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THE ROLE OF PROTEASOME IN THE DEVELOPMENT, MAINTENANCE AND MANAGEMENT OF CHRONIC PAIN

Presentata da: Dott.ssa Laura Rullo

Coordinatore Dottorato	**Supervisore**
Prof.ssa Maria Laura Bolognesi	Prof.ssa Patrizia Romualdi

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Abstract

Chronic pain is defined as pain that persists past normal healing time and hence lacks the acute warning function of physiological nociception. Generally, it lasts or recurs for longer than 3 months and represents one of the most frequent causes for which patients search medical care. Albeit several mechanisms seem to be involved in the pathogenesis of this pathological condition, and new potential targets have been also identified for its treatment, opioids still represent the gold standard analgesics. However, their use is often hampered by the development of several adverse side effects, including the development of analgesic tolerance and opioid-induced hyperalgesia (OIH). Although these phenomena are not yet completely understood, much evidence showed that many molecular mechanisms, including changes in opioid receptors, neurotransmitter release, and glia/microglia activation, are involved in their appearance, as well as in the development of chronic pain.

Over the last years, beside the above-mentioned mechanisms, a crucial role has also been proposed for oxidative stress phenomena and proteasome function in the development of chronic pain and side effects related to its treatment. In this regard, substantial data showed how the production of reactive species as well as a dysfunction of proteasome machinery could participate to the development of analgesic tolerance, OIH and in chronic pain symptom appearance (central sensitization, hyperalgesia, and allodynia), probably through the alterations of cellular signaling or neuroinflammation response. Indeed, the use of proteasome inhibitors or antioxidant drugs seems to be useful to prevent/counteract the appearance of these phenomena.

Based on this evidence and in order to better elucidate these aspects, the aim of this PhD thesis was to investigate the effects of a series of opioid drugs on cell oxidative stress, antioxidant enzymatic machinery and proteasome expression and activity in vitro. In addition, the involvement of the proteasome complex in the development of chronic pain conditions was investigated utilizing an experimental model of oxaliplatin-induced neuropathy (OXAIN), *in vivo.*

For these purposes, we assessed the ability of four different opioids analgesic drugs to produce oxidative stress, to affect cell antioxidant response as well as to alter the proteasome β2 and β5 subunit proteolytic activities and gene expression in SH-SY5Y cell line. In order to highlight a possible correlation between changes of these parameters and the different analgesic drug pharmacological profile, cells were exposed to morphine or fentanyl (full MOR agonists),

buprenorphine (partial MOR agonist) or tapentadol (bifunctional MOR/NRI analgesic). In addition, based on the peculiar binding profile of buprenorphine, acting as MOR agonist at low concentrations and as NOP agonist at high ones, we also investigated the effects of this opioid drug on proteasome parameters.

Data showed that the selected drugs alter differently ROS production levels. In particular, results revealed that the ROS increasing effect of morphine is not shared by the other investigated opioid drugs, thus suggesting that the different pharmacological profile could influence this parameter. On the contrary, all the selected compounds were able to alter in the same manner the antioxidant machinery. Our data also indicated that morphine, fentanyl, buprenorphine, and tapentadol produced different alterations of β2 trypsin- like and β5 chymotrypsin- like activities. In fact, while morphine and fentanyl were able to increase the proteolytic activity after prolonged exposure, a different picture was observed for buprenorphine and tapentadol, with buprenorphine reducing proteasome activity after prolonged exposure intervals and tapentadol not inducing significant alterations at any assessment interval. However, we observed that buprenorphine was able to affect proteasome activity in opposite directions, depending on its concentrations. In fact, and similarly to morphine and fentanyl, it decreased proteasome activity at the lower concentration whereas an increase of the same parameter was observed at higher ones. In light of the above mentioned peculiar pharmacological profile of buprenorphine, the obtained data seems to suggest that the level of MOR agonism could be strongly related with proteasome activation.

Moreover, given the strong correlation between proteasome and oxidative stress, if taken together the obtained results could suggest that a lesser MOR activation can be related to a lower ROS production that does not request a *de novo* synthesis of proteolytic subunits and an increase of their activation.

Regarding the *in vivo* studies, the involvement of proteasome in neuropathic pain was evaluated measuring β2 trypsin-like and β5 chymotrypsin-like activities of 20S Proteasome in the spinal cord (lumbar, thoracic and cervical portions) and supraspinal CNS regions (SSCx, TH and PAG) of OXAIN suffering rats. Moreover, to evaluate and discern the involvement of the constitutive and inducible (immune) proteasome complex, the gene expression of both β5 (constitutive) and LMP7 (or β5i, inducible) proteasome subunits was assessed in the CNS supraspinal areas. Data revealed that rats treated with oxaliplatin (2.4 mg kg-1 i.p., daily for 10 days) showed a significant increase in chymotrypsin-(β5) like activity of the proteasome 20S,

in the thalamus (TH) and somatosensory cortex (SSCx). In addition, a selective up-regulation of β5 and LMP7 (β5i) subunit gene expression was also assessed in the SSCx. Furthermore, our study revealed that oprozomib, a selective β5 subunit proteasome inhibitor, was able to normalize the spinal prodynorphin gene expression upregulation induced by oxaliplatin, as well as to revert mechanical/thermal allodynia as well as mechanical hyperalgesia observed in oxaliplatin-treated rats. These results underline the role of proteasome in the OXAIN and also suggest new pharmacological targets to counteract this neuropathy.

Data presented in this thesis provide original evidence about the ability of the selected opioids to alter in different manner the activities of proteasome as well as to determine a different degree of oxidative stress in cells. Moreover, they highlight the involvement of proteasome in phenomena related to OXAIN development. Even though the modulation of this degradation complex as well as the oxidative stress process seems to be a promising pharmacological target for the treatment of chronic pain, further studies are required to better clarify their role.

1.General background

1.1 What is pain?

The definition of pain was strongly influenced by the beliefs in the course of the centuries.
The ancient Greeks considered pain like an emotional experience or as godly punishment for disbelievers (Bial and Cope., 2011). Indeed, the etiology of word pain itself comes from the Latin “poena", which means punishment and in turn from ancient Greek “poine”, which means “penalty”.
In 1662, Renè Descartes in “Treatise of Man” refused the idea that pain come from outside and for the first time described this condition as internal mechanical process. For Descartes the body was a machine, and pain represented a disturbance within the “machine” that passed through nerves to the brain.

Figure 1: Pain representation from “Treatise of a man”, (Renè Descartes.,1662)

Starting from that time, a lot of theories were proposed to describe the mechanisms underlying pain. Among these, particular attentions received the “Specificity Theory of Pain”, which postulated the presence of dedicated pathways for each somatosensory modality and the “Gate Control Theory of Pain”, which for the first time proposed the idea that non-noxious stimuli are able to close the ‘gate’ to noxious stimuli and therefore able to suppress pain sensations (Moayedi and Davis, 2013; Melzack and Wall, 1965).

The culmination of centuries of ideas and work that have explored the concept of pain has found its expression in the definition of pain established by the International Association for the Study of Pain (IASP) which actually defined pain as *"*An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (Raja et al., 2020). This last definition of pain, released by IASP in June 2020, comprises key notes underlining that pain is always a personal experience that is influenced to varying degrees by biological, psychological, and social factors and that, although pain usually serves an adaptive role, it may have adverse effects on functions and social and psychological well-being (Raja et al., 2020).

Although pain represent a complex experience, its classification seems to be important in order to ensure an adequate pain management. According to the World Health Organization (WHO), anatomic, etiologic, duration, and pathophysiological are the most commonly used classification systems.

The anatomical classification provides information about the specific body region or area experiencing pain and identifies three different kind of pain visceral, somatic and mixed (Orr at al., 2017).

The etiological classification has the function to describe the causative factor of pain and classifies pain as malignant or non-malignant (cancer or no cancer-related) (Orr at al., 2017).

The duration classification gives information about the time for which patients experience pain. According to this classification pain can be defined as transient, acute, chronic (Table.1) (Orr at al., 2017). The *transient* pain is elicited by the activation of nociceptive transducers (nociceptors) in skin or other tissues of the body in the absence of any tissue damage, that ends when the offending physical disturbance is no longer affecting the body. *Acute* pain usually follows trauma to tissue, has short duration (3-6 months) and is associated with temporal reductions in intensity, is evoked by significant injury of body tissue and activation of nociceptive transducers at the site of local tissue damage.

Chronic pain is commonly triggered by an injury or disease and its duration continues after ordinary timeframe for healing of tissues. Chronic pain has often an unclear pathophysiology and it is associated with significant modifications in the neuronal pathways involved in the transmission and processing of pain signals, which are supporting factors independent of nociceptors' action. The injury may exceed the body's capability for healing, because of the loss of the body part, the extensiveness of the trauma, or the involvement of the nervous system in the injury itself. The nervous system may be damaged by the original injury in such a way as to be unable to restore itself to a normal state (Silverthon, 2007). This kind of pain can

significantly affect the quality of life of patients and is often associated with several negative psychological condition (Orr et al.,2017).

<u>The pathophysiological classification is</u> based on the pathophysiological mechanism of injury to the body resulting in pain. Through this classification is possible identify the nociceptive, inflammatory and neuropathic pain **(Figure 2)**.

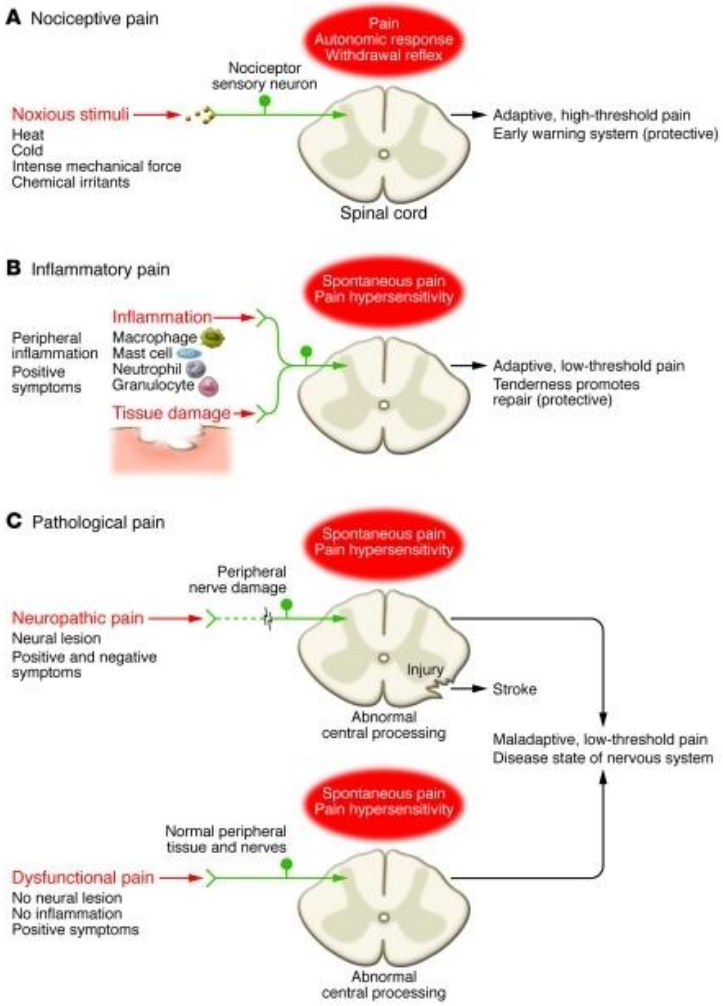

***Figure 2**:* Pain classification. **a)** nociceptive pain; **b)** inflammatory pain; **c)** neuropathic pain (Woolf et al.,2010)

The first kind of pain is generally defined as an early-warning physiological protective system, essential to detect and minimize contact with damaging or noxious stimuli **(Figure 2a)** (Woolf., 2010). The two major categories of nociceptive pain are somatic and visceral. Somatic pain is caused by the activation of nociceptors (pain receptors) in either surface tissues (skin, mucosa of mouth, nose, urethra, anus, etc.), while visceral pain is caused by the activation of nociceptors located in the viscera (the internal organs of the body that are enclosed within a cavity, such as thoracic and abdominal organs). It can occur due to infection, distension from fluid or gas, stretching or compression, usually from solid tumors.

The second kind of pain is also adaptive and protective. This pain is caused by activation of the immune system by tissue injury or infection **(Figure 2b)** (Woolf., 2010).

Finally, there is the pain that is not protective, but maladaptive and which generally resulting from abnormal functioning of the nervous system. This pathological condition **(Figure 2c)**, which is not a symptom of some disorder but rather a disease state of the nervous system, can occur after damage to the nervous system (neuropathic pain), but also in conditions in which there is no such damage or inflammation (nociplastic pain) (Woolf.,2010; Chimenti et al., 2018).

1.2Pain signaling

The sensation of pain is associated with the activation of a specific pathway which generally remains silent during homeostasis in the absence of pain and are activated when there is a potential of noxious stimulus. Fundamentally, pain transmission is strictly dependent on the balance of the excitatory and inhibitory influences that act on the neuron circuits of the somatosensory system. There are two main routes that conduct signal transmissions: ascending and descending pathways (Reddi et al., 2013).

The activation of these pathways is crucial for the pain perception and is generally characterized by three different events: transduction, transmission and modulation (Yam et al., 2018).

These physiological events, which basically represent the pain mechanism, involve several areas of both peripheral and central nervous system and can be strongly influenced by subjective phenomena like sociological, psychological and genetic factors of the individual (Coghill, 2010).

For instance, it has recently been suggested that neonates experiencing painful procedures, such as heel punctures, not only have a heightened pain response at the time due to the incomplete development of pain inhibition pathways, but also have increased pain responses later in life

(Beggs, 2015). The latter highlights how early life experiences can shape the nociceptive system in adulthood. Furthermore, this complex mix of factors means that two individuals receiving the same noxious stimuli can experience two very different sensory experiences, rendering the task of creating an effective therapy even more challenging.

Basing on this evidence, the experience of pain could be described along two main axes: the sensory-discriminative dimension, comprising spatial, temporal, and intensity properties and the affective-motivational dimension that is strongly related to the unpleasantness of the stimulus and to the behavioral reactions that it evokes (Hofbauer et al., 2001). Each of these dimensions can undertake multiple intertwined paths that, in turn, can interact with additional dimensions, making the system intrinsically very complex. The fact that it is a personal experience implies a subjective value that is not easily quantifiable, in other words it is difficult to measure and evaluate a condition of pain in its entirety (Hartrick and Rozek, 2011).

1.2.1The ascending pain pathway

The nociception process originates in periphery, where intense thermal, mechanical or chemical stimuli are detected by a subpopulation of specialized peripheral nerve fibers, called nociceptors (Basbaum and Jessell, 2000). Nociceptors possess biophysical and molecular properties that make able them to selectively detect and respond to potentially injurious stimuli, indeed they express a combination of ion channels that tuned to respond with high threshold only when stimulus intensities reach the noxious range (Basbaum et al., 2009; Woolf and Ma, 2007).The cell bodies of nociceptors are located into the dorsal root ganglia (DRG) for the body, where their afferents go alongside the spinal cord and synapse with spinal neurons. Conversely, trigeminal ganglion neurons have their cell bodies located into the trigeminal ganglion and they project either directly to the brain stem or to the upper regions of the spinal cord (Capra and Dessem, 1992; Erzurumlu et al., 2010).

The nociceptors, basing on anatomical and functional criteria, can be divided in two main groups (Meyer, 2008). The first includes medium diameter myelinated (Aδ) afferents that mediate acute, well-localized “first” or fast pain. These myelinated afferents differ considerably from the larger diameter and rapidly conducting Aβ fibers that respond to innocuous mechanical stimulation. The second class of nociceptor includes small diameter unmyelinated “C” fibers that convey poorly localized, “second” or slow pain (Dubin and Patapoutian, 2010). Furthermore, electrophysiological studies have showed that Aδ nociceptors can be subdivided

into two major classes: Type I (HTM: high threshold mechanical nociceptors) and Type II. The first class respond to both mechanical and chemical stimuli but have relatively high heat thresholds (>50C). Type I fiber probably mediates the first pain provoked by pinprick and other intense mechanical stimuli Differently, the second class of Aδ nociceptors have a much lower heat threshold, but a very high mechanical threshold. Activity of this afferent is likely in the mediation of the "first" acute pain response to noxious heat (Basbaum et al., 2009).

Like the myelinated afferents, the unmyelinated C fibers are also heterogeneous. The majority of C fibers are polymodal and include a population that is both heat and mechanically sensitive (CMHs) (Costigan et al.,2009). Of particular interest are "silent nociceptors", unmyelinated afferents which are heat responsive but mechanically insensitive and that develop mechanical sensitivity only in the setting of injury (Schmidt et al., 1995). However, it is interesting to note that not all C fibers are nociceptors. Indeed, some of these fibers respond to cooling, and in particular it has been, also, showed that a population of unmyelinated afferents responds to innocuous stroking of the hairy skin, but not to heat or chemical stimulation thus suggesting that these fibers mediate the pleasant touch (Olausson et al., 2008). Based on molecular and neuroanatomical characterisation, C-fibers can be further classified in peptidergic neurons and non-peptidergic neurons, with the former that release neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) (Woolf and Ma, 2007; Basbaum et al. 2009; Lawson 2002; Snider, McMahon, 1998). Primary afferent nerve fibers project to the dorsal horn of the spinal cord, which is organized into anatomically and electrophysiological distinct ten layers called "Rexed laminae" (Basbaum and Jessell, 2000). Small caliber, unmyelinated C fibers, and medium caliber, myelinated Aδ convey nociceptive information principally to superficial (laminae I/II) and deep (V/VI) laminae of the dorsal horn, as well as to the circumcanular lamina X. On the other hand, large caliber, myelinated, rapidly-conducting Aβ fibers transmit information to deeper laminae (III–VI) (Millan, 2002). The spinal cord is characterized by several neuronal cell types, which make connections with primary afferents. These neurons have different properties, which depend on the precise synaptic inputs received and they respond to different types of sensory information. A rough classification includes nociceptive-specific (NS) cells, that are mainly found superficially and synapse with Aδ- and C-fibers only, firing action potentials when a painful stimulus is detected at the periphery.

