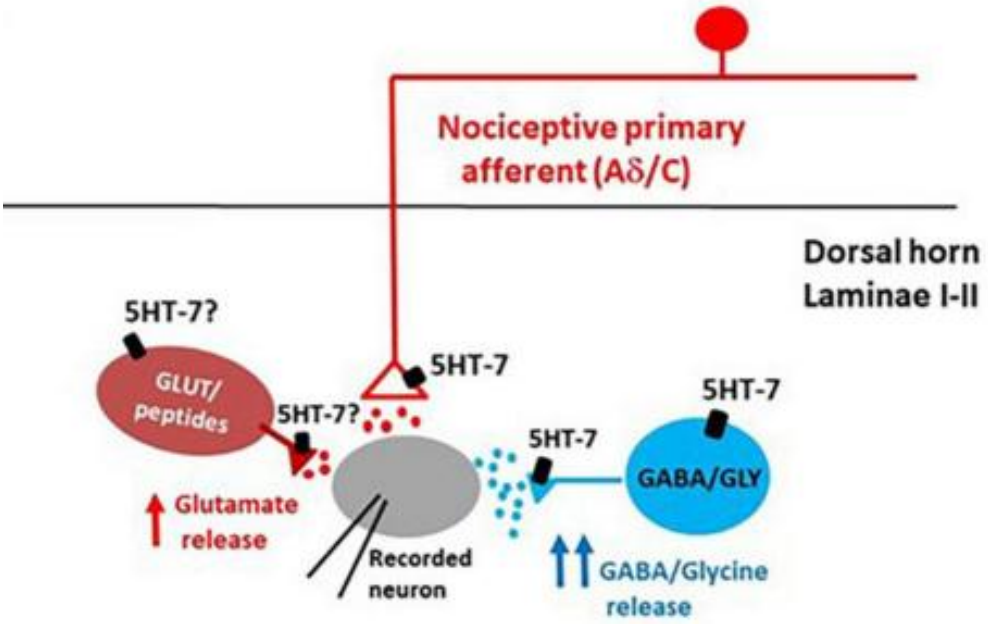


**Figure 10** Schematic diagram depicting the hypothetical dorsal horn circuit involved in 5-$HT_7$R-mediated synaptic modulation (see section "Discussion").

The neurons likely interact with two different cell populations, inhibitory interneurons (indicated in light blue) and, probably,with some excitatory interneurons (indicated in dark red). On the the soma and and axon terminals of both interneurons, we hypothesize the presence of functional 5-$HT_7$Rs.

The role of 5-$HT_7$ receptor in *in vivo* systems is not clear.
Studies performed in rodents have reported both anti- and pro-nociceptive (Castellano et al., 2012, Bardoni et al. 2019) actions of these receptors.
Gonzalez et al. 2005, assessed the role of rat spinal 5-HT7Rs in the formalin test after local administration of a 5-$HT_7$ r antagonist, demonstrating that 5-$HT_7$Rs play a pronociceptive action in a dose-dependent manner.
In according with Gonzalez's lab, Chaparro et al. 2012 suggested that spinal 5-HT, released from the serotonergic projections in response to formalin injection, activates pre- or post-synaptic 5-$HT_7$ receptors at the spinal cord promoting the development and maintenance of secondary allodynia and hyperalgesia.
In addition, Amaya-Castellanos et al., 2011, suggested that the activation of spinal 5-$HT_7$Rs exerts a pronociceptive effect in neuropathic rats. In their study, the results showed that systemic or spinal treatment with SB-269970 could reduce tactile allodynia in a dose-dependent fashion in L5/L6 SNL models.

In contrast, several studies suggest that 5-$HT_7$Rs could be involved in nociceptive inhibition upon injury.
In the SNL animal model of chronic neuropathic pain, antinociceptive effects of spinally and systemically administered 5-$HT_7$agonists and a reduced 5-$HT_7$ protein content in DH afterinjury were shown.
Brenchat et al. (2010) demonstrated that acute or repeated administration of AS-19 or E-57 431, a new highly selective potent 5-$HT_7$R agonist, induced a clear-cut inhibition of mechanical allodynia and thermal hyperalgesia in a neuropathic pain model in mice. They also reported in this model an increased level of the receptor in the dorsal horn of the spinal cord (Brenchat et al., 2010).

An anti-nociceptive action of 5-$HT_7$Rs has been observed also in other models of pain hypersensitivity induced by capsaicin injection (Brenchat et al., 2009) or chronic constriction injury (Viguier et al., 2012).
Similarly, in mice, 5-$HT_7$ agonists LP-211 and LP-44 induce analgesic effects on formalin-induced orofacial pain (Demirkaya et al., 2016).

GABAergic interneurons seem to be especially involved in 5-$HT_7$-mediated anti-nociception since the intrathecal administration of the $GABA_A$ antagonist bicuculline prevented the anti-hyperalgesic effect of 5-$HT_7$ agonists in rats subjected to the constriction injury of the sciatic nerve (Viguier et al., 2012, 2013).
These functional differences may be explained by different factors (i) change in receptor expression and/or protein content sincean increased 5-$HT_7$ expression was involved in inhibitory antinociceptive effects and the facilitatory pronociceptive effect was observed with reduced 5-$HT_7$ protein content- (ii) animal pain models used; (iii) low selectivity of some of the 5-$HT_7$ agonists employed in the studies; (iv) cross-talk with other receptors and/or neurotransmitters; (v) route of drug administration

Besides the relatively extensive amount of research on the involvement of serotonin in nociceptive transmission in the healthy rodent and in the neuropathic pain rodent, some important gaps remain in the current knowledge on the topic that should be addressed in future studies.
Firstly, further clarification on the expression of the 5-HT receptors on the different cell types within the dorsal horn may help the interpretation of behavioral or electrophysiological results and eliminate speculation both in the healthy rodent and after the induction of neuropathic pain.
Secondly, present literature on changes in 5-HT expression in the DH after injury is rather conflicting, which may be due to different pain models used. Studies aimed at, or including, the evaluation of changes in 5-HT content indescending serotonergic terminals in the DH after injury may provide more clarity.
Thirdly, identifying changes in 5-HT receptor expression and function upon injury will help pinpoint pharmacological targets for a more successful use of serotonergic drugs in the treatment of chronic neuropathic pain.
Lastly, there are some limitationsin the published studies obtained from behavioral tests and these results should be critically evaluated. .
Indeed, many of the behavioral studies evaluate evoked pain using reflex based tests, such as the tail flick, hot plate and paw withdrawal tests.
To assess spontaneous pain or better evaluate the effects of interventions that involve supraspinal mechanisms known to be involved in pain, future studies should extend the behavioral test repertoire to include not only evoked pain tests but also tests related to cognitive and emotional aspects of pain
Diffuse noxious inhibitory control should also be tested as a way of evaluating descending inhibition and serotonergic involvement herein. Furthermore, serotonin is also involved in the regulation of locomotion. The use of serotonergic drugs at concentrations that induce motor effects may lead to incorrect interpretation of test results. Future studies utilizing serotonergic drugs should include some form of locomotion testing or incorporate a pilot study to select a correct dosage that does not induce locomotion effects

For all these reasons, although our results show a prevalent and strong effect of LP-211 in potentiating synaptic inhibition in naïve mice, it is difficult to predict how these modulatory mechanisms could be altered in conditions of chronic pain.
In summary, the complexity of these results reflects the complexity of responses elicited by 5-HT within the dorsal horn. Diverse responses can occur not only as theresult of activation of the many different receptor subtypes, but also from the involvement of various receptor isoforms the

interactions between different 5-HT receptor subtypes, the amount of serotonin release, andthe level of receptor expression in primary nociceptors and dorsal horn interneurons

Finally, the exact role of 5-$HT_7$Rs in the spinal cord may be better understood once more selective agonists will beavailable. The examination of dissociated spinal neurons will be also helpful to eliminate the potential confounding effects of activating nearby inhibitory and excitatory networks and to isolate the direct effects of 5-$HT_7$Rs on dorsal horn neurons.