Then, there are proprioceptive cells which receive input exclusively from Aβ-fibers and only respond to touch. A third type of neuron, termed wide dynamic range (WDRs), receive input from all three types of sensory fiber, and therefore respond to the full range of stimulation (D'Mello and Dickenson, 2008). Another population of neurons located in laminae I-III are

called interneurons because of their axons that remain and arborize locally. Interneurons can be excitatory or inhibitory, where the formers have as main neurotransmitter glutamate, whereas the inhibitory ones use GABA and/or glycine. Essentially, there are two scenarios: primary afferent fibers can either stimulate projection neurons directly, thus relaying the message to the brain or indirectly via excitatory (EXINs)/ inhibitory interneurons (ININs). These can increase or decrease the response of NS cells and WDRs, thus influencing the output of the dorsal horn (Todd, 2010). It has been shown that non-neuronal cell within the spinal cord (astrocytes and microglia), are also able to influence pain transmission through the dorsal horn, particularly under pathological conditions. The major output from the dorsal horn to the brain is constitute by projection neurons within laminae I and V (Basbaum and Jessell, 2000). This neuronal tract, named 'nociceptive ascending pathways', includes the spinothalamic and spinoreticulothalamic tracts, which carry pain messages to the thalamus and brainstem, respectively **(Figure 3)**. The first is particularly relevant to the sensory-discriminative aspects of the pain experience whereas the second seems to be more relevant to poorly localized pains. More recently, particular attention has been also concentrated on spinal cord projections to the parabrachial region of the dorsolateral pons (Becerra et al., 2001; Baliki et al., 2010) and the periaqueductal grey matter (PAG) (Dunckley et al., 2005) because the output of this region provides for a very rapid connection with the amygdala and nucleus accumbent, regions generally considered to process information relevant to the aversive properties of the pain experience (Basbaum et al., 2009). From these brainstem and thalamic loci, information achieves cortical structures.

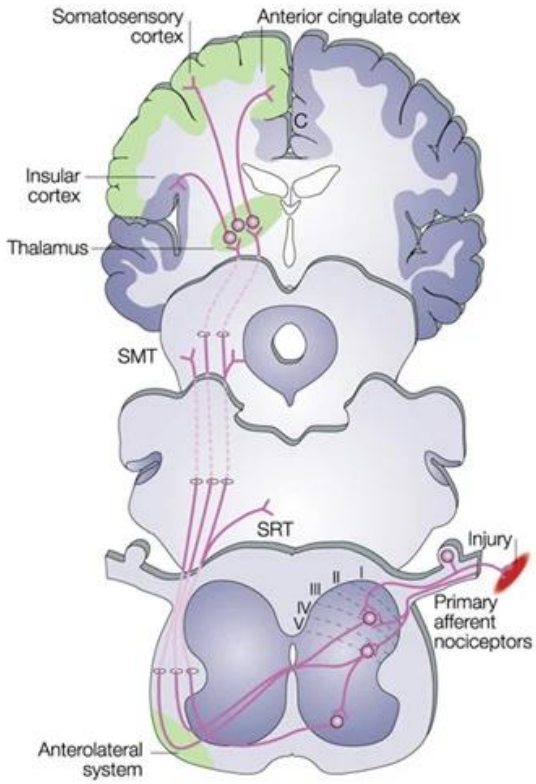

Figure 3: Anatomy of ascending pain pathway (Fields et al., 2004)

Among the areas of the CNS which are most commonly activated by nociceptive impulses are the primary somatosensory cortex, the secondary somatosensory cortex, the anterior cingulate cortex, the prefrontal cortex, the insular cortex, the thalamus, the hypothalamus, the cerebellum and the basal ganglia (Apkarian et al., 2005). Neuronal activation in these areas has been demonstrated with anatomy and electrophysiology studies showing an afferent nociceptive connection to these regions (Apkarian et al., 2005; Friedman et al., 1986; Apkarian et al., 1998; Craig et al., 1991; Dum et al., 2009; Saab et al., 2003; Monconduit et al., 2005).Rather, pain results from activation of a distributed group of structures, some of which are more associated with the sensory discriminative properties (such as the somatosensory cortex) and others with the emotional aspects (such as the anterior cingulate gyrus and insular cortex) **(Figure 4)**.

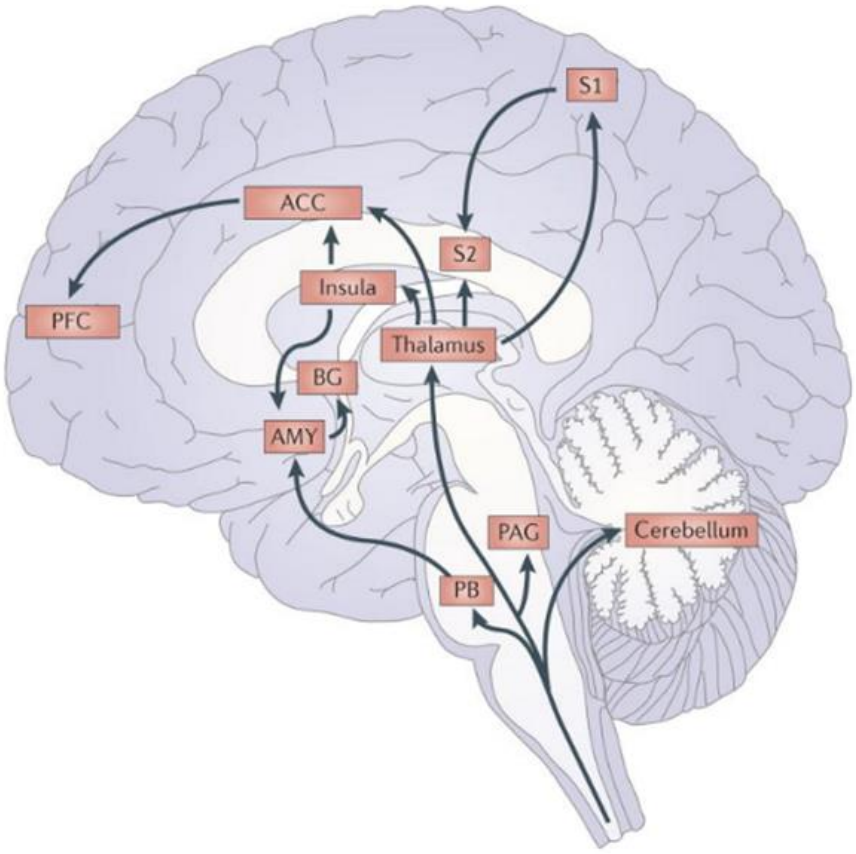

Figure 4*:* Brain areas commonly activated by nociceptive stimuli. Painful impulses are transmitted to the CNS by numerous spinal pathways, including the spinothalamic pathway, the spinoparabrachial pathway, the spino-reticulum-thalamus-cortical pathway. From the thalamus, nociceptive information is transmitted to numerous brain areas: anterior cingulate cortex (ACC), primary somatosensory cortex (S1), secondary somatosensory cortex (S2), insula. Information from the amygdala (AMY) is transmitted to the basal ganglia (BG). Other areas involved are the prefrontal cortex (PFC), the cerebellum and the periaqueductal grey matter (PAG). (Bushnell et al., 2013)

1.2.2 The descending pain pathway

The afferent pain pathway is subjected to the control of the descending pathways (Melzack and Wall, 1965; Millan, 2002). Descending control of spinal nociception originates from many brain regions and plays a fundamental role in both acute and chronic pain. Descending control was considered as an "analgesia system" but is now being replaced with a more nuanced model in which pain input is prioritized relative to other competing behavioral needs and homeostatic demands (Heinricher et al., 2009). It has become clear that certain mechanisms could either enhance or impede the passage of nociceptive information. According to this view, descending pathways can exert both "descending inhibition" (DI) and "descending facilitation" (DF) on spinal nociceptive transmission, even though there is no absolute anatomical separation between these processes (Millan, 2002; Woolf, 2004). The balance between inhibition and facilitation is dynamic, and depends on different behavioral, emotional and pathological conditions. An intense level of stress or fear is associated with a reduction in the response to pain (Rhudy and Meagher, 2000), while inflammation, nerve injury or disease are associated with hyperalgesia, which can be partially ascribed to descending faciliatory mechanisms (Ossipov et al., 2010). Descending control arises from a number of supra-spinal sites, including the PAG, the rostral part of the ventromedial marrow (RVM), the more lateral and caudal dorsal reticular nucleus (DRt) and caudal ventrolateral medulla (cVLM) (Heinricher et al.,2009). From PAG the neurons do not project directly to the spinal cord. The RVM, that includes the *nucleus raphe magnus* and the adjacent reticular formation represents the main descending projection. The neurons of this area receive a dense innervation from the PAG and project to the dorsal horn through the dorsolateral funiculus, forming synapses with spinal cord neurons of the dorsal horn, in both superficial and deep layers (Heinricher et al.,2009; Fields et al., 1985; Almeida et al.,1999). Terminals of descending pathways originating in the RVM and other brainstem nuclei (for example, nucleus raphe magnus, A5, A6 and A7 nuclei) interact with afferent fibers, interneurons and projection neurons in the dorsal horn (Millan et al.,2002) **(Figure 5)**.

Electrophysiological recordings in the RVM have reported two types of neurons. One class of RVM neuron, the 'ON' cell, shows a burst of activity beginning just before withdrawal from a noxious stimulus. The other major cell class, the 'OFF' cell, has the opposite firing pattern, pausing during withdrawal from noxious heat. Several studies suggest that 'ON' cells facilitate, while 'OFF' cells inhibit, pain transmission (Heinricher et al., 2009).

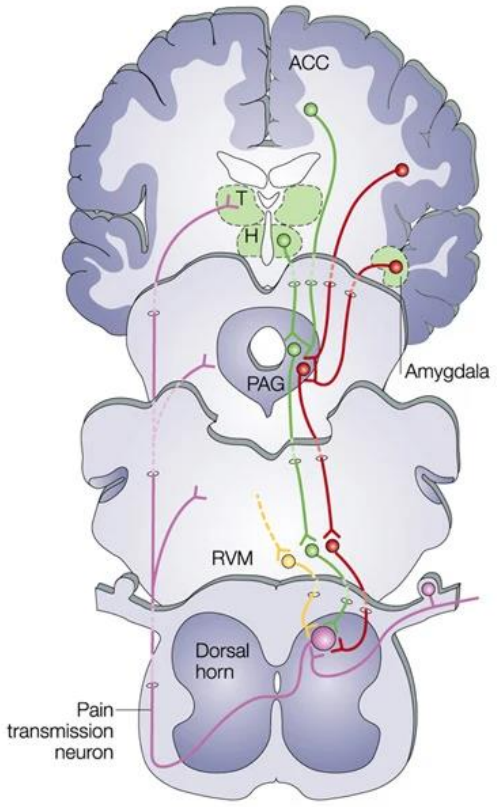

Figure 5: Anatomy of descending pain pathway (Fields et al., 2004)

The endogenous opioid system, the descending noradrenergic system, and serotonergic neurons represent three components of the descending system that play critical roles in modulating pain transmission (Vanegas and Schaible, 2004). However, among these components a great interest is focused on the monoaminergic pathway, which implies a complex interaction between primary nociceptive afferents, dorsal horn projection neurons, local interneurons, and is mainly mediated by noradrenaline neurotransmitters (NA), serotonin (5-HT) and dopamine (Bannister and Dickenson, 2016).

The predominant source of serotonergic input to the spinal cord arises within the vicinity of the RVM and, most prominently, from the *nucleus raphe magnus*. Serotonin causes hyperpolarization of afferent nociceptive fiber terminals and dorsal horn projection neurons when interacting with 5-HT1 and 5-HT2 receptors, and it produces excitation in spinal GABAergic interneurons when interacting with 5-HT3 receptors (Millan et al.,2002). Similarly, noradrenaline causes hyperpolarization of projection neurons when interacting with α-2A receptors and over terminals of primary afferent fibers when it interacts with α-2B/C receptors, whereas it induces excitation of dorsal horn inhibitory interneurons via α-1A receptors (Millan

et al.,2002). The dopaminergic pathways originate mainly from neurons A11 of the posterior periventricular thalamus (Björklund and Skagerberg, 1979; Millan, 2002). Their activation results in a reduced response to noxious stimuli mediated by D2 receptors (D2R), with concomitant inhibition of neurotransmitter release from primary afferents. In fact, it has been shown that administration of D2R agonists reduces the response to pain, whereas administration of D2R antagonists increases the nociceptive response in models of persistent pain (Morgan and Franklin, 1991; Magnusson and Fisher, 2000; Taylor et al., 2003). Conversely, activation of D1 / D5 receptors results in facilitated transmission of nociception (Yang et al., 2005).

1.3 Chronic Pain

Chronic pain was defined as pain that persists past normal healing time and hence lacks the acute warning function of physiological nociception. Generally, chronic pain is pain that lasts or recurs for longer than 3 months (Treed et al.,2019). It represents one of the most frequent causes for which patients search medical care (Mantyselka et al 2001). Although mortality rates are highest for other pathologies the chronic pain seems to be one of the main sources of human suffering and disability. Indeed, though physiological pain serves an important protective function, pain can take on a disease character in pathological states such as inflammation, neuropathy, cancer, viral infections, chemotherapy and diabetes. Individuals with chronic pain manifest hyperalgesia (increased sensitivity to painful stimuli), disease-induced, therapy-resistant deviations from normal tactile sensation, such as paresthesia and dysesthesias. Since the impact of this condition on the patients' life and in order to create a classification system that is applicable in primary care and in clinical settings for specialized pain management recently, pain has been recognized as a disease itself, by the International Classification of Diseases (ICD) and a systematic classification of chronic pain was developed by a task force of IASP for the 11th *ICD.*

This classification distinguishes chronic primary (pain can be conceived as a disease) chronic secondary pain (pain initially manifests itself as a symptom of another disease)and defines the most common clinically relevant groups of chronic pain which are represented by (1) chronic primary pain; (2) chronic cancer-related pain; (3) chronic postsurgical or posttraumatic pain; (4) chronic neuropathic pain; (5) chronic secondary headache or orofacial pain; (6) chronic secondary visceral pain; and (7) chronic secondary musculoskeletal pain. There is some overlap between these groups of chronic pain conditions (e.g., neuropathic pain caused by cancer or its treatment) in ICD-11 (e.g., chronic headaches) (Treed et al.,2015). These pain diagnoses have been implemented in the 11th version of ICD that was released by WHO in June 2018. The classification provides precise definitions and further characteristic features of the respective diagnoses according to the content model of the WHO for ICD-11, including the severity of pain, its temporal course, and evidence for psychological and social factors **(Figure 6)** (Treede et al, 2019).

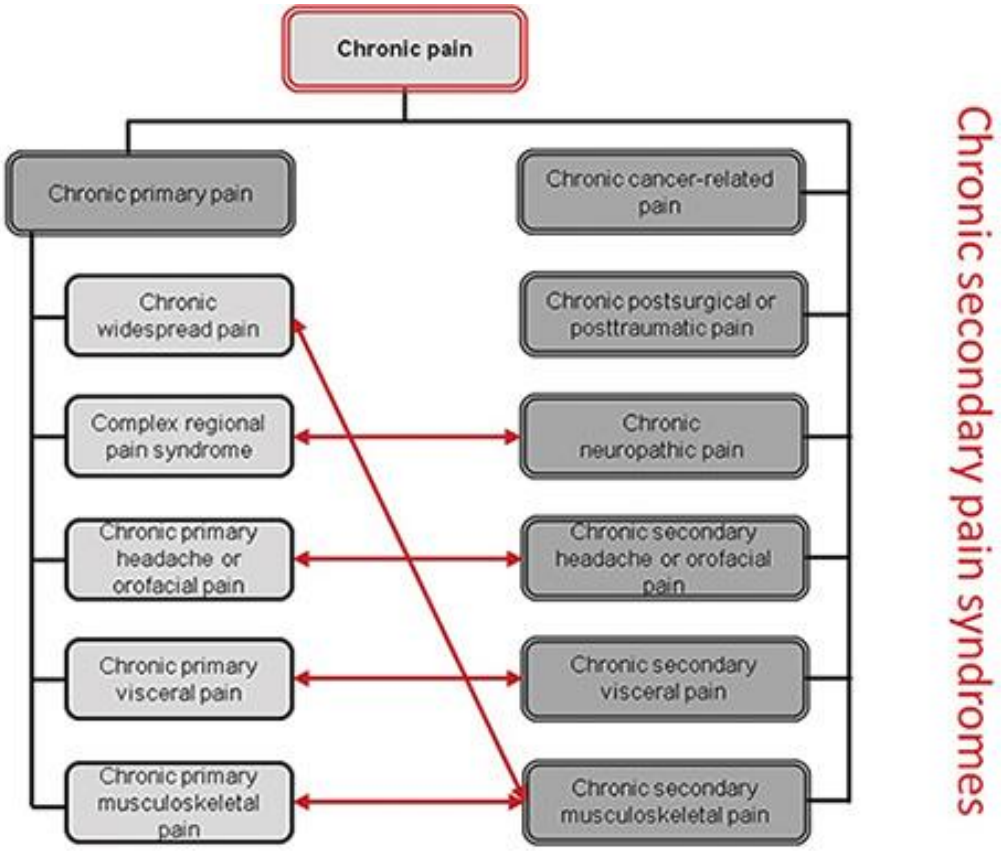

Figure 6. Structure of the IASP Classification of Chronic Pain (Treed et al.,2019)

1.4 Neuropathic pain

The IASP initially describes with term of neuropathic pain “the pain initiated or caused by a primary lesion or dysfunction in the nervous system” (Merskey et al.,1994). However, in a new definition, proposed by Jensen and colleagues, neuropathic pain is stated as ‘the pain initiated or caused by a lesion or disease of the somatosensory system’ (Jensen et al.,2011). This new definition substitutes the word “dysfunction” with “disease” in order to underline the difference between neuropathic pain and the pain caused by the plastic changes that occur in response to an intense nociceptive stimulation. Similarly, the replacement of the “nervous system” concept with the term “somatosensory system” seems to be necessary to differentiate neuropathic pain from the pain caused by an eventual lesion in other specific areas of the nervous system. Definitely, the new definition assumes that the neuropathic pain could be caused by a lesion and/or disease of the peripheral and central somatosensory system (Jensen et al., 2011). Damage to this system can be attributed to traumatic lesions, inflammation, infections, cancer infiltrations, as well as can be also consequences derived from pharmacological treatments (for example, chemotherapy and anti-retroviral therapy) (Dworkin et al., 2003; Woolf and Mannion, 1999). Although the nerve trauma is necessary, it is not sufficient for the development of a neuropathic pain condition, which occurs only in a minority of patients with a neurological lesion. Indeed, it is due to secondary peripheral and central changes in the nociceptive systems (Bouhassira, 2019). The nervous system has high adaptive capacities (Plsek and Greenhalg, 2001), however maladaptive plasticity phenomena can occur within the nociceptive system which causes a neuropathic pain condition (Costigan et al., 2009).

Several experimental evidence have shown that the physiopathological mechanisms of neuropathic pain are associated with nociceptor sensitization, spontaneous and ectopic firing of afferent nociceptive fibers and with changes in the molecular expression of ion channels, neurotransmitters and receptors in the nociceptive axons, as well as in the dorsal root ganglia neurons. Within the framework of neuropathic pain, the sensitization of second-order nociceptive neurons (central sensitization) and changes in the neurotransmitters, neuropeptides and receptor expression represent the main changes established at the spinal level. In addition, many other changes could be responsible of the dysregulation of the inhibitory interneurons in the dorsal horn and the descending modulatory pathways, the synthesis and release of proinflammatory cytokines and consequent glia cell reactivation and, finally, a morphological and functional reorganization of the afferent projections in the dorsal horn (Zimmerman et al.,2001; Campbell et al.,2006).

Clinically negative and positive sensory phenomena coexist in neuropathic pain. Negative phenomena usually include neurological sensory deficits in the painful area, together with other deficits (motor, cognitive etc.), depending on the location of the lesion: deficits in various somatosensory qualities such as touch, hypoesthesia or anesthesia, thermal hypoesthesia, hypoalgesia at the point stimulus and loss of vibratory sensation. These symptoms are unpleasant but not painful. Positive spontaneous symptoms such as paresthesia and dysesthesia (e.g. tingling, numbness, pins and needles), paroxysmal pain and continuous superficial pain also occur (Bouhassira, 2019). Other positive symptoms that arise instead, because induced by a stimulus, include hyperalgesia and allodynia (Nickel et al., 2012). Hyperalgesia is an increased response to pain evoked by a harmful over-threshold stimulus and is the result of abnormal processing of inputs from nociceptors. Allodynia is the sensation of pain caused by a non-harmful stimulus and can be triggered in two ways: by the action of low-threshold myelinated Aβ fibers on an altered area of the central nervous system or by a reduction in the threshold of terminal peripheral nociceptors.

Once neuropathic pain has arisen, sensory hypersensitivity typically persists for prolonged periods, even if the original cause may have disappeared, as occurs after a trauma to the nervous system. However, pain associated with acute neural damage turns into chronic neuropathic pain only in a minority of patients (Costigan et al., 2009). Despite the estimates of the risks, it remains to be clarified why a patient, unlike others, may develop chronic pain following an injury and an understanding of this aspect represents a crucial point in order to develop new therapeutic strategies. Epidemiological studies on the prevalence of neuropathic pain indicate a high incidence also associated with risk factors such as gender, age and anatomical site of the lesion. It is also certain that emotional and cognitive factors influence how patients react to chronic pain, but it is less clear whether these factors also contribute to an increased risk of developing pain (Haythornthwaite et al., 2003). The most common conditions associated with neuropathic pain include postherpetic neuralgia, trigeminal neuralgia, painful radiculopathy, diabetic neuropathy, HIV infection, leprosy, amputation, pain in peripheral nerve injuries, chemotherapy treatment and stroke (in the form of central post-stroke pain) (Colloca et al., 2017).

Several experimental studies have showed that an important feature of neuropathic pain is represent by inflammation, even though inflammatory pain and neuropathic pain are classically considered as distinct entities. In this regard, numerous evidence suggest the involvement of neuroimmune interactions in the maintenance of this pathological condition. As reported, lesions in peripheral nerves and inflammation of skin, muscle joints and internal organs leads

to sodium and calcium ions influx in the nociceptors, resulting in depolarization and sensitization, respectively. Peripheral sensitization activates intracellular cascades that induce ionic channel and membrane receptor phosphorylation, subsequently enhancing nociceptor sensitivity to algogenic mediators, and also molecular changes including overexpression of voltage-gated ion channels, receptors to algogenic mediators and neurotransmitters–neuromodulators (Cregg et al.,2010; Zoga et al.,2010). Sensitization and hyperexcitability of injured peripheral nociceptors also induce hyperexcitability of nociceptive spinal neurons, triggering within an increase of electric activity, the expansion of their receptor field and a threshold decrease to afferent inputs as a result of over expression of ionic channels and receptors. These plastic changes in spinal nociceptive neurons induce the phenomenon known as "central sensitization". Indeed, the hyperexcitability of peripheral nociceptors determines a greater release of neurotransmitters such as glutamate, SP, calcitonin gene-related peptide, ATP in spinal cord at the level of the second-order nociceptive neurons. The above mentioned neurotransmitters, interacting with N-methylD-aspartate receptor (NMDA), α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid receptor (AMPA), metabotropic receptors of glutamate (mGluR), neurokinin-1 receptor (NK1R) and purinergic receptors (P2X), cause depolarization of spinal nociceptive projection neurons and the generation of painful signals along the nociceptive spinothalamic pathway (Gu et al.,2003; Neugebauer et al.,2002; Ruscheweyh et al.,2002; Polgar et al.,2008). This chemical neurotransmission induces an influx of calcium ions that activates calcium-dependent intracellular cascades and determines an overexpression ionic channel and membrane receptor and phosphorylation sensitization in the spinal nociceptive neurons of second order (Dolen et al.,2012; Ultenius et al., 2006). Moreover, the neurotransmitters and neuromodulators released from nociceptive primary afferent fibers in the dorsal horn interact with NMDA, AMPA, SP receptor (NK1), purinergic receptors and calcitonin gene-related peptide receptors of microglia and astrocytes (Aronica et al.,2001; Hansson and Ronnback, 2004; Pocock and Kettenmann,2007) Microglial activation induces the release of cytokines (IL1, IL6, TNF-alpha), prostaglandins (PGE2), chemokines (MCP-1 or CCL2) and nitric oxide, which in a paracrine manner amplifies the microglia reactivity favoring the increase of these mediators and long-lasting neuroplastic changes in the spinal cord (Malmberg et al., 1997).

1.4.1 Chemotherapy-induced peripheral neuropathy

Chemotherapy-induced peripheral neuropathy (CIPN) represents one of the most dose-limiting side effect of antineoplastic drugs. This sensory neuropathy is generally characterized by the development of the typical symptoms of neuropathic pain which include allodynia, loss of sensation, paresthesia, numbness, tingling, and gait disturbance (Wu et al., 2019). The development of CIPN can result in a relevant loss of functional abilities and negatively impact patients' quality of life, leading to lowering of the dose and discontinuation of assumption, and ultimately affecting overall survival rates (Flatters et al.,2017). Some chemotherapeutic drugs such as taxanes and oxaliplatin have been associated with a higher prevalence and duration of CIPN, which can persist for up to six months or two years from the end of chemotherapy treatment (Wu et al., 2019). As reported in several studies, different molecular mechanisms and various components of the PNS and SNC seems to be involved in the development of CIPN **(Figure 7)** (Brandolini et al., 2019).

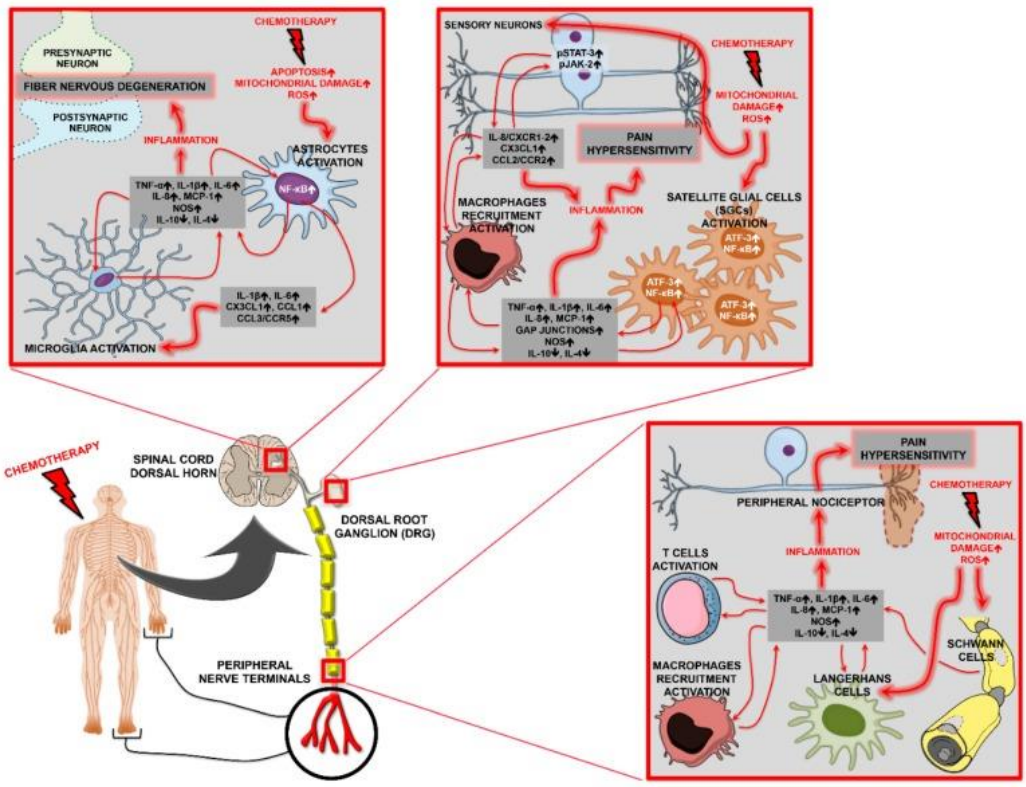

Figure 7. Molecular mechanisms involved in the development of CIPN (Brandolini et al., 2019)

It has been shown that at the neuronal level antineoplastic drugs are able to damage microtubules affecting microtubule-based axonal transport, damage mitochondrial function, alter ionic homeostasis, or directly target DNA (Jordan et al.,2004), leading to peripheral nerve degeneration or small fiber neuropathy. Moreover, some drugs like taxanes and vinca alkaloids exhibit an antiproliferative effect by disrupting mitotic spindles and causing cell cycle arrest (Jordan et.,2004). For what concern platinum agents are known to cause CIPN by damaging the DRG through mitochondrial dysfunction and apoptosis and also causing DNA damage or oxidative stress (Grisold et al.,2012). New drugs, such as bortezomib are also correlated with high incidences of CIPN by affecting tubulin polymerization (Grisold et al.,2004; Fukuda et al.,2017). A crucial role in CIPN development seems to be played by glial cells. Indeed, chemotherapy lead to the activation of apoptosis inducing alterations of Schwann cells, satellite cells in the DRG, and astrocytes in the spinal cord (Han et al.,2013). Loss of glia cells results in a decrease in the protection and sustainment of nerve fibers and consequent defects in the propagation of the action potential (Boyette-Davis et al.,2015). Several findings indicate that CIPN can also cause morphological changes that are responsible of the involvement of inflammation and immune responses. Moreover, the formation of mitochondrial DNA adducts and defects in electron transport chain proteins, following a chemotherapy treatment, leads to mitochondrial dysfunction (Salvemini et al.,2011; McDonald et al.,2002). This event is accompanied by dysregulation in the redox balance and an increase in ROS within cells (Salvemini et al.,2011). These reactive species can trigger perturbations in peripheral neurons, such as mitochondrial apoptosis, inflammation, and subsequent nerve degeneration (Salvemini et al.,2011; McDonald et al.,2002). ROS can also damage biomolecules such as phospholipids, resulting in demyelination, oxidized proteins, and an increase in carbonyl by-products, which can activate transient receptor potential vanilloid (TRPV) channels, impair antioxidant enzymes, and destroy microtubules (Salvemini et al.,2011).

Intracellular ROS can also cause hyperexcitability of peripheral nociceptor by increasing pro-inflammatory mediators (interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), bradykinin, and nerve growth factors) (Salvemini et al.,2011; Wang et al.,2004). All these functional and metabolic changes participate to the development and maintenance of peripheral neuropathic injuries in neurons (Salvemini et al.,2011).

1.5 Opioids, analgesia and pain management

1.5.1 Endogenous Opioid system

The opioid system represents one of the most important endogenous systems involved in neurotransmission. It consists of specific peptidergic ligands with their corresponding receptors. This system is widely distributed in the CNS and periphery and is involved in the modulation of different functions. Indeed, in addition to the well-known modulation of the nociceptive transmission, this system is also involved in the regulation of gastrointestinal, endocrine and autonomic functions, in reward and dependence mechanisms as well as in memory and learning processes.

The existence of the opioid system is crucial to understand the pharmacological actions of the alkaloid morphine and its derivatives, the opiates (Romualdi and Candeletti, 2016).

The word opiate is related to the natural substances chemically similar to morphine contained in opium and to those synthetically derived from it. In fact, in the 1980s according to the chronological acquisition of knowledge about central analgesics, a distinction between opiates (exogenous drugs) and opioids (the endogenous substances that represent the natural ligands of opioid receptors) was done. However, few years later, the English terminology adopted the word "opioids" to indicate both endogenous opioid peptides/receptors and exogenous opiate drugs.

1.5.1.1 Opioid peptides

Structurally the opioid neuropeptides are short sequences of amino acids and represent the opioid receptors' natural endogenous ligands. All classical opioid peptides may be considered belonging to three families: the enkephalins, the endorphins and the dynorphins. Each family derives from a distinct precursor: the proenkephalin, the proopiomelanocortin (POMC) and the prodynorphin. The POMC produces the opioid peptide β-endorphin and also α-MSH and ACTH hormones. The proenkephalin produces few copies of met-enkephalin and one leu-enkephalin. The prodynorphin produces three main opioid peptides: dynorphin A, dynorphin B and α-neoendorphin, all sharing the leu-enkephalin sequence as in the first five aminoacids. Another precursor, named pronociceptin, has been then cloned and isolated; it produces a peptide called nociceptin or orphanin FQ (F: phenylalanine, Q: glutamine, as the first and last

aminoacids, according to the single-letter code aminoacid nomenclature) (Meunier, 1995; Reinscheid et al., 1995). Pronociceptin also contains nociceptin 2, and another peptide named nocistatin which showed different characteristics from the other opioid peptides.

In addition to these peptides other aminoacid sequences able to interact with opioid receptors have been isolated: the deltorphins and dermorphins, isolated from the amphibian's skin, and endomorphins, tetrapeptides isolated from rodent brains. These peptides have a characteristic atypical structure and display high selectivity towards the μ-opioid receptor (Zadina et al., 1997).

In the CNS, β-endorphin is present in the arcuate nucleus, in the nucleus of the solitary tract, the dorsal parvocellular area of the hypothalamic paraventricular nucleus, in the ventral septum, nucleus accumbens, medial thalamus medial amygdala, in the periaqueductal gray matter in the locus coeruleus and bulbopontine reticular area. In addition to the CNS and hypophysis, β-endorphin is also produced in some peripheral tissues such as pancreas, gastric antrum mucosa, placenta, testis and the adrenal medulla.

The enkephalins are widely distributed in the CNS, peripheral nerve plexuses, in the adrenal medulla; in the CNS they are present in short interneurons in areas involved in nociceptive transmission modulation (lamina I and II of the spinal cord, spinal trigeminal nucleus, periaqueductal gray matter), in the control of affective behavior and memory (nucleus accumbens , amygdala, hippocampus, locus coeruleus , anterior olfactory nucleus, cerebral cortex), in the control of motor activity (substantia nigra, caudate) , in the regulation of autonomic nervous system (medulla oblongata) and of neuroendocrine functions (hypothalamus).

Although often present in distinct neuronal populations, in the CNS dynorphins show a distribution quite similar to enkephalins. They have been localized in the lamina II of the spinal cord, the anterior hypothalamic nucleus whose axons project to the posterior hypophysis, in the reticular formation, the caudate, the hippocampus and in different regions of the cerebral cortex. Nociceptin is widely present both in the CNS and in peripheral tissues.

The presence of endomorphins has been demonstrated in the outer layers of the spinal cord dorsal horns, in the spinal trigeminal nucleus, the nucleus ambiguous, the nucleus accumbens, the septum, in the thalamic nuclei, hypothalamus, amygdala, locus coeruleus and in the PAG.

The three main families of endogenous peptides are identified with enkephalins, dynorphins and βendorphin which derive from proenkephalin (PENK), prodynorphin (PDYN) and proopiomelanocortin (POMC), respectively. PENK is the source of [Met5]- and [Leu5]-enkephalins and several longer peptides. Endogenous opioid peptides such as dynorphin A,

dynorphin B and α- and β-neoendorphin can be generated from PDYN. While, POMC is the precursor of β-endorphin, α-endorphin and other non-opioid peptides. Several evidence showed that the endogenous opioid peptide are produced and secreted by the neurons of many regions involved in the nociceptive response such as the thalamus, PAG, limbic system, cortex and in the spinal cord. Moreover, also the autonomic nervous system centers seem to be innervated by central and peripheral opioidergic neurons (Przewłocki and Przewłocka, 2001).