## REFERENCES


1. Amaya-Castellanos E., Pineda-Farias J. B., Castañeda-Corral G., Vidal-Cantú G. C., Murbartián J., Rocha-González H. I., et al. (2011). Blockade of 5-HT7 receptors reduces tactile allodynia in the rat. *Pharmacol. Biochem. Behav.* 99 591–597. 10.1016/j.pbb.2011.06.005

2. Bannister K., Lockwood S., Goncalves L., Patel R., Dickenson A. H. (2017). An investigation into the inhibitory function of serotonin in diffuse noxious inhibitory controls in the neuropathic rat. *Eur. J. Pain* 21 750–760. 10.1002/ejp.979

3. Bardoni R. (2019). Serotonergic modulation of nociceptive circuits in spinal cord dorsal horn. *Curr. Neuropharmacol.* 17 1133–1145. 10.2174/1570159X17666191001123900

4. Bardoni R., Shen K.-F., Li H., Jeffry J., Barry D. M., Comitato A., et al. (2019). Pain inhibits GRPR neurons via GABAergic signaling in the spinal cord. *Sci. Rep.* 9:15804. 10.1038/s41598-019-52316-0

5. Betelli C., MacDermott A. B., Bardoni R. (2015). Transient, activity dependent inhibition of transmitter release from low threshold afferents mediated by GABAA receptors in spinal cord lamina III/IV. *Mol. Pain* 11:64. 10.1186/s12990-015-0067-5

6. Bickmeyer U., Heine M., Manzke T., Richter D. W. (2002). Differential modulation of I(h) by 5-HT receptors in mouse CA1 hippocampal neurons. *Eur. J. Neurosci.* 16 209–218. 10.1046/j.1460-9568.2002.02072.x

7. Blattner K. M., Canney D. J., Pippin D. A., Blass B. E. (2019). Pharmacology and therapeutic potential of the 5-HT7 receptor. *ACS Chem. Neurosci.* 10 89–119. 10.1021/acschemneuro.8b00283

8. Breivik H, Eisenberg E, O'Brien T. (2013). The individual and societal burden of chronic pain in Europe: the case for strategic prioritisation and action to improve knowledge and availability of appropriate care. *BMC Public Health*. 13:1229

9. Brenchat A., Nadal X., Romero L., Ovalle S., Muro A., Sánchez-Arroyos R., et al. (2010). Pharmacological activation of 5-HT7 receptors reduces nerve injury-induced mechanical and thermal hypersensitivity. *Pain* 149 483–494. 10.1016/j.pain.2010.03.007

10. Brenchat A., Romero L., García M., Pujol M., Burgueño J., Torrens A., et al. (2009). 5-HT7 receptor activation inhibits mechanical hypersensitivity secondary to capsaicin sensitization in mice. *Pain* 141 239–247. 10.1016/j.pain.2008.11.009

11. Cao S, Li J, Yuan J, Zhang D, Yu T. 2020. Fast localization and sectioning of mouse locus coeruleus. *Biomed Res Int* 2020:4860735.

12. Cardenas C. G., Mar L. P., Vysokanov A. V., Arnold P. B., Cardenas L. M., Surmeier D. J., et al. (1999). Serotonergic modulation of hyperpolarization-activated current in acutely isolated rat dorsal root ganglion neurons. *J. Physiol.* 518 507–523. 10.1111/j.1469-7793.1999.0507p.x

13. Chapin E. M., Andrade R. (2001). A 5-HT(7) receptor-mediated depolarization in the anterodorsal thalamus. II. Involvement of the hyperpolarization-activated current I(h). *J. Pharmacol. Exp. Ther.* 297 403–409.

14. Ciranna L., Catania M. V. (2014). 5-HT7 receptors as modulators of neuronal excitability, synaptic transmission and plasticity: physiological role and possible implications in autism spectrum disorders. *Front. Cell Neurosci.* 8:250. 10.3389/fncel.2014.00250

15. Comitato A., Lacivita E., Leopoldo M., Bardoni R. (2021). "Serotonergic modulation by 5-HT7 receptors in mouse spinal cord dorsal horn," in *Proceedings of the 44th AISD National Meeting* (Bari: Università di Bari; ).