In addition to the peptide's families mentioned above another group of peptides, named endomorphins, has been identified in the brain. By comparison with other opioid peptides, endomorphins have a characteristic atypical structure and display high selectivity towards the μ-opioid receptor (Zadina et al., 1997). Anatomical studies demonstrated a distinct anatomical distribution of endomorphins (endomorphin-1 is present mainly in the brain and endomorphin-2 in the spinal cord) and their synthesis in separate cellular systems.

Another endogenous opioid peptide identified in 1990s is the nociceptine. This peptide derived from its prohormone, pronociceptin, and showed high selectivity for ORL1 receptors (It is present in neurons widely distributed throughout brain and spinal cord.

1.5.1.2 Opioid receptors

There are three types of opioid receptors that, according to the most common nomenclature are named as μ, δ and κ. Over the last fifteen years they have also been named as OP3, MOR or MOP (for μ receptor); OP1, DOP or DOR (for δ); OP2, KOP or KOR (for κ).

In addition, another receptor, initially named ORL-1 or OP4 and currently named as NOP, has been identified as the binding site for the endogenous ligand nociceptin. Despite a high structural homology with the opioid classical receptors, it exhibits a different pharmacology. In fact, NOP activation seems able to cause effects that are different from those classically described for the opioid system, at least at supraspinal level. In this regard several studies hypothesized that the nociceptin/NOP system might be able to exert a functional antagonism toward the classical opioid system.

Despite a lot of in vitro and in vivo pharmacological studies have suggested the existence of subtypes for each of the three receptors, the data from molecular biology do not corroborate their existence.

It can be assumed that several phenomena, such as the heterodimerization between the different types of receptors (or between these and other 7TM receptors) or the alternative splicing of their

mRNAs, may underlie the discrepancies between the different pharmacological properties of natural and synthetic ligands on one side and the molecular evidence on the other.

All above mentioned opioid receptors belong to the seven transmembrane domains receptors superfamily, coupled to G protein (GPCRs) α i/o subunit and their activation inhibits the adenylate cyclase (AC) effector with consequent inhibition of cAMP production.

Presynaptic μ opioid receptor stimulation decreases the depolarization-dependent neurotransmitter release, by inhibiting the N-type Ca^{2+} channels, whereas μ postsynaptic receptors stimulation produces hyperpolarization by activating K^+ channels and by inhibiting L type Ca^{2+} channels. In this way opioids tend to inhibit neuronal transmission. For many years it has been reported that opioid receptors were coupled only to inhibitory $G_{\alpha i/o}$ proteins, sensitive to pertussis toxin (PTX). Moreover, it has been shown that μ, δ and κ opioid receptors interacting with five different isoforms of $G_{\alpha i/o}$ ($G_{i1\text{-}3}$ and $G_{oA\text{-}B}$) are also able to regulate the signal transduction through different effectors, such as adenylate cyclase (AC1, 5, 6, 8), ion channels and MAP kinases (mitogen-activated protein kinases).

In addition, as reported, all three opioid receptors may transduce inhibitory signals through pertussis toxin- insensitive G proteins, like $G_{\alpha z}$ proteins, the unique term of the $G_{\alpha i}$ to be insensitive to PTX, because of a missing cysteine residue in the carboxy-terminal portion, which is the site for the ADP- ribosylation catalyzed by PTX.

$G_{\alpha z}$ is expressed in nervous tissue and colocalized with the opioid receptors in neuronal cell lines; it is coupled with the μ receptor in the PAG, where it mediates the supraspinal analgesia. Besides AC activity inhibition, $G_{\alpha z}$ is able to regulate the MAPK, Ca^{2+} and K^+ channels activity and to interact with several recently identified effectors, belonging to the RGS family (Regulator G protein Signaling), such as GRIN (G- Protein - Regulated Inducers of neurite outgrowth), Rap1GAP (Rap1 - specific GTPase Activating Protein).

In addition to $G_{\alpha z}$, opioid receptors can transduce by other G proteins, insensitive to PTX, such as $G_{\alpha 14}$ and $G_{\alpha 16}$, activating a signal transduction cascade mediated by phospholipase C stimulation (PLCβ), which is usually activated by $G_{\alpha q}$ protein, with activation of the JNK (c-Jun N-terminal Kinase) influencing cellular growth, consequently.

Their distribution, limited to hematopoietic cells and peripheral tissues, suggests a role in the immunomodulatory activity of opioids (Romualdi and Candeletti, 2015).

It has been also suggested the ability of opioids to modulate some of their actions, such as analgesia, tolerance and dependence, through the subunit $G_{\alpha s}$ and the recent demonstration that μ receptors in CHO cells transduce the signal for caveolae formation through a $G_{\alpha s}$ represents a confirms of it.

In primary cultures of trigeminal sensory neurons, the regulation of μ receptor localization and its signaling can be modulated also by integrins that may switch the coupling with a G protein to another (for example, from a $G_{\alpha i}$ to a $G_{\alpha s}$).

The $G_{\beta\gamma}$ complex, after uncoupling with α subunit, plays a very important role in the diversification of the opioid-activated signal transduction, as well as for other GPCRs.

It has been demonstrated that a high number of effectors and proteins involved in signal transduction may functionally interact with $G_{\beta\gamma}$ subunits, determining a wide range of biological responses, also in the opposite direction to those triggered by the $G_{\alpha s}$ subunit: for example, the AC2-induced cAMP production might be regulated by $G_{\beta\gamma}$ and $G_{\alpha i}$ in opposite directions.

The GIRK channels (G protein coupled inwardly rectifying K^+ channels) of type 3 are activated by direct interaction with the βγ subunits after stimulation of GPCR; this phenomenon is considered part of the mechanism of opioid inhibition of nociceptive transmission (Romualdi and Candeletti, 2015).

Generally, μ, δ and κ opioid receptors are coupled to $G_{\alpha o}$ and $G_{\alpha i2}$ proteins, showing differences related to the type of tissue or cells in which they are expressed. For example, the μ receptor expressed in DRG sensory neurons transduces through a $G_{\alpha o}$ subunit, while when it is localized in brain areas is preferentially coupled to proteins $G_{\alpha i1\text{-}3}$. The κ receptor activates the $G_{\alpha o}$ subunit preferentially, while the receptor δ activates $G_{\alpha i1}$ proteins. At last, it is possible that following the binding of different agonists, exhibiting different intrinsic activity, the receptor can show different conformations that determine the formation and stabilization of a specific receptor/G protein complex, thus inducing a related signal transduction. In recent years it has also been proposed that dimerization could occur between different types of opioid receptors, generating homo-and hetero-dimers with each other, and with other 7TM GPCR. Dimerization can cause changes in ligand affinity, receptor transduction mechanisms and cellular trafficking; nevertheless, the functional significance of this phenomenon is still uncertain.

The availability of ligands able to interacts with these alternative forms of opioid receptors selectively will allow the development of analgesics endowed with less tolerance and fewer side effects, together with the possibility to study their tissue localization, dynamics of assembly and their trafficking. The different transduction pathways undergo adaptation following prolonged receptor exposure to agonists.

1.5.2 Opioids and modulation of pain

The ascending spino-thalamic-cortical and the descending brain-spinal systems represent the main CNS neuronal pathways involved in the transmission, modulation and control of nociception. The strategic localization of opioid receptors and their transduction mechanisms, award to the endogenous opioid system a crucial role in the modulation of nociceptive input and the ability to produce analgesia.

When tissue damage occurs in the periphery, physiological pain signals, or nociceptive signals, travel down primary afferent Aδ and C-fibers to the spinal cord dorsal horn. These afferent fibers induce the release of excitatory neurotransmitters into the synapse, which are responsible of the activation of the neuron cell bodies that send axons up ascending tracts to the brain (Holden et al.,2018). The laminae II and III of the dorsal horn (substantia gelatinosa, SG) contain small interneurons that produce and release opioid peptides (enkephalins, dynorphins). Opioids are able to modulate / inhibit transmission between primary afferent fibers and spino-thalamic neurons through opioid receptors located at two levels: presynaptically, on the spinal the primary neuron and/or postsynaptically on the second-order (spino- thalamic) neuron (Clementi and Fumagalli, 2018).

The descending inhibitory system originates from neurons in the PAG that receive impulses from the cortex, hypothalamus and from the thalamus in particular.

The PAG neurons project to some nuclei of the medulla oblongata, such as the nucleus raphe magnus (NRM), the nucleus reticularis magnocellularis (NRMC) and the nucleus reticularis paragigantocellularis (NRPG). Arising from these nuclei, descending aminergic fibers running along the dorsal lateral funiculus of the spinal cord terminate in the SG where this descending system modulates the afferent nociceptive transmission directly or through the activation of opioids interneurons. Basing on this evidence is clear that the analgesic effects of opioids and opiates are strongly related to their ability to directly inhibit the ascending transmission of nociceptive impulses from spinal cord dorsal horns and to activate the descending pain control pathway from the thalamus to the dorsal horn. The activation of μ receptors always causes analgesia, for example through the removal of GABAergic inhibition on PAG neurons projecting, downstream to the spinal cord, in the descending system. However, activation of κ receptors can produce, inhibiting the neurons descending from the PAG, both analgesia or hyperalgesia.

The presence of opioid receptors on the peripheral terminals of sensitive neurons suggested the possibility that opioids are involved in the nociceptive modulation even at the periphery. This

idea is supported by recent studies which displayed an increase ofμ and κ receptors on the terminals of primary afferents and migration of immune cells from the vessels to the inflamed tissue during the inflammatory response. In this condition, the release of opioid peptides induced by leukocytes determine the interaction with the up-regulated opioid receptors and the consequent induction of analgesia through the decrease of the sensory endings excitability and/or of the release of pro-inflammatory neuropeptides (Clemente and Fumagalli, 2018).

1.5.3 Exogenous opioids

Opioids are a group of analgesic agents commonly used in clinical practice for the treatment of chronic pain.

Morphine is considered to be the archetypal opioid analgesic and the agent to which all other molecules generally used in pain treatment are compared. Many evidence suggest that as long ago as 3000 bc the opium poppy, *Papaver somniferum*, was cultivated for its active components. However, the modern opioid pharmacology was truly born not until morphine was isolated from opium in 1806 by Sertürner. The definition the chemical formula for morphine in the 1847 led to the more precise and diffused clinical use of morphine (Blakemore et al.,2002). Although morphine represents the most widely known extract of *P. somniferum*, other substances can be isolated from it such as codeine, papaverine and thebaine. Following the isolation of morphine, simple chemical manipulations of these basic opiate alkaloids have resulted in the production of numerous semi-synthetic opioids such as buprenorphine, nalbuphine, naloxone and oxycodone that are generally useful in clinical medicine. Moreover, during the 20th century a number of synthetic opioids were also discovered. These latter molecules can be divided into four chemical groups: the morphinan derivatives (levorphanol, butorphanol), the diphenylheptane derivatives (methadone, propoxyphene), the benzomorphan derivatives (pentazocine, phenazocine) and the phenylpiperidine derivatives (pethidine, alfentanil, fentanyl, sufentanil and remifentanil) (Pathan et al.,2012). Opioids can also be classified basing on their effect at opioid receptors. According with these classification opioids can be considered as agonists, partial agonists and antagonists. Agonists interact with a receptor to produce a maximal response from that receptor. Contrarily, antagonists bind to receptors but produce no functional response, while at the same time preventing an agonist from binding to that receptor (naloxone). Partial agonists bind to receptors but elicit only a partial functional response no matter the amount of drug administered (buprenorphine). Among the numerous

opioids drugs this thesis will be focused on Morphine, Fentanyl, Buprenorphine and tapentadol that as briefly described below show a different pharmacological profile.

1.5.3.1 Morphine

Morphine is a natural alkaloid derived from the opium poppy *Papaver somniferum*, and is the most widely used opioid drugs to treat moderate to severe pain. The chemical structure of morphine is derived from phenanthrene molecule consisting of five condensed rings (**Figure 8**). The partially hydrogenated phenanthrene core incorporates benzene ring (A-ring) and two partially unsaturated cyclohexane rings (B- and C-rings). It consists of two hydroxyl function groups at positions 3 and 6 (C3 phenolic and C6 alcoholic hydroxyl groups) and amino group at position 17. Its full systematic name is 7,8-didehydro-4,5-epoxy-17-methyl(5α,6α)-morphinan-3,6-diol (Braenden et al.,1955; Andersen et al.,2003).

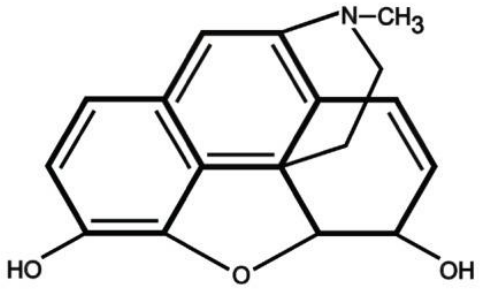

Figure 8. Chemical Structure of morphine (Skrabalova et al.,2013)

Morphine is almost 100% absorbed from the gastrointestinal tract after oral administration (Christrup, 1997) and is rapidly distributed to highly perfused tissues (Spector and Vesell, 1971). Approximately 15–35% of morphine is bound to plasma proteins, mostly to albumin and less to α_1-acid (Ederoth et al., 2004, Leow et al., 1993, Milne et al., 1992).

The mean bioavailability of this drug is low (20–30%) after oral administration and is related to extensive hepatic first-pass metabolism (Gutstein and Akil, 2005, Hanks et al., 2001, Hasselstrom and Sawe, 1993, Osborne et al., 1990). The conjugation with glucuronic acid represent the primary metabolic pathway leading the formation of two main metabolites the morphine-3-glucuronide (M3G) and the analgesically active M6G (Christrup,

1997, Gutstein and Akil, 2005). The major enzymes involved in this process are diphospho-glucuronosyltransferases (UGT) 2B7 and UGT1A3. The former is involved in the formation of M6G and M3G, while the latter UGT1A3 is only involved in the formation of M3G (Coffman et al., 1997, Green et al., 1998). Induction of UGT2B7 would lead to an increased metabolism of morphine with consequent increased of M6G and M3G levels.

This molecule is a full agonist at the μ opioid receptor and its effects, mainly associated with the activation of this receptor, include analgesia, respiratory depression, reduced gastrointestinal motility, nausea, and sedation (Gutstein and Akil, 2005). Some evidence reported that morphine is also able to bind to the κ opioid receptors, known to be involved in the modulation of peripheral analgesia and dysphoria (Pfeiffer et al., 1986, Wang et al., 2010), and to the δ opioid receptors that are involved in supraspinal and spinal analgesia as well as reduced gastrointestinal motility and secretion (Porreca et al., 1984).

The analgesic effects of this drug are determined by the presynaptic block of the Ca2 + channels that control the release of excitatory neurotransmitters (substance P, glutamate, acetylcholine, noradrenaline) at the level of first-order nociceptive fibers and opening of the K + channels in the cell body of second order neurons, reducing their excitability.

High morphine doses can induce respiratory depression by direct inhibition of the bulbopontine centers of respiration leading to death (Martin, 1983). In fact, it has been reported that this opioid drug depresses respiratory activity within half an hour of the intramuscular administration of an analgesic dose, and it takes more than 2 hours for the resumption of normal respiratory function. The antitussive action is exerted by direct intervention on the bulbar centers that modulate the cough reflex.

At the gastrointestinal level, morphine and its analogues cause constipation, resulting from an increase in muscle tone and a reduction in the defecation reflex (Dooley et al., 1988). Morphine and opiates generally have an emetic effect, as a result of direct stimulation of the chemosensitive bulbar area which in turn stimulates the vomiting center.

Morphine exerts a constricting effect on the pupil which is dose-dependent. Myosis appears to be due to an indirect effect on the mesencephalic nuclei that control the parasympathetic innervation of the pupil and, at the same time, to a direct effect on iris receptors.

1.5.3.2 Fentanyl

Initially synthesized in Belgium in the late 1950s, fentanyl was marketed the next decade as an intravenous anesthetic under the trade name of Sublimaze®. The analgesic action of fentanyl surpasses that of morphine by approximately 100-fold. In fact, it has been observed that a dose of 100 micrograms exerts an analgesic action comparable to that of 10 milligrams of morphine **(Figure 9)**.

Fentanyl (N-phenyl-N- [1- (2-phenylethyl) - 4-piperidinyl] propenamide) belongs to the phenylpiperidine class, synthesized starting from morphine. Structurally it manifests a non-basic amide tertiary nitrogen atom in place of the quaternary C corresponding to the C-13 of morphine. The basic nitrogen included in the piperidine ring is linked to the phenylethyl radical which had already led to a significant increase in activity in other series of morphine-like compounds. This peculiarity derives from its close resemblance to the phenylalanine residue present in endomorphins.

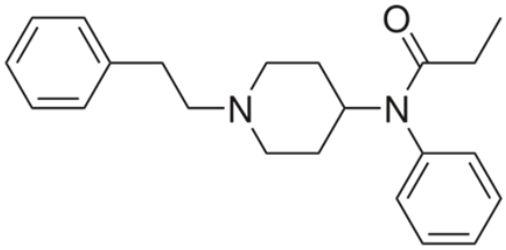

Figure 9. Chemical Structure of fentanyl

Like morphine, fentanyl also acts as an agonist against the μ receptor, resulting in the inhibition of the enzyme adenylate cyclase and the consequent decrease in AMPc (Wandless et al., 1996). As regards the pharmacokinetics of this drug, it has been shown that it is able to act rapidly after intravenous or intramuscular administration (3-5 minutes) and is characterized, by virtue of its marked fat solubility, by a short duration of action, ranging from 5 and 20 minutes, depending on the dose. It binds in a high percentage to plasma proteins and undergoes a significant tissue redistribution; from this it is clear that the elimination rate of the drug is quite variable. It is metabolized in the liver by dealkylation and hydroxylation reactions, and its

metabolites are mainly excreted in the urine. The high activity of fentanyl, even at very low dosages, has allowed its administration also through transdermal patches or oral formulations, thus significantly improving patient compliance. Duragesic® for example is a transdermal patch that releases fentanyl and is used in the treatment of chronic pain (Romualdi et al., 2016). Fentanyl is generally used as an analgesic supplement in general anesthesia, as an anesthetic agent alone or in the treatment of chronic pain. In addition to exerting an important analgesic action, it can induce a dose-dependent respiratory depression which manifests itself with a reduction in respiratory rate and tidal volume, an increase in CO2 pressure and, in the most severe cases, apnea. Respiratory depression may persist even after the analgesic action has ceased; therefore, patient monitoring should continue beyond the end of the surgery. With the exception of the bradycardic effect, the drug, at therapeutic dosages, produces minimal effects on the cardiovascular system; however, hypotension may appear, albeit transient, if the opiate is administered intravenously in association with barbiturates. Overall, the drug exerts a sympatholytic action, therefore, other possible side effects include the release of the anal sphincter and sialorrhea.

1.5.3.3 Buprenorphine

Within the class of opioid drugs, we find 2S) -2 - [(-) - (5R, 6R, 7R, 14S) - 9α-cyclopropylmethyl 4,5-epoxy -6,14-ethane-3-hydroxy-6-methoxymorfinan-7-yl] -3,3-dimethylbutan-2-ol, also known as buprenorphine **(Figure 10)**.

Buprenorphine was synthesized in the late 1960s with the aim of identifying new analgesic molecules based on the structure of morphine. Among the various compounds synthesized, buprenorphine seemed to have promising characteristics given the high therapeutic index demonstrated (Cowan et al., 1977).

In this regard, the ratio between lethal dose (LD50) and dose necessary to reach 50% of the effect (EC50) has been shown to be at least three times higher than that of morphine, therefore considered a safe analgesic both in animals and in human (Orwin et al., 1976). Buprenorphine is a semi-synthetic opiate belonging to the class of oripavine derivatives; it is a molecule with a complex chemical structure, containing several chiral centers; it is highly lipophilic (logP = 4.98) and shows a high volume of distribution in all tissues, including the brain. Chemically, the oripavine derivatives represent the only exception among all the opioid agonists that normally present themselves as molecular simplifications of morphine. In fact, buprenorphine and ethorphine (opioid agonist 1000 times more powerful than morphine and used only in the

veterinary field for large animals), are a "molecular complication" of morphine, presenting an extra ring on the C ring.

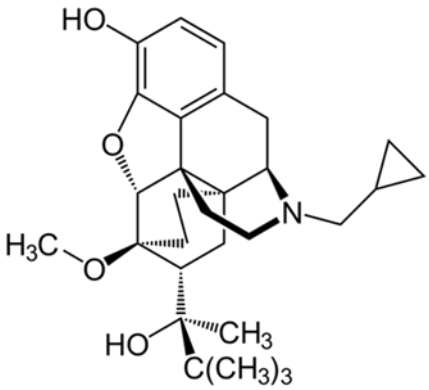

Figure 10. Chemical Structure of buprenorphine

It is mainly metabolized in the liver where it undergoes N-dealkylation at the nitrogen level 17 becoming the active metabolite nor-buprenorphine. Both buprenorphine and nor-buprenorphine (NBN) can subsequently both undergo a rapid phase 2 reaction, i.e. they are glucuronidated on the phenolic site by glucuronisyl transferases (UGT, UGT2B7 and UGT1A1) at the level of the hepatocyte cytosol and transforming respectively into buprenorphine-3-glucuronide (B3G) and nor-buprenorphine-3-glucuronide (N3G) (Bruce et al., 2006).

Initially, metabolism from buprenorphine to nor-buprenorphine was considered an inactivating pathway, as the de-alkylated metabolite in rats had 1/50 of the analgesic potency after intravenous administration and 1/4 following intracerebroventricular administration compared to the drug of origin (Ohtani et al., 1995). On the contrary, the most recent evidence suggests that de-alkylation is instead a path of bio-activation. Indeed, nor-buprenorphine is a potent opioid agonist with high affinity for MOR, δ (DOR) and κ (KOR) receptors (Huang et al., 2001; Ohtani et al., 1995). In rats, this molecule causes dose-dependent respiratory depression and is 10 times more potent than buprenorphine (Ohtani et al., 1997; Megarbane et al., 2006). In a study carried out by Brown and colleagues, published in 2011, it was demonstrated through the use of animal models that both B3G and N3G have mild analgesic effects and bind to opioid receptors. Buprenorphine and its three major metabolites have distinct profiles; respectively

B3G is a MOR, DOR and N / OFQ specific receptor agonist called NOP, while N3G is a LAD and NOP ligand. All metabolites, except N3G, are analgesic and contribute to the pharmacological profile of buprenorphine (Brown et al., 2011; Butler, 2013). The potential contribution of these metabolites to the effects of buprenorphine adds complexity to the understanding of its pharmacology. This molecule, in fact, has a complex pharmacology (Lutfy et al., 2004) and is considered an agonist of the low-dose MOR receptor, antagonist of the KOR and DOR receptor and high-dose agonist of the NOP receptor (Wnendt et al., 1999; Bloms-Funke et al., 2000; Hawkinson et al., 2000; Huang et al., 2001).

The in vitro profile of buprenorphine, obtained by receptor binding assays, reported the following values: the molecule has a Ki = 1.5 nM for the MOR receptor, a Ki = 6.1 nM for the DOR receptor and a Ki = 2.5 nM for the KOR receptor, where in both cases it acts as an antagonist. Furthermore, with a Ki = 77.4 nM it binds the NOP receptor as an agonist (Khroyan et al., 2015). These characteristics have identified buprenorphine as a molecule with a distinct pharmacological profile from other opiates, which is why it has some unique characteristics in clinical applications **(Figure 11)**.

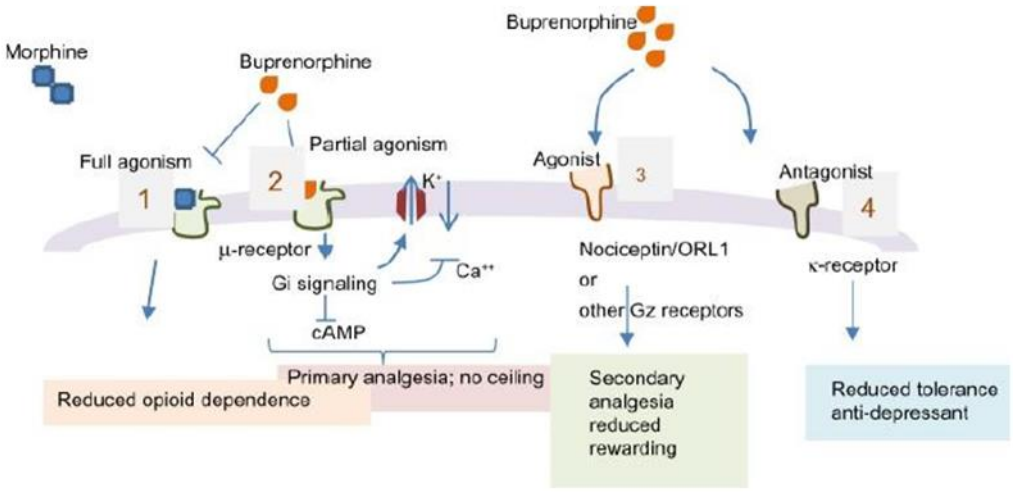

Figure 11. Buprenorphine interaction with opioid receptors and its effect (Khroyan et al., 2015)

The high potency (about 25 times more powerful than morphine) and the low dissociation constant (dissociation half-life of 2-5 h), explain the various actions of this molecule (Virk et al., 2009). In fact, buprenorphine has a long duration of action attributable to the slow

dissociation from MOR receptors (Gudin and Fudin, 2020) and its prolonged therapeutic effect can be used for the treatment of opiate addiction and the treatment of pain. Furthermore, this substance, if administered with full agonists, such as morphine, antagonizes the action of these drugs. The high affinity for the KOR receptor in some studies seems to reduce the onset of respiratory tolerance and depression (Dahan et al., 2006; Karp et al., 2014). At high doses, buprenorphine is also an agonist of the NOP receptor and in animal models the activation of this receptor at the level of the dorsal horns of the spinal cord has an analgesic action (Davis, 2012). From the dose-response curves, in nociceptive assays, it is evident that, depending on the nature of the stimulus and the intensity, this molecule produces a sub-maximal effect (roof effect) or dose-response bell curves. At low concentrations, therefore, buprenorphine is an effective and safe analgesic and has no ceiling effect (Walsh et al., 1994; Chou et al., 2009), but at higher doses the antinociceptive effect of the drug is often reduced, producing dose-bell curves response (Lizasoain et al., 1991; Cowan, 2003).

The high safety profile means that buprenorphine is considered one of the preferred drugs in different pain conditions, including neuropathic pain and chronic cancer pain. Buprenorphine is used in the clinic for the management of moderate to severe pain and the results indicate a marked decrease in pain intensity (Khanna and Pillarisetti, 2015). In conditions of neuropathic pain, 40% of patients with central neuropathic syndromes, usually considered refractory to opioid analgesia, showed improvements following treatment with buprenorphine. Unlike other MOR agonists, this substance appears to block secondary hyperalgesia induced by central sensitization in humans (Induru et al., 2009) and has been shown in some circumstances even more effective than fentanyl (Likar,2006; Andresen et al.,2011). The typical dose for analgesia is 0.3-0.6mg for intravenous or intramuscular administration, and its analgesic effect lasts about 6h (McNicholas et al., 2004). However, the significant reduction in long-term pain requires the maintenance of opiate plasma levels for a long time, in order to prolong the analgesic action and reduce adverse effects. In this regard, the introduction of slow-release systems, such as transdermal systems, has offered a number of advantages by providing a safe, convenient and reliable method for administering the active ingredient. Through clinical and preclinical studies, it emerged that the main side effects of buprenorphine are similar to those of MOR agonists (such as nausea, vomiting, constipation) but the intensity of these effects is significantly reduced. It has been shown that buprenorphine is able to cause respiratory depression but to a lesser extent than other opiate analgesics (Heel et al., 1979; Yassen et al., 2008). Due to its slow dissociation from MOR receptors, the onset of this effect is slower than that caused by morphine. A plateau is reached for this condition, so that even by increasing the doses, a sub-

maximal effect is always obtained (Gudin and Fudin, 2020). Although some pharmacological aspects are not fully understood in vivo, buprenorphine has a significantly superior safety profile than other opioids and is becoming the drug of choice in the treatment of opiate addiction. (Davis et al., 2012). The clinical utility of opiates is often hindered by the development of tolerance following chronic treatment (Way et al., 1969).

Although tolerance to the antinociceptive effect of buprenorphine has been demonstrated, onset appears to be slower than with morphine.

In a retrospective study involving 900 cancer patients, buprenorphine was found to produce lower analgesic tolerance than fentanyl (Koppert et al., 2005; Vanderah et al., 2000; Likar and Sittl, 2005; Louis et al., 2006). Multiple mechanisms have been proposed to explain the tolerance associated with MOR agonists such as morphine (Kress, 2009; Pergolizzi et al., 2008) and among these a proposed mechanism for tolerance is the increased activity of the Nociceptin / Orphanine peptide (N / OFQ) in the brain. To confirm this, an increase in nociceptin levels in the cerebro-ventricular fluid, in the periaqueductal gray matter and in the amygdala of morphine-tolerant rats was already highlighted in 1999 (Yuan et al., 1999). From this study it was proposed that morphine leads to an acceleration of the biosynthesis and release of N / OFQ, where this antagonizes the antinociceptive effect of morphine, thus contributing to the phenomenon of tolerance. Therefore, the slower onset of tolerance in buprenorphine could depend on its concomitant ability to activate the NOP receptor. Buprenorphine could also control secondary hyperalgesia thanks to the activation of the NOP receptor and the antagonism on the KOR receptor (Vanderah et al., 2000; Lutfy et al., 2004).

1.5.3.4 Tapentadol

One of the non-racemic compounds having a dual mechanism of action and developed for the treatment of pain is (-) - (1R, 2R) -3- (3-Dimethylamino-1-ethyl-2-methyl-propyl) -phenol hydrochloride (Tapentadol HCl) **(Figure 12)**, which, in rat and mouse models of acute and chronic pain, has demonstrated analgesic, anti-hyperalgesic and anti-allodynic properties (Thomas et al., 2007).

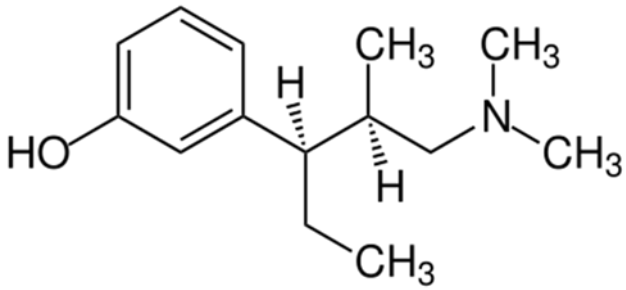

Figure 12. Chemical Structure of (-) -(1R,2R)-3-(3-Dimetilammino-1-etil-2-metil-propil)-fenolo

The molecule passively diffuses across the blood brain barrier (BEE), following its concentration gradient. The analgesic properties of this compound are related to a single enantiomer which does not require metabolic activation to exert its action (Tzschentke et al., 2006). Tapentadol has no analgesically active metabolites, therefore both of its mechanisms of action reside in the racemic mixture. Many tests show that important pharmaceutical compounds act by interacting with multiple targets and in recent years it has been recognized that multi-pathophysiological medical conditions are more effectively treated through complementary multi-modal mechanisms of action. In this regard, tapentadol, as a centrally acting analgesic, is both a MOR agonist and a noradrenaline reuptake inhibitor (NRI) (Tzschentke et al., 2007). This is the first example of a new pharmacological class called MOR-NRI (Pergolizzi et al., 2011), and as such it has an analgesic effect, even if the pain conditions that these two classes of drugs treat are different (Tzschentke et al. al., 2007). In particular, the two mechanisms of action are complementary: 1) μ-opioid agonism is mainly effective against moderate to severe acute pain; 2) the blockade of noradrenaline reuptake is particularly favorable in the treatment of chronic pain (Carter et al., 2002). This implies that a compound with both activities is able to show efficacy in a broader spectrum of painful conditions,

counteracting both acute pain and chronic pain (Tzschentke et al., 2007). The dual mechanism of action induces analgesia through different ways:

1- in the spinal cord the agonism on the MOR receptors interrupts the ascending pain signals, blocking the intracellular influx of Ca2 +, with consequent inhibition on the release of glutamate (presynaptic MOR), and inhibiting the activity of the postsynaptic neuron due to hyperpolarization of the membrane (Postsynaptic MOR)
2- in the rat periaqueductal gray matter, MOR agonists inhibit the release of γ-aminobutyric acid (GABA), with consequent disinhibition of the descending inhibitory pathways, which results in an increase in the release of noradrenaline (Fields et al, 1991; Osborne et al., 1996; Vaughan and Christie, 1997). Therefore, at the supraspinal level, tapentadol-mediated activation of MOR receptors alters the balance between the release of norepinephrine (inhibitory) and that of serotonin (inhibitory and inducer) in the descending pain pathway (Suzuki et al., 2004). As a result, there is an increase in the levels of noradrenaline in the synaptic junction and the activation of α2-adrenergic receptors on the postsynaptic membrane. This determines the inhibition of pain transmission to the thalamus, a structure involved in the conscious processing of pain **(Figure 13)**. The combination of these two mechanisms of action in a single molecule can represent an advantage, in conditions that often involve both a nociceptive and a neuropathic component of pain. Furthermore, the synergy of the two mechanisms (MOR / NRI) may explain the lower incidence of side effects typical of opioids, observed for tapentadol compared to other classical opiates such as oxycodone (a drug used as a comparison in clinical trials). As demonstrated by studies on preclinical models of acute and neuropathic pain, there is an effective synergism of action between the two mechanisms exploited by tapentadol at the receptor level (Schröeder et al., 2010).

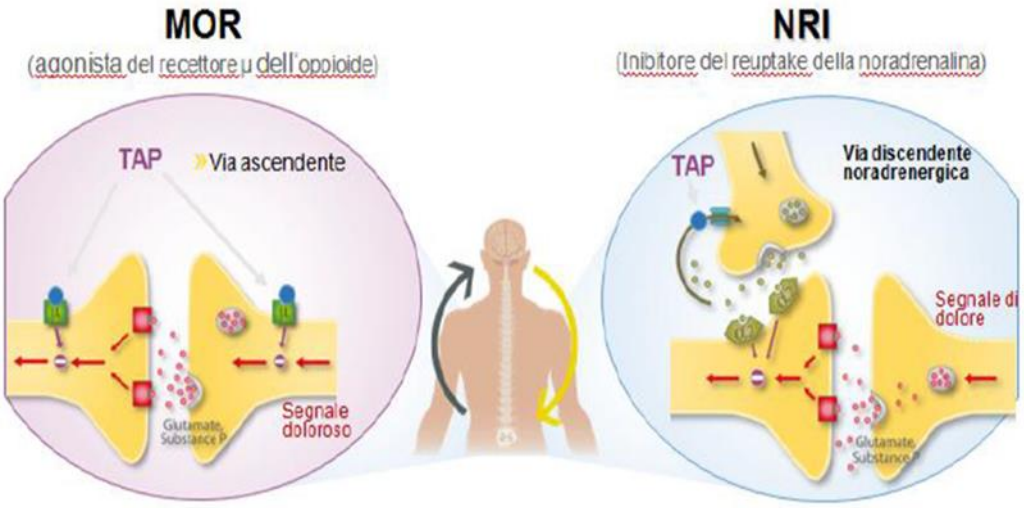

Figure 13. Mechanism of action of tapentadol (Tzschentke et al., 2009)

Furthermore, the reduced effect of tapentadol on serotonergic transmission (Pergolizzi et al., 2011) reduces the side effects induced by an increase in serotonin in the enteric nervous system (constipation, nausea and vomiting).