16. Cortes-Altamirano J. L., Olmos-Hernandez A., Jaime H. B., Carrillo-Mora P., Bandala C., Reyes-Long S., et al. (2018). Review: 5-HT1, 5-HT2, 5-HT3 and 5-HT7 receptors and their role in the modulation of pain response in the central nervous system. *Curr. Neuropharmacol.* 16 210–221. 10.2174/1570159X15666170911121027

17. Costa L., Sardone L. M., Bonaccorso C. M., D'Antoni S., Spatuzza M., Gulisano W., et al. (2018). Activation of serotonin 5-HT7 receptors modulates hippocampal synaptic plasticity by stimulation of adenylate cyclases and rescues learning and behavior in a mouse model of fragile X syndrome. *Front. Mol. Neurosci.* 11:353. 10.3389/fnmol.2018.00353

18. Costa L., Sardone L. M., Lacivita E., Leopoldo M., Ciranna L. (2015). Novel agonists for serotonin 5-HT7 receptors reverse metabotropic glutamate receptor-mediated long-term depression in the hippocampus of wild-type and Fmr1 KO mice, a model of Fragile X Syndrome. *Front. Behav. Neurosci.* 9:65. 10.3389/fnbeh.2015.00065

19. Costa L., Spatuzza M., D'Antoni S., Bonaccorso C. M., Trovato C., Musumeci S. A., et al. (2012). Activation of 5-HT7 serotonin receptors reverses metabotropic glutamate receptor-mediated synaptic plasticity in wild-type and Fmr1 knockout mice, a model of Fragile X syndrome. *Biol. Psychiatry* 72 924–933. 10.1016/j.biopsych.2012.06.008

20. Daniele C. A., MacDermott A. B. (2009). Low-threshold primary afferent drive onto GABAergic interneurons in the superficial dorsal horn of the mouse. *J. Neurosci.* 29 686–695. 10.1523/JNEUROSCI.5120-08.2009

21. Demirkaya K., Akgün ÖM., Şenel B., Öncel Torun Z., Seyrek M., Lacivita E., et al. (2016). Selective 5-HT7 receptor agonists LP 44 and LP 211 elicit an analgesic effect on formalin-induced orofacial pain in mice. *J. Appl. Oral Sci.* 24 218–222. 10.1590/1678-775720150563

22. Dogrul A., Seyrek M., Akgul E.O., Cayci T., Kahraman S., Bolay H. 2012 Systemic paracetamol-induced analgesic and antihyperalgesic effects through activation of descending serotonergic pathways involving spinal 5-$HT_7$ receptors. *Eur. J. Pharmacol.* 677(1-3):93–101.

23. Dogrul A., Seyrek M. 2006 Systemic morphine produce antinociception mediated by spinal 5-HT7, but not 5-HT1A and 5-HT2 receptors in the spinal cord. *Br. J. Pharmacol.*149(5):498–505

24. Doly S., Fischer J., Brisorgueil M.-J., Vergé D., Conrath M. (2005). Pre- and postsynaptic localization of the 5-HT7 receptor in rat dorsal spinal cord: immunocytochemical evidence. *J. Comp. Neurol.* 490 256–269. 10.1002/cne.20667

25. Garraway S. M., Hochman S. (2001). Pharmacological characterization of serotonin receptor subtypes modulating primary afferent input to deep dorsal horn neurons in the neonatal rat. *Br. J. Pharmacol.* 132 1789–1798. 10.1038/sj.bjp.0703983

26. Goaillard J.-M., Vincent P. (2002). Serotonin suppresses the slow afterhyperpolarization in rat intralaminar and midline thalamic neurones by activating 5-HT(7) receptors. *J. Physiol.* 541 453–465. 10.1113/jphysiol.2001.013896

27. Hantman A. W., Perl E. R. (2005). Molecular and genetic features of a labeled class of spinal substantia gelatinosa neurons in a transgenic mouse. *J. Comp. Neurol.* 492 90–100. 10.1002/cne.

28. Hayashida, K.; Clayton, B.A.; Johnson, J.E.; Eisenach, J.C. 2008 Brain derived nerve growth factor induces spinal noradrenergic fiber sprouting and enhances clonidine analgesia following nerve injury in rats. *Pain 136*, 348–355.

29. Hayashida, K. Eisenach, J.C. 2010 Spinal alpha 2-adrenoceptor-mediated analgesia in neuropathic pain reflects brain-derived nerve growth factor and changes in spinal cholinergic neuronal function. *Anesthesiology 113*, 406–412.

30. van Hecke O, Torrance N, Smith BH. (2013). Chronic pain epidemiology and its clinical relevance. *Br J Anaesth* 111(1):13-8.

31. Hughes D. I., Sikander S., Kinnon C. M., Boyle K. A., Watanabe M., Callister R. J., et al. (2012). Morphological, neurochemical and electrophysiological features of parvalbumin-expressing cells: a likely source of axo-axonic inputs in the mouse spinal dorsal horn. *J. Physiol.* 590 3927–3951. 10.1113/jphysiol.2012.235655

32. Kvachnina E., Dumuis A., Wlodarczyk J., Renner U., Cochet M., Richter D.W., Ponimaskin E. 2009 Constitutive G s-mediated, but not G 12-mediated, activity of the 5-hydroxytryptamine 5-HT 7 (a) receptor is modulated by the palmitoylation of its C-terminal domain. *Biochimica et Biophysica Acta (BBA)*-Mol. Cell Res. 1793(11):1646–1655.

33. Kimura, M. Suto, T. Morado-Urbina, C.E. Peters, C.M. Eisenach, J.C. Hayashida, K. 2015 Impaired pain-evoked analgesia after nerve injury in rats reflects altered glutamate regulation in the locus coeruleus. *Anesthesiology 123*, 899–908.

34. Larkman P. M., Kelly J. S. (1997). Modulation of IH by 5-HT in neonatal rat motoneurones in vitro: mediation through a phosphorylation independent action of cAMP. *Neuropharmacology* 36 721–733. 10.1016/s0028-3908(97)00021-x

35. Lenglet S., Louiset E., Delarue C., Vaudry H., Contesse V. (2002). Involvement of T-type calcium channels in the mechanism of action of 5-HT in rat glomerulosa cells: a novel signaling pathway for the 5-HT7 receptor. *Endocr. Res.* 28 651–655. 10.1081/erc-120016981

36. Leopoldo M., Lacivita E., Berardi F., Perrone R., Hedlund P. B. (2011). Serotonin 5-HT7 receptor agents: structure-activity relationships and potential therapeutic applications in central nervous system disorders. *Pharmacol. Ther.* 129 120–148. 10.1016/j.pharmthera.2010.08.013

37. Lippiello P., Hoxha E., Speranza L., Volpicelli F., Ferraro A., Leopoldo M., et al. (2016). The 5-HT7 receptor triggers cerebellar long-term synaptic depression via PKC-MAPK. *Neuropharmacology* 101 426–438. 10.1016/j.neuropharm.2015.10.019

38. Liu J., Akay T., Hedlund P. B., Pearson K. G., Jordan L. M. (2009). Spinal 5-HT7 receptors are critical for alternating activity during locomotion: in vitro neonatal and in vivo adult studies using 5-HT7 receptor knockout mice. *J. Neurophysiol.* 102 337–348. 10.1152/jn.91239.2008

39. Llorca-Torralba M, Borges G, Neto F, Mico JA, Berrocoso E. 2016 Noradrenergic locus coeruleus pathways in pain modulation. *Neuroscience* 338:93–113.

40. Loughlin, S.E. Foote, S.L. Grzanna, R. 1986 Efferent projections of nucleus locus coeruleus: Morphologic subpopulations have different efferent targets. *Neuroscience* , *18*, 307–319.

41. Mannion RJ, Woolf CJ. (2000). Pain mechanisms and management: a central perspective. *Clin J Pain.* 16 S144-56.

42. Meuser T., Pietruck C., Gabriel A., Xie G.-X., Lim K.-J., Pierce Palmer P. (2002). 5-HT7 receptors are involved in mediating 5-HT-induced activation of rat primary afferent neurons. *Life Sci.* 71 2279–2289. 10.1016/s0024-3205(02)02011-8

43. Millan MJ. (1999). The induction of pain: an integrative review. *Prog Neurobiol.* 57 (1):1–164.

44. Millan MJ. Descending control pain. (2002). *Prog Neurobiol.* 66 (6):355–474.

45. Norum J. H., Hart K., Levy F. O. (2003). Ras-dependent ERK activation by the human G(s)-coupled serotonin receptors 5-HT4(b) and 5-HT7(a). *J. Biol. Chem.* 278 3098–3104. 10.1074/jbc.M206237200

46. Pain and Disability: Clinical, Behavioral, and Public Policy Perspectives. Washington (DC): National Academies Press (US); 1987. ISBN-10: 0-309-03737-9