The power and efficacy of its analgesic action have been demonstrated in rodent models of nociceptive and neuropathic pain (Kogel et al., 2011). Preclinical studies with MOR antagonists and α2-adrenergic antagonists have also shown the real involvement of the two mechanisms in the analgesia induced by tapentadol (Kogel et al., 2011). The synergy that is established between the individual mechanisms of action involved, outlines a wide therapeutic range, as well as a high efficacy in neuropathic pain models (Schröeder et al., 2011).

Receptor binding assays have shown that tapentadol exhibits only moderate affinity for the MOR receptor (Ki = 0.1μM in rats) (Tzschentke et al., 2006), approximately 50 times lower than that of morphine, and this may explain the reduced side effects induced by the new analgesic (Tzschentke et al., 2009).

In this context, it is also important to underline that, from studies conducted on animal models of chronic constriction injury (CCI), emerged that the onset of tolerance to the analgesic effect of tapentadol in repeated administration is delayed compared to what happens for repeated administration of morphine. Specifically, while tolerance to the analgesic effect of morphine is established quickly, tapentadol shows a delayed onset of the development of tolerance, which is complete only after 51 days of treatment (Tzschentke et al., 2007); effect that seems to be

mainly attributable to the NRI component of its action (Tzschentke et al., 2006).An in vitro study, previously conducted in our laboratory, highlighted the ability of tapentadol to modify the mRNA levels of the opioid receptors MOR and NOP (Caputi et al., 2014). In particular, the gene expression of the MOR receptor appeared significantly up-regulated after 24 and 48 hours of exposure. These results showed a similar trend to that reported following treatment with fentanyl in the same cell line (Caputi et al., 2013). In this regard, it has been shown that the molecular mechanisms that contribute to opiate tolerance also involve changes in gene expression (Martini and Whistler, 2007). This could suggest that for tapentadol, as well as for fentanyl, the slow development of tolerance reported in vivo could be related to the ability of these two drugs to induce up-regulation of the MOR gene. Furthermore, the data presented in this 2014 study using engineered cells over-expressing the MOR receptor, showed that tapentadol, like morphine, does not cause internalization of these opioid receptors (Caputi et al., 2014).

The most frequently reported adverse events in clinical trials related to tapentadol appeared mainly to be related to the opiate activity of the drug, including the potential for addiction and abuse, and are comparable to the side effect profile of strong opiates. Nausea, vomiting and constipation are among the most common side effects induced by opioid analgesics and represent the main cause of discontinuity in therapy (Deidre et al., 2012).

The best pharmacological profile of tapentadol was also found compared to other opioid analgesics, such as: fentanyl, hydromorphone, morphine and oxymorphone (Hartrick and Hernandez, 2012). The reduced respiratory depression hypothesized for tapentadol has not yet been experimentally demonstrated, therefore extreme caution is advised, especially when combined with other sedative drugs (Deidre et al., 2012).

1.6 Molecular mechanisms of opioid-induced tolerance and hyperalgesia

Although opioid analgesics represent the most used drugs for the pharmacologic treatment of moderate to severe pain, their use are often hampered by the development of two opioid-related phenomena. The first is tolerance, which is manifested clinically by the need for increasing opioid dosages over time to maintain the same level of pain relief. The second is the opioid-induced hyperalgesia (Sjogren et al., 1998) that is characterized by a paradoxic increase in atypical pain, typically unrelated to the original nociceptive stimulus, that is determinated by a prolonged administration of opioids.

Pharmacologically, the opioid-induced tolerance is defined as a shift to the right in the dose-response curve. Although, this phenomenon could be related to the progression of pathological diseases (Collin et al., 1993; Foley,1993) also other factors such as pharmacokinetic or pharmacodynamic changes could corroborate the increased opioid needs.

The two major molecular mechanism proposed to explain opioid tolerance involve changes in opioid receptors. The former support the idea by which the prolonged exposure to opioids induce receptors changes that result in decreased receptor activation, or desensitization. The latter suggests that opioid receptor down-regulation is at least partially responsible for the development of tolerance (DuPen et al., 2007)

The desensitization mechanism induces changes in the opioid receptors' physiology. Indeed, when the opioid is bound to the receptor, the associated G protein becomes "activated." Activation of G proteins eventually leads to decreasing excitability along the cell membranes of neurons in the pain pathways. This action occurs through a reduction in cyclic adenosine monophosphate (cAMP), leading to a suppression of Na+ and Ca+ channels and resulting in analgesia. Moreove, sometimes, through the opioid receptor desensitization, an alterations in the G protein–mediated mechanism can produce the decrease of analgesia (Ferguson et al., 1998; Luttrell and Lefkowitz, 2002; Perry and Lefkowitz, 2002; Raehal and Bohn,2005; Shen and Crain, 1990; Terman et al., 2004; Wang et al., 2005; Yoburn et al., 2003). In animal models, it has been shown that this mechanism occurs when opioids activate the intracellular regulatory proteins or enzymes, such as GPCR kinases, β-arrestins, and adenylyl cyclase which decouple the opioid receptor from the G protein or produce a switch in coupling of the receptor to a non-analgesic G protein, inducing a decrease of analgesic effect (Romualdi and Candeletti, 2015)

The second mechanism that seems to be implicated in the development of opioid tolerance occurs via internalization of the opioid receptor from the cell membrane. The density of opioid receptors located on the cell membrane is governed by endocytosis that is responsible of the closing of the cell membrane around the receptor which is then drawing into the body of the cell. When the receptor is inside the intracellular environment it loses its function and is effectively downregulated. Studies showed that the lack of one of these down-regulators (β-arrestin2) in rats, is able to induce a prolongation of the morphine-induced analgesic effect. (Bohn et al., 2002; Bohn et al., 1999). Despite this evidence, some researchers have also demonstrated that an increase of internalization could be also related to the decrease of tolerance. In fact, the internalization by getting desensitized receptors off the membrane causes resensitization through new or recycled receptors being substituted (Finn and Whistler, 2001). The opioid agonists (e.g., morphine, methadone, fentanyl) as demonstrated, differ in their capacity to desensitize or down-regulate opioid receptors (Arden et al., 1995; Sim-Selley et al., 2000; Yabaluri and Medzihradsky, 1997). Some of these differences have been attributed to the "intrinsic efficacy" of the opioid agonist (Chavkin and Goldstein ,1982; Mercadante,1999). Generally, continuous treatment with opioids having lower intrinsic efficacy, like morphine, causes larger tolerance (Saeki and Yaksh, 1993). In addition, to the mechanisms above mentioned recent evidence suggest the possible role of oxidative stress in the opioids tolerance (Skrabalova et al.,2013). Moreover, it not really clear how this phenomenon participates to its development.

Opioid-induced hyperalgesia (OIH) is a paradoxical condition manifesting clinically as hyperesthesia (i.e., dramatically increased sensitivity to painful stimuli) and/or allodynia (i.e., pain elicited by a normally nonpainful stimulus). Several studies showed that it occurs both in human and animals treated chronically with opioids. Generally, the abnormal pain originates from an anatomically region different from that original pain arises (Ossipov et al., 2005). To date, several mechanisms associated with OIH have been identified. Among these the activation of NMDA plays a crucial role. In fact, the involvement of this receptor has been demonstrated that this condition could be blocked by the administration of NMDA receptor antagonists , MK801 (King et al., 2005; Mao, 2006; Ossipov et al 2005). Other studies have displayed that hyperalgesia results from increased excitatory peptide neurotransmitters, such as cholecystokinin (CCK), which are released from neurons in the RVM and activate spinal pathways that up-regulate spinal dynorphin. Both of these substances act as pronociceptive agents (Dourish et al., 1988; Gardell et al., 2002; Vanderah et

al., 2000; Vanderah et al., 2001; Xu et al., 1992). These and other excitatory neurotransmitters are imply in the central sensitization that result in hypersensitivity of the spinal cord to nociceptive inputs from the periphery.

1.7 Opioids and oxidative stress

1.7.1 Free radicals and oxidative stress

The concept of oxidative stress was introduced in biological and medical research around 1985 when several evidence showed that oxidation and reduction reactions in living cells were involved in many fundamental regulatory processes, defined as "redox signaling" and "redox control". Indeed, the maintenance of cellular homeostasis through redox mechanisms is crucial for cellular survival (Sies et al., 2017).

Oxygen is involved in multiple biochemical activities, such as: signal transduction, gene transcription and regulation of the activity of guanylate cyclase. Free radicals are the result of aerobic metabolism and are produced by cells as products of physiological and biochemical processes (Uttara et al., 2009).

In fact, more than 5% of oxygen (O2) is converted into reactive chemical species called "reactive oxygen species" (ROS) such as superoxide anion (O2 • -), hydroxyl radical (OH •) and peroxide of hydrogen (H2O2) by univalent reduction of O2. In this way the cells, in aerobic conditions, are always exposed to the insult of ROS (Uday et al., 1990). It is important to note that lower levels of ROS normally produced under physiological conditions, play a crucial role in the control of cell proliferation and survival (Trachootham et al.,2008). However, excessive amounts of ROS seem to be involved in the oxidative tissue damage.

To protect the cell from the toxic effect of ROS (Uday et al., 1990) there are molecules such as ubiquinone, albumin and thiol compounds that function as free-radical scavengers and that reacting with the ROS determine their inactivation (Uttara et al., 2009). Theseprocess is also favorite by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase and peroxidase (GPX) and that acting with free-radical scavengers corroborate to the removal of radical species (Uttara et al., 2009). However, the imbalance between ROS production and antioxidant defenses determined a condition knownas "oxidative stress". This condition is able to cause a damage at the level of many biomolecules (lipids, proteins and nucleic acids) and lead the development of different pathological conditions (Sies et al.,2017).

1.7.2 Oxidative stress as a possible consequence of opioids treatment

Some studies displayed a close correlation between the oxidative stress and its implication in the treatment with opioids. Among, opioids drug, morphine has received more attention than other opioids in this context. In fact, several evidence showed that acute and chronic exposure to morphine may induce, in both human and rodent, a significant decrease in level of the antioxidant tripeptideglutathione (GSH) in the brain and liver (Goudas et al.,1999; Zhang et al.,2004; Ozmen et al.,2007). It was also demonstrated that chronic morphine treatment affects enzyme activities of SOD, CAT and GPx, which as previously indicate represent the major enzymes involved in the endogenous antioxidant defense. In particular, the activities of these enzymes result decreased after morphine exposure (Zhang et al.,2004; Payabvash et al.,2006; Zhou et al.,2001).

The activity of antioxidants and antioxidant enzymes is strongly related to the ROS production which seems to be increased both after short-term or long-term treatment even with low doses of morphine in vascular endothelial cells (Lam et al.,2007; Hisiao et al.,2009) and macrophages (Bhat et al.,2004).

The most important oxygen species involved in cell processes mediated by morphine is peroxynitrite that is produced from its precursors, superoxide and nitric oxide. These reactive species as reported by some evidence, are implicated in the development of pain, OIH and antinociceptive tolerance (Salvemini et al.,2009). It has been displayed that the inhibition of the formation of peroxynitrite precursors blocked the development of morphine antinociceptive tolerance (Muscoli et al.,2007). The enzymes involving in the formation of peroxynitrite and its precursors are nitric oxide synthase (NOS), spinal manganese superoxide dismutase (MnSOD) and NADPH oxidase. Generally, an activation of NOS inducesthe production of NO, nitration and subsequent inactivation of MnSOD which leading to peroxynitrite formation and activation of NADPH-oxidase (Salvemini et al.,2009). The inactivation of NOS or inhibition of nitration and inactivation of MnSOD hampered the development of morphine-induced antinociceptive tolerance (Muscoli et al.,2007). Indeed, in a model of NOS-deficient mice morphine was not able to induce antinociceptive tolerance (Heinzen et al.,2004). Macrophage injury induced by the production of superoxide as a consequence of of NADPH oxidase is apparently mediated by activation of μ-opioid receptor and subsequent activation of the phospholipase D pathway and increase in intracellular Ca^{2+} concentration (Bhat Et al.,2004). Moreover, other cell processes such as neuroimmune activation of NMDA receptors can

contribute to peroxynitrite formation and morphine dependence and tolerance (Bhat et al.,2004; Salvemini et al.,2009; Murray et al.,2007).

Basing on these evidence it is clear that morphine-induced ROS formation and decrease in the activity of antioxidant enzymes can lead to oxidative damage of different types of biomolecules, including DNA, lipids and proteins (Ozmen et al.,2007). In this context the individuation of molecules with the ability to normalize morphine-induced depletion of GSH, alterations in enzyme activities of SOD, CAT and GSHPx and reduction of cell viability could be important in order to prevent many phenomena related to the use of opioids drugs.

1.8 Proteasome, oxidative stress and pain

1.8.1 Protein degradation and Ubiquitin Proteasome system

The intracellular protein levels depend on the balance between synthesis and degradation processes that are both pivotal to ensure the correct cell functioning. In particular, protein degradation is finely regulated by two major mechanisms: lysosomal digestion (Dikic et al.,2017; Appelqvist et al., 2013) and degradation through the ubiquitin-proteasome system (UPS) (Peth et al., 2010; Inobe et al.,2014; Collins et al.,2017). The 26S Proteasome is a dynamic protein complex, extremely abundant in cells (Collins et al., 2017; Goldberg et al., 2003) and endowed with the capacity to degrade different intracellular proteins (about 90% of the entire non-lysosomal degradation) when they are conjugated with Ubiquitine (Ubq). This latter represents the "label marker" allowing a highly specific proteolysis to prevent uncontrolled protein degradation (Goldberg et al., 2003; Hochstrasser et al., 1996). Moreover, several evidence showed that beside the degradation function of mutated or damaged proteins, UPS participates in the regulation of many cellular processes, such as cell growth and proliferation, cell cycle control through the proteolysis of specific regulatory proteins (Schwartz et al.,1999), DNA repair, and regulation of the immune and inflammatory responses (Wang et al., 2006; Kammerl et al., 2016). The conjugation of selected substrates with Ubq molecules occurs through the action of three different enzymes: E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligase enzyme) which work sequentially to label proteins for different fates (Glickman et., 2002; Gallestegui et al., 2010) **(Figure 14)**. It is interesting to note that the E1 enzyme, through the ATP molecule hydrolysis, is able to form a high-energy thio-esther bond with Ubq involving the thiol group of the E1 enzyme active site. Thus, through a trans-estherification reaction, the activated portion of Ubq is transferred to a cysteine residue on the conjugating enzyme E2. This latter allows the binding of the activated Ubq to the protein substrate which is specifically bound to the enzyme E3 (Glickman et al., 2000).

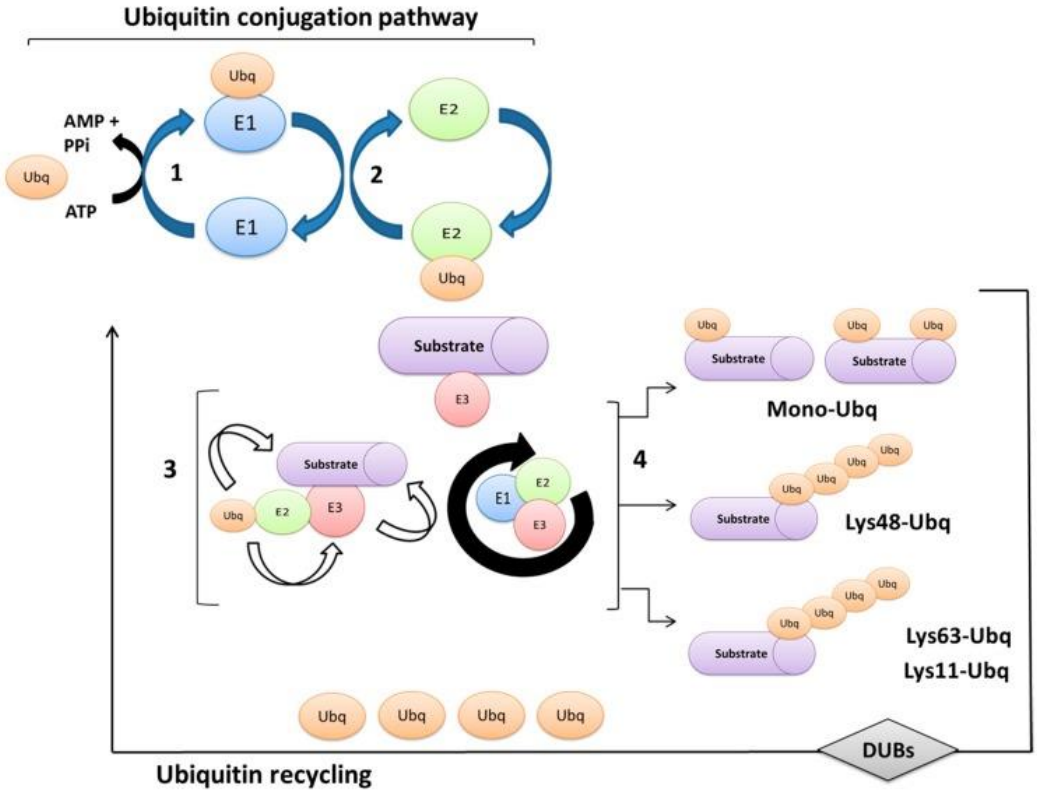

Figure 14. Schematic representation of ubiquitination process (Caputi et al.,2019)

The E3 enzymatic class represents the main class involved in the whole ubiquitination process. Indeed, it ensuring the efficiency of the entire process guaranteeing the selective recognition of the substrate. Proteins can be modified by the conjugation of a single Ubq molecule to one or several lysine residues, thus, resulting in mono- or poly-ubiquitinated products. However, because Ubq itself contains lysine residues that act as sites of self-conjugation, poly-ubiquitin chains can also be subsequently produced (Pickart et al., 2001). The ubiquitin post-translational modifications, both mono- or poly-ubiquitination, direct the conjugated substrates to different cellular fates. In particular, has been observed that the mono-ubiquitination process is particularly involved in the histone regulation (Pham et al.,2000; Robzk et al., 2000) and in endocytosis, for these reasons it regulates the activity of several proteins located at the plasma membrane (Strous et al.,1996; Rotin et al., 2000). The most studied poly-ubiquitin chain is linked to the lysine-48 residue, and it is known as a "protein destroyer" due to its ability to tag proteins for the 26S Proteasome degradation (Hershko et al.,1998; Gadhave et al., 2016). The poly-ubiquitination of protein on the lysine-63 instead induced a degradation process via

lysosomal pathway (Lehman et al., 2009), and it is also involved in DNA repair (Huang et al., 2006); the chain linked to the lysine-11 appears to be directly implicated in cell cycle regulation even though its function is not entirely clear (Bremm et al.,2011; Kulathu et al., 2012). Finally, the lysine-6 poly-ubiquitination seems to be associated with DNA repair and also with mitochondrial homeostasis (Caputi et al., 2019). The ubiquitination is a reversible process independently of the residue on which it takes place. This phenomenon is favored by the action of the de-ubiquitination enzymes (DUBs) which hydrolyze individual linkages to cleave Ubq chains from their substrates (Clague et al., 2013).