47. Pan, H.L. Wu, Z.Z. Zhou, H.Y. Chen, S.R. Zhang, H.M. Li, D.P. 2008Modulation of pain transmission by G-protein-coupled receptors. *Pharmacol. Ther. 117*, 141–161

48. Pearlstein E., Mabrouk F. B., Pflieger J. F., Vinay L. (2005). Serotonin refines the locomotor-related alternations in the in vitro neonatal rat spinal cord. *Eur. J. Neurosci.* 21 1338–1346. 10.1111/j.1460-9568.2005.03971.x

49. Pertovaara A (2013) The noradrenergic pain regulation system: a 1727 potential target for pain therapy. *Eur J Pharmacol* 716:2–7.

50. Proudfit, H.K.; Clark, F.M. 1991. The projections of locus coeruleus neurons to the spinal cord. *Prog. Brain Res. 88*, 123–141.

51. Raia SN. Carr DB.,Cohen M., Vader K. (2020). The revised International Association for the Study of Pain definition of pain: concepts, challenges, and compromises. *Pain* 161 1976-1982

52. Rocha-González H. I., Meneses A., Carlton S. M., Granados-Soto V. (2005). Pronociceptive role of peripheral and spinal 5-HT7 receptors in the formalin test. *Pain* 117 182–192. 10.1016/j.pain.2005.06.011

53. Rothstein, J.D. Dykes-Hoberg, M. Pardo, C.A.; Bristol, L.A.; Jin, L. Kuncl, R.W Kanai, Y. Hediger, M.A Wang, Y. Schielke, J.P. et al. 1996 Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron 16*, 675–686

54. Singewald, N.; Philippu, A. 1998 Release of neurotransmitters in the locus coeruleus. *Prog. Neurobiol. 56*, 237–267

55. Siwiec M., Kusek M., Sowa J. E., Tokarski K., Hess G. (2020). 5-HT7 receptors increase the excitability of hippocampal CA1 pyramidal neurons by inhibiting the A-type potassium current. *Neuropharmacology* 177:108248. 10.1016/j.neuropharm.2020.108248

56. Smith K. M., Boyle K. A., Madden J. F., Dickinson S. A., Jobling P., Callister R. J., et al. (2015). Functional heterogeneity of calretinin-expressing neurons in the mouse superficial dorsal horn: implications for spinal pain processing. *J. Physiol.* 593 4319–4339. 10.1113/JP270855

57. Tang Z.-Q., Trussell L. O. (2015). Serotonergic regulation of excitability of principal cells of the dorsal cochlear nucleus. *J. Neurosci.* 35 4540–4551. 10.1523/JNEUROSCI.4825-14.2015

58. Tokarski K., Kusek M., Hess G. (2011). 5-HT7 receptors modulate GABAergic transmission in rat hippocampal CA1 area. *J. Physiol. Pharmacol.* 62 535–540.

59. Turk DC, Wilson HD, Cahana A. (2007) Treatment of chronic non-cancer pain. *Lancet* 377:2226–2235.

60. Viguier F., Michot B., Hamon M., Bourgoin S. (2013). Multiple roles of serotonin in pain control mechanisms–implications of 5-HT7 and other 5-HT receptor types. *Eur. J. Pharmacol.* 716 8–16. 10.1016/j.ejphar.2013.01.074]

61. Viguier F., Michot B., Kayser V., Bernard J.-F., Vela J.-M., Hamon M., et al. (2012). GABA, but not opioids, mediates the anti-hyperalgesic effects of 5-HT7 receptor activation in rats suffering from neuropathic pain. *Neuropharmacology* 63 1093–1106. 10.1016/j.neuropharm.2012.07.023

62. Yang E. J., Han S. K., Park S. J. (2014). Functional expression of 5-H$^{T7}$ receptor on the substantia gelatinosa neurons of the trigeminal subnucleus caudalis in mice. *Brain Res.* 1543 73–82.

63. Yasaka T., Tiong S. Y. X., Hughes D. I., Riddell J. S., Todd A. J. (2010). Populations of inhibitory and excitatory interneurons in lamina II of the adult rat spinal dorsal horn revealed by a combined electrophysiological and anatomical approach. *Pain* 151 475–488. 10.1016/j.pain.2010.08.008