1.8.2 Structure and function of 26S Proteasome

Structurally, the 26S Proteasome consists of more than 33 different protein subunits that assemble to form the two main components of the complex: a core particle also named 20S (CP) with catalytic activity and one or two 19S particles (RP)with regulatory activity **(Figure 12)** (Förster and Sakata, 2013). Although, at beginning proteasome was considered only a cytoplasmic system with enzymatic activity useful to the "recycling" of misfolded or short-lived proteins (Adams, 2003) several evidence have instead shown that the substrates of this complex can include molecules such as transcription factors, cell cycle regulators, signal molecules and anti-apoptotic proteins known to be involved in the control of many cellular mechanisms (Adams, 2003).

Core particles 20S: The core particle 20S (CP), also named 20S proteasome, can be found in cells both associated that dissociated with different regulatory subunits such as 19S subunits (PA700), 11S (PA28, REG or PA26) and PA200 which are essential to performer its catalytic activity (Tanaka, 2009; Kim et al., 2011; Bai et al., 2014). Although this particle is constitutively present in all viable domains, including bacteria, it seems to play a crucial role especially in eukaryotes. The high structural and functional analogy between the various species demonstrates how the core particle has undergone evolutionary modifications in order to perform its function also in the more complex organisms (Förster and Sakata, 2013; Gallastegui and Groll, 2010). The 20S complex present a quaternary structure that is highly preserved in each species and has a molecular weight of about 700 kDa (Förster and Sakata, 2013). It consists of four overlapped hetero-heptameric rings that give it the typical barrel shaped. In the eukaryotes, the constituent rings are composed of different subunits; in particular, the two inner

rings are constituted by 7 beta subunits (β1-β7) while the two outermost are composed by 7 α subunits (α1-α7) **(Figure 15)**. The whole complex thus appears to have a clear symmetry of the α / β / β / α type. The specific location of these structures within the 20S proteasome allows us to identify three cylindrical compartments placed in series, in the centerof which is located the cavity hosting the proteolytic active site (Kim etal.,2011). The latter is precisely located in the correspondence of the β subunits which, like the α, belong to the superfamily of N-terminal nucleophilic hydrolases (Ntn). The main feature of this enzymatic class is the ability to use only one N-terminal residue, consisting of the triad Thr / Ser / Cys, for catalysis. Specifically, threonine plays a critical role in the proteolytic process since it is a species capable of donating and accepting protons. During hydrolysis, threonine, through the donation of a proton to its own amino group in position α, generates a nucleophilic species capable of attacking the substrate's carbonylcarbon. At the end of the enzymatic reaction the structure of threonine is restored though the transfer of the proton to the nitrogen group of the cleavaged peptide (Förster and Sakata, 2013).

In the eukaryotes' 20S proteasome the threonine is present exclusively at the extremity of N-terminal of the β1, β2 and β5 subunits of each β ring (Kim et al., 2011). Therefore, is evident that the active sites responsible for the proteolysis are six, three for each ring, and everyone show high specificity for its substrates. Indeed, the chymotrypsin-like active site, of the β5 subunit, hydrolyzes the substrate's chain having hydrophobic residues while basic and acid residues are lysed by the activity of the β2 and β1 subunits that show the trypsin-like and caspase-like active sites respectively (Förster and Sakata, 2013). The entry of specific substrates into the internal cavity, where degradation occurs, is controlled by a narrow channel located in the center of the outer α ring. The passage through this channel of the unfolded polypeptides is prevented or favorite by the closing or opening state of the gate formed by the N-terminal tails of the α subunits (Groll et al., 2000,2003).

***Regulatory particles 19S*:** The catalytic action of the core particle, in the 26S proteasome, is regulated by the 19S multiprotein complex (RP or PA700) which can bind only one or both the α rings of the 20S proteasome (Tanaka, 2009). The 19S portion has a molecular weight of about 900 kDa and consists of 19 different protein subunits which assembling in a specific way leads to the formation of two sub-complexes: base and lid (Glickman et al., 1998). According to their functional and structural characteristics the different subunits can be divided into two groups. The former, named Rpt1-6 (Regulatory Particle Triple – A protein), contains six subunits with ATP-asic activity while the latter is constituted by thirteen subunits without ATP-asic activity and called Rpn1-13 (Regulatory Particle Non – ATPase). The base subcomplex is formed by Rpt1-6 and Rpn1, Rpn2, Rpn13 and often also from the Rpn10. The C-terminal ATP-asic domains of the subunits Rpt, which are part of the AAA-ATPase family, form a hetero-hexameric ring that directly binds the α structures of the core 20S (Kwak et al., 2011).

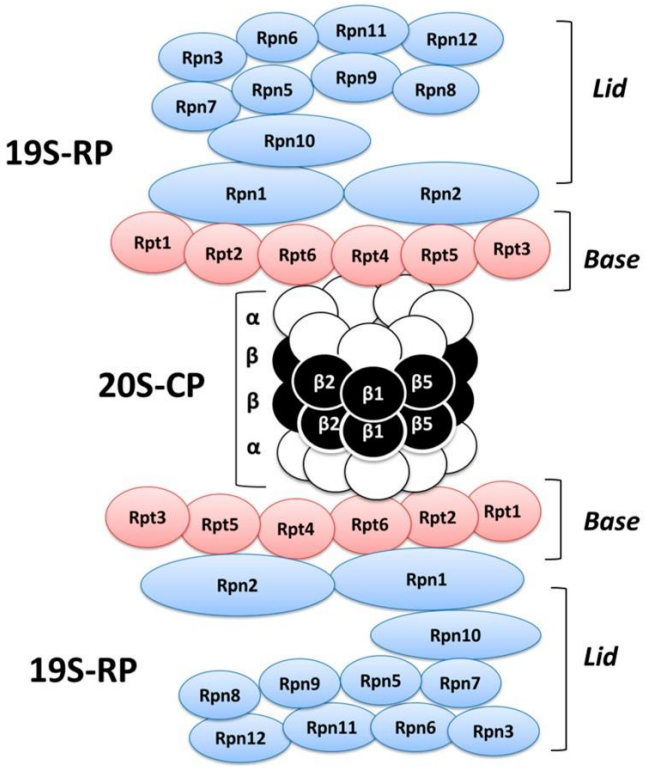

Figure 15. Schematic representation of 26S Proteasome complex (Caputi et al.,2019)

1.8.2 Proteasome and oxidative stress

The 26S proteasome represents, in physiological conditions, the main cellular degradation machinery that allows, through an ATP-dependent mechanism, the degradation of ubiquitinated substrates. However, it has been shown that the onset of oxidative stress could also activate this enzymatic complex in order to degrade mildly oxidized proteins and protect in this way the cell from oxidative damage (Ding et al.,2003; Grune et al.,2004).Although the degradation of oxidatively damaged proteins can occur by ubiquitin/ATP-dependent mechanism, several studies suggest that the activation of 20S proteasomes may be more critical for the removal, through a process ubiquitin/ATP-independent, of damaged proteins (Goldberg et al.,2003; Jung and Grune, 2008). This could be in part related to the fact that the 20S proteasome is more resistant to oxidative stress than the 26S proteasome. Indeed, as reported, 20S complex can maintain its proteolytic activity even upon treatment with moderate to high concentrations of H_2O_2, contrarily the 26S proteasome seems to be more vulnerable to the oxidative damage (Reinheckel et al., 1998; Reinheckel et al., 2000). In fact, several evidence displayed that when the oxidative challenge persists, or acute oxidative stress is applied, it is possible to observe a partial inhibition of 26S activity which lead to an increase of ubiquitinated substrates in the cell (Seifert et al., 2010; Wang et al., 2010). The inhibition of 26S proteasomes seems to be strongly related to the ability of oxidative stress to trigger 26S disassembly (Grune et al., 2004; Sitte et al., 2000) and to cause the production of oxidation products such as protein aggregates or oxidized lipids (Seifert et al., 2010; Wang et al., 2010). The disassembly of the 20S core from the 19S particle allows the liberation of 20S complexes and in this way increases cellular capacity to removal of oxidized proteins via a mechanism ATP/ubiquitin-independent. Once the oxidative stress is removed, the reassembly of the 26S proteasome occurs and the degradation of ubiquitinated substrates can resume, leading to cellular recovery.

During prolonged exposure to oxidative stress (more than 12 h following stress induction), proteasome activities are inhibited, and *de novo* proteasome synthesis is activated (Ding et al., 2003; Hussong et al., 2010). The increase of the synthesis both standard and inducible proteasome subunits lead to the formation of more functional 20S and i20S proteasomes, respectively. The newly produced 20S and i20S complexes can associate with PA28 and/or 19S regulatory complexes respectively to form diverse functional proteasome complexes for ubiquitin/ATP- independent and/or dependent degradation of oxidized proteins (Seifert et al.,2010; Pickering et al.,2010) **(Figure 16)**.

As suggested by many studies the activated 20S, i20S, and i26S proteasomes are more efficient to degrade oxidized proteins than the standard 26S proteasome (Seifert et al.,2010; Pickering et al.,2010) and the production of immunoproteasomes seems to be of particular importance in order to induce a cellular response against oxidative stress (Hussong et al.,2010).

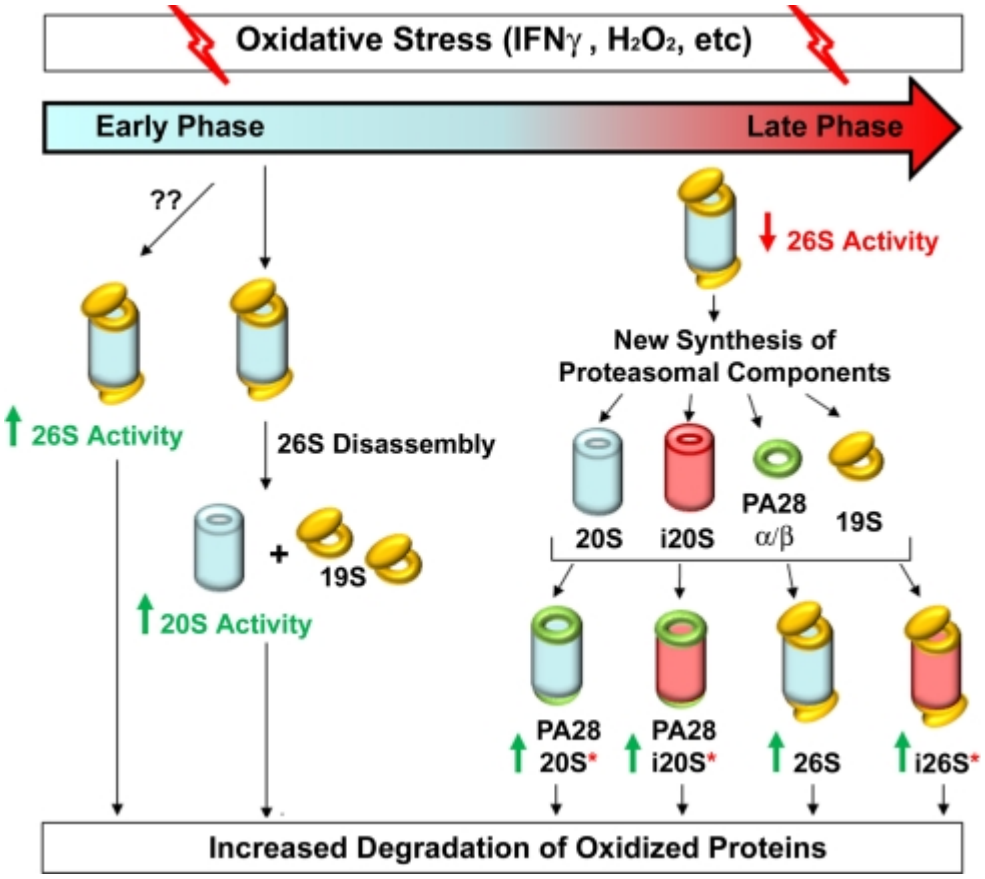

Figure 16. Model of oxidative stress dependent regulation of proteasomes (Aiken et al.,2011)

1.8.3 Involvement of Proteasome in opioids receptor signaling associated with analgesia and neuropathic pain

Some evidence indicates the involvement of UPS in many molecular phenomena associated both with analgesia that the development of neuropathic pain. In this regard, it has been shown that the down-regulation of MOR and DOR induced by an opioid agonist can be attenuated by proteasome inhibitors, and to a lesser extent by lysosomal inhibitors (Chaturvedi et al., 2001). Moreover, it is also noting that in the absence of agonist ligands proteasome inhibitors are able to increase MOR and DOR levels, thus, suggesting a prominent role of UPS either in basal and in agonist-induced turnover of opioid receptors (Chaturvedi et al., 2001).Other findings, have been reported that prolonged morphine exposure promotes the G_{β} down-regulation, an effect that is totally suppressed by MG-115 or lactacystin proteasome inhibitors (Moulédous et al., 2005) and which as suggested by authors is likely related to the proteasome degradation of G_{β} protein that seems to participate in the so-called "hypertrophy of the cAMP system" caused by the prolonged morphine-induced MOR activation. The involvement of this complex system in the signaling pathway of opioids receptor has been supported by other evidence showing that overnight exposure to [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin (DAMGO) or [D-Pen2,D-Pen5]encephalin (DPDPE), selective MOR and DOR agonists respectively, produces a significant decrease of regulator G protein signaling protein 4 (RGS4) which acts as GTPase that modulates opioid receptor signaling, and causes a profound loss of opioid receptors in SH-SY5Y cells (Wang et al.,2011). The RGS4 down-regulation appears completely blocked by MG-132 pretreatment or by the specific proteasome inhibitor lactacystin and, accordingly, the protein remains strongly poly-ubiquitinated suggesting that these two drugs are able to the RGS4 poly-ubiquitination which normally acts as a signal degradation for UPS. Contrarily, the opioid receptor loss was not counteracted by MG-132 likely because other pathways are involved in their degradation (Wang et al.,2011). This hypothesis has been demonstrated for DOR that is well known to undergo endocytic trafficking to lysosomes (Tsao et al., 2000). However, conflicting data exist about the mechanism of agonist-induced opioid receptor down-regulation. Indeed, Chaturvedi et al. demonstrated that pretreatment with proteasome inhibitors, but not with lysosomal, attenuates the agonist-induced MOR and DOR down-regulation and that in the absence of agonist the proteasome inhibitors increase the steady-state levels of both opioid receptors (Chaturvedi et al., 2001). Although the exact mechanism by which chronic opioid agonists, including morphine, activate UPS machinery is still poorly understood, even though the involvement of UPS in neuropathic pain is envisioned (Moss et al., 2002; Ossipov

et al., 2007). In fact, an increase of proteasome activity seems to be often associated with the neuropathic pain conditions. In this regard, it has been demonstrated that pain behavioral signs induced by spinal nerve ligation (SNL) are accompanied by the increase of dynorphin A levels in the spinal cord and that proteasome inhibitors are able to decrease painful signs together with dynorphin level normalization (Ossipov et al., 2007). Moreover, authors have also demonstrated that proteasome inhibitors directly modulate the dynorphinergic system, since mouse insulinoma MIN6 cells exhibit a reduction in dynorphin secretion after epoxomicin and MG-132 exposure (Ossipov et al., 2007). In addition, the single intrathecal injections of epoxomicin reduced capsaicin-evoked calcitonin gene-related peptide (CGRP) release from tissues of both sham-operated and SNL rats, thus, demonstrating the potential usefulness of proteasome inhibitors in the prevention and normalization of neurotransmitter release (Ossipov et al.,2007). All these results converge on the involvement of UPS in the development and maintenance of neuropathic pain condition. In addition, it has recently showed that an increase of proteasome activity could be related to the phenomenon of analgesic tolerance. Indeed, it has been demonstrated that the co-administration of MG-132 with morphine prevents the development of morphine tolerance through the prevention of both spinal glutamate transporter down-regulation and spinal glutamate uptake activity decrease (Yang et al., 2008).

2. AIMS OF THE RESEARCH

Chronic pain represents one of the major health issues in our society. Although mortality rates are highest for other pathologies, this condition seems to be one of the main sources of human suffering and disability that profoundly impacts patients' quality of life. Despite research advancement and the suggestions of new targets for acute and chronic pain treatment, opioids still represent the gold standard analgesics. However, their use is often hampered by the development of several adverse side effects including the development of analgesic tolerance and opioid-induced hyperalgesia (OIH). Although these phenomena are not yet completely understood, molecular changes in opioid receptors, neurotransmitter release alterations, as well as glia and microglia activation have been suggested as possible mechanisms involved both in the development of chronic pain conditions and in the appearance of side effects related to chronic opioid treatment (Du Pen et al.,2007; Chu et al., 2008, Lee et al., 2011; Hutchinson et al., 2011; Roeckel et al.,2016; Micheli et al.,2018).

Moreover, some evidence also suggests the potential role of oxidative stress in the above mentioned phenomena and pathological conditions (Skrabalova et al.,2013; Little et al 2012). Indeed, substantial data showed how the production of reactive species, probably through a neuroinflammation process, could participate to the development of analgesic tolerance, OIH as well as to chronic pain (Salvemini et al.,2009; Muscoli et al.,.2007). In this context, the degradation of non-functional oxidized proteins represents a fundamental cellular process necessary for maintaining antioxidant defense machinery and to protect cells from oxidative damage. This process is carried out by the proteasome which represents the major enzymatic complex involved in the ubiquitinated/oxidated protein degradation (Ding et al.,2003; Grune et al.,2004). In this regard, the contribution of proteasome activation in central sensitization, hyperalgesia, and allodynia phenomena, which all represent the characteristic features of neuropathic pain condition, has been demonstrated (Moss et al.,2002; Ossipov et al.,2007). Moreover, the use of proteasome inhibitors, like MG132 and epoxomicin, has been proposed for their ability to prevent/revert both tolerance and opioid-induced hyperalgesia (Yang et al.,2008; Ossipov et al.,2007) suggesting the involvement of this degradation complex also in the development of these two phenomena. Based on this evidence, the aims of this PhD thesis were to investigate the effects of a series of opioid drugs on cell oxidative stress, antioxidant enzymatic machinery and proteasome expression and activity *in vitro*. In addition, the involvement of the proteasome complex in the development of chronic pain conditions was

investigated *in vivo* utilizing an experimental model of oxaliplatin-induced neuropathy (OXAIN), in the rat.

For these purposes, the ability of four different opioids analgesic drugs to produce oxidative stress, to affect cell antioxidant response as well as to alter the proteasome β2 and β5 subunit proteolytic activities and gene expression was assessed in SH-SY5Y cell cultures.

In an attempt to find possible correlations between changes of these parameters with the analgesic drug pharmacological profile, cells were exposed to morphine or fentanyl (full MOR agonists), buprenorphine (partial MOR agonist) or tapentadol (bifunctional MOR/NRI analgesic). In addition, taking into account the peculiar binding profile suggested for buprenorphine, acting as MOR agonist at low concentrations and as NOP agonist at high ones, the effects of this opioid drug on proteasome parameters were investigated in both experimental conditions.

With regards to the *in vivo* studies, β2 trypsin-like and β5 chymotrypsin-like activities of 20S proteasome were evaluated in the spinal cord (lumbar, thoracic and cervical portions) and supraspinal CNS regions known (SSCx, TH and PAG) of OXAIN suffering rats.

To evaluate and discern the involvement of the constitutive and inducible (immune) proteasome complex, the gene expression of both β5 (constitutive) and LMP7 (or β5i, inducible) proteasome subunits was assessed in the supraspinal areas. Finally, given the observed reduction of neuropathic pain induced by intrathecal proteasome inhibitors (Moss et al.,2002) that are also capable to counteract the increase of spinal dynorphin A peptide levels associated with neuropathic pain conditions, (Ossipov et al.,2007), we have also investigated the gene expression of spinal prodynorphin in OXAIN-suffering rats as well as the behavioral and neurochemical effects of oprozomib, a second-generation proteasome inhibitor compound.

3. Material and methods

3.1 In vitro studies

3.1.1 Cell culture

Human SH-SY5Y neuroblastoma cells purchased from ICLC-IST (Genoa, Italy), were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin and 2 mM glutamine. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and were allowed to reach 80% confluence before starting treatments. All reagents employed for cell culture were purchased from Lonza (Milan, Italy).

3.1.2 Cell treatments

SH-SY5Y cells were exposed in the first experimental set to 10μM Morphine, 0.1μM Fentanyl, 0.25μM Buprenorphine or 10μM Tapentadol. The doses used for the cell treatment were chosen according with previous studies (Caputi et al., 2015; Kiraly et al., 2015; Caputi et al., 2013) and based on the analgesic potency of the selected opioid drugs respect to morphine.
In the second experimental set, SH-SY5Y cells were exposed to two different doses of Buprenorphine 0.02μM (>MOR agonist) or 2.5μM (>NOP agonist).
All biochemical analyses were performed at 2, 5, 24, 48 hours for both experimental sets. Four/six biological replicates per treatment were utilized in each experiment.

3.2 In vivo studies

3.2.1 Animals

Sprague Dawley rats (220–250 g; Envigo, Varese, Italy) were used. Animals were housed in the Centro Stabulazione Animali da Laboratorio (CeSal, University of Florence), four rats were housed per cage (size 26 × 41 cm^2); animals were fed with a standard laboratory diet and tap water *ad libitum* and kept at 23 ± 1 °C with a 12 h light/dark cycle (light at 7 A.M.). All animal manipulations were carried out according to the Directive 2010/63/EU of the European Parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85–23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 498/2017-PR) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines (McGrath et al., 2015). Five animals per group were utilized in each experiment. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.2.2 Oxaliplatin-induced neuropathic pain model and oprozomib administration

Oxaliplatin (2.4 mg kg^{-1}; Carbosynth, Compton, UK) was injected intraperitoneally (i.p.) for 5 consecutive days every week for 2 weeks for a total of 10 injections (from day 1 to day 5 and from day 8 to day 12) (Resta et al.2018). Oxaliplatin was dissolved in a 5% glucose solution. Oprozomib (30–100 mg kg^{-1}), suspended in 1% carboxymethylcellulose sodium salt (CMC; Sigma-Aldrich, Milan, Italy) was acutely administered *per os* (p.o.) on day 15, at the end of oxaliplatin injection (day 15) when neuropathy was well established. Control animals received an equivalent volume of vehicles.

3.2.3 Assessment of mechanical hyperalgesia (Paw pressure test)

The nociceptive threshold of rats was determined by an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton et al. (Leighton et al., 1988). Briefly, a constantly increasing weight was applied to a small area of the dorsal surface of the hind paw using a blunt conical probe by a mechanical device. Mechanical weight (expressed in g) was increased until vocalization or withdrawal reflex occurred while rats were lightly restrained. An arbitrary cut-off value of 100 g was adopted.

3.2.4 Assessment of thermal allodynia (cold plate test)

Given that cold hypersensitivity represents a hallmark of oxaliplatin-induced neuropathy (Ling et al., 2007) the thermal allodynia was assessed using the cold-plate apparatus (Ugo Basile, Varese, Italy). With minimal animal-handler interaction, rats were taken from home-cages, and placed onto the cold surface maintained at a constant temperature of 4 °C ± 1 °C. Ambulation was restricted by a cylindrical Plexiglas chamber (diameter: 10 cm, height: 15 cm), with open top. Pain-related behavior (paw lifting or licking) was observed, and the time (s of the first sign was recorded. The cut-off time latency was set at 30 s.

3.2.5 Assessment of mechanical allodynia (Von Frey test)

Mechanical allodynia was measured with an electronic Von Frey hair unit (Ugo Basile, Varese, Italy) as described by Sakurai and colleagues (Sakurai et al.,2009). Briefly, rats were placed in 20 × 20 cm Plexiglas boxes equipped with a metallic screen-mesh floor, 20 cm above the bench. A habituation of 15 min was allowed before the test. The withdrawal threshold was evaluated by applying a force ranging from 0 to 50 g with a 0.2 g accuracy. The punctuate stimulus was delivered to the mid-plantar area of each posterior paw from below the meshy floor through a plastic tip and the withdrawal threshold was automatically displayed on the screen. The paw sensitivity threshold was defined as the minimum pressure required to elicit a robust and immediate withdrawal reflex of the paw. Voluntary movements associated with locomotion were not taken as a withdrawal response. Stimuli were applied on each posterior paw with an interval of 5 s. The measure was repeated five times and the final value was obtained by averaging the five measures.

3.2.6. Assessment of motor coordination (Rota-rod test)

The Rota-rod apparatus (Ugo Basile, Varese, Italy) consisted of a base platform and a rotating rod with a diameter of 6 cm and a non-slippery surface. The rod was placed at a height of 25 cm from the base. The rod, 36 cm in length, was divided into 4 equal sections by 5 disks. Thus, up to 4 rats were tested simultaneously on the apparatus, with a rod-rotating speed of 10 revolutions per minute. The integrity of motor coordination was assessed on the basis of the time the animals kept their balance on the rotating rod for a maximum of 10 min (600 s). After a maximum of 6 falls from the rod, the test was suspended and the time elapsed was recorded.

3.2.7 Tissue collection

On day 15, at the end of the last behavioral test, animals were sacrificed using a guillotine. Thalamus (TH), the somatosensory cortex (SSCx) the periaqueductal gray (PAG) and the spinal cord (dissected in lumbar, thoracic and cervical portions) were collected and immediately frozen in liquid nitrogen for further ex vivo biochemical analysis.

3.3 Biochemical Analysis

3.3.1 Intracellular reactive oxygen species production

Intracellular reactive oxygen species was measured with the 2,7-dichlorofluorescin diacetate (DCFH-DA) assay, OxiSelect™ Intracellular ROS Assay Kit (Cell Biolabs). According with manufacturer's instruction SH-SY5Y cells were seeded at a density of 3×10^4 cells/well in 96-well plates. 10 μM DCFH-DA was then dissolved in medium containing 1% FBS and added to each well. Cells were incubated for 2 h in order to allow cellular incorporation. Thereafter, the original medium was discarded, and opioids drugs was added to the cell medium, culturing for 2, 5, 24, 48 hours. Then DCF fluorescence intensity was read at 37 °C in a fluorescence plate reader with an emission wavelength of 525 nm and an excitation wavelength of 485 nm. Results are expressed as the percentage of DCF fluorescence intensity in relation to the untreated control.

3.3.2 Protein extraction

Samples were homogenized in lysis buffer (150 mM NaCl, 50 mM HEPES pH 7.5, 5 mM EDTA, 2 mM ATP, 1% Triton; Sigma-Aldrich) and centrifuged at 14,000 × g at 4 °C for 15 min. Homogenates were aliquoted and kept at -80 °C. Protein concentration was determined by using Pierce TM BCA protein assay kit (Thermo Scientific, Italy).

3.3.2.1 SOD activity assay

The SOD activity was determined by using the SOD assay kit-WST (Sigma Aldrich) according to the manufacturer's protocol. The assay utilized the Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt) which forms a water-soluble formazan dye upon reduction with a superoxide anion and this reduction rate is inversely proportional to SOD activity. The assay was carried out according to the manufacturer's instructions. The samples (20 µl) mixed with 200 µl WST working solution (1 ml WST solution, 19 ml of buffer solution) were allowed to react with 20 µl of enzyme working solution (15 µl of enzyme solution, 2.5 ml of dilution buffer). Distilled water (ddH2O, 20 µl) was used as the sample substitute for blank 1 (S1) wells and 20 µl of dilution buffer as the substitute of enzyme working solution for blank 2 (S2) wells. In blank 3 (S3) wells, only 20 µl of each ddH2O and dilution buffer were added to the 200 µl WST working solution. The reaction mix was then incubated at 37 °C for 20 min. The decrease in absorbance was measured at 450 nm.
The SOD activity was measured as follows:

SOD activity (%)={[(AbsS1−AbsS3) (Abssample−AbsS2)]/(AbsS1−Absblank)} ×100

3.3.2.2 Proteasome activity

The proteasome trypsin- and chymotrypsin-like activities were analyzed monitoring the cleavage of two fluorogenic substrates using 25 µg of lysate proteins. The substrates benzyloxycarbonyl-Ala-Arg-Arg-7-amino-4-methylcoumarin (Z-ARR-AMC) and succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC) (both purchased from Merck Millipore, Milan, Italy) were used according to the manufacturer's instructions to

measure trypsin- (β2) and chymotrypsin-like (β5) activities, respectively. The assay is based on the detection of the fluorophore 7-amino-4- methylcoumarin (AMC) after cleavage from the labeled substrates Z-ARR-AMC or LLVY-AMC. All samples were run in triplicate and added to the substrates, then incubated for 2 h at 37 °C. The free AMC fluorescence was quantified at 380 nm excitation and 460 nm emission wavelengths using a plate reader fluorometer (GENios Tecan). According to the manufacturer's instructions, an AMC standard curve was generated for reference by preparing a dilution series of AMC standard reagent in the concentration range of 0.04–12.5 μM and runin triplicate. The assay was validated by analyzing proteasome positive control incubated with the inhibitor lactacystin and two independent experiments were carried out for each analyzed tissue.

Data are expressed as percentage of relative fluorescence (RFU) and are reported as mean ± SEM of five rats per group.

3.3.3 RNA isolation and qRT-PCR

Total RNA was isolated using the TRIZOL reagent (Life Technologies, USA) according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 2006) and its integrity was checked by 1% agarose gel electrophoresis. In brief, the amounts of RNA were determined by measuring optical densities and only RNA samples with an OD260/OD280 1.8 < ratio < 2 were used. Total RNA was reverse transcribed using GeneAmp RNA PCR kit (ThermoFisher Scientific, USA) in a final volume of 20 μl, according to manufacturer's instructions. Quantitative real-time PCR was performed on a StepOne Real-Time PCR System (Life Technologies, USA) using TaqMan Gene Expression Master Mix (ThermoFisher Scientific, USA), to analyze theβ2 (Hs 01002946_m1, FAM), β5 (Hs 00605652_m1, FAM), SOD1 (Hs 00533490_m1, FAM), β5 (Rn01488742_m1, FAM-MGB) and the LMP7 (or β5*i*) (Rn00589926_m1, FAM-MGB) proteasome subunits gene expression. All samples were run in triplicate and were normalized to the endogenous reference gene glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) (Hs 03929097_g1, VIC) or (Rn01775763_g1, VIC-MGB). Primers and probe sequences used for TaqMan gene expression were purchased from ThermoFisher Scientific, USA.

To analyze pDYN gene expression the SYBR Green PCR MasterMix was used (Life Technologies, USA). Each sample was run in triplicate and all data were normalized to GAPDH. The primers used for PCR amplification in SYBR Green PCR MasterMix were

designed using Primer 3 and are here reported: GAPDH Forward 5′-AGACAGCCGCATCTTCTTGT-3′; GAPDH Reverse 5′-CTTGCCGTGGGTAGAGTCAT-3′; pDYN Forward 5′-CCTGTCCTTGTG TTCCCTGT-3′; pDYN Reverse 5′-AGAGGCAGTCAGGGTGAGAA-3′. Relative abundance of each mRNA species was calculated by Delta–Delta Ct (ΔΔCt) method and converted to relative expression ratio ($2^{-\Delta\Delta Ct}$) for statistical analysis. Data are expressed as means ± SEM (each sample run in triplicate) and represented as fold change in mRNA levels.

3.4 Statistical analysis

All experimental results were expressed as mean ± SEM of four/six biological replicates (*in vitro* studies) or five animals (*in vivo* studies) and data were analyzed by *t*-test or by one-way or two-way ANOVA followed by Bonferroni's multiple comparison post hoc test, as appropriate. P values < 0.05 were considered statistically significant. Experiments and data analysis were carried out in a blind fashion. Statistical analysis was performed using GraphPad Prism software package (v8 for Windows, GraphPad Software, San Diego CA, USA, www.graphpad.com).

4. Results

4.1 In vitro results

4.1.1 ROS production after exposure to opioid analgesic drugs at different time points

Overall ANOVA indicated a significant effect of treatment ($F_{(4,84)} = 11.47$, $p<0.0001$), time ($F_{(3,84)} = 87.66$, $p<0.0001$) and interaction ($F_{(12,84)} = 3.62$, $p=0.0002$) on ROS production. Bonferroni's multiple comparison test showed that all the selected opioid drugs were not able to induce significant alteration of the intracellular ROS both after the exposure at 2 or 5 hours **(Figure 17)**.

The statistical analysis revealed a significant increase of ROS after the treatment with morphine at 24 and 48 hours respect the relative vehicle groups (24h: 10µM Morphine 202.73 ± 21.60 vs Vehicle 148.20 ± 8.91, $p<0.05$; 48h: 10µM Morphine 265.92 ± 27.13 vs Vehicle 158.06 ± 10.00, $p<0.0001$) **(Figure 17)**.

In addition, the results displayed that in the comparison between morphine and other opioids drugs, tapentadol is able to induce lower ROS production in SHSY5Y cells at 24 and 48 hours (24h: 10µM Tapentadol 151.46 ± 9.11 vs 10µM Morphine 202.73 ± 21.60, $p<0.05$; 48h: Tapentadol 180.1 0± 15.97 vs 10µM Morphine 265.92 ± 27.13, $p<0.0001$) **(Figure 17)**. Nevertheless, this effect has been showed also for the other compounds at the longest time of exposure 0.1 µM Fentanyl 180.06 ± 10.02 vs 10µM Morphine 265.92 ± 27.13, $p<0.0001$; 0.25µM Buprenorphine 192.32 ± 12.41 vs 10µM Morphine 265.92 ± 27.13, $p<0.0001$). Finally, it is interesting to note that ROS level appeared significantly higher in the vehicle group after 24 and 48 hours in comparison the respective 2h vehicle group (24h Vehicle group 148.20 ± 8.91 vs 2h Vehicle group 100.00 ± 2.17, $p<0.05$; 48h Vehicle group 158.06 ± 10.00 vs 2h Vehicle group 100.00 ± 2.17, $p<0.05$) **(Figure 17)**.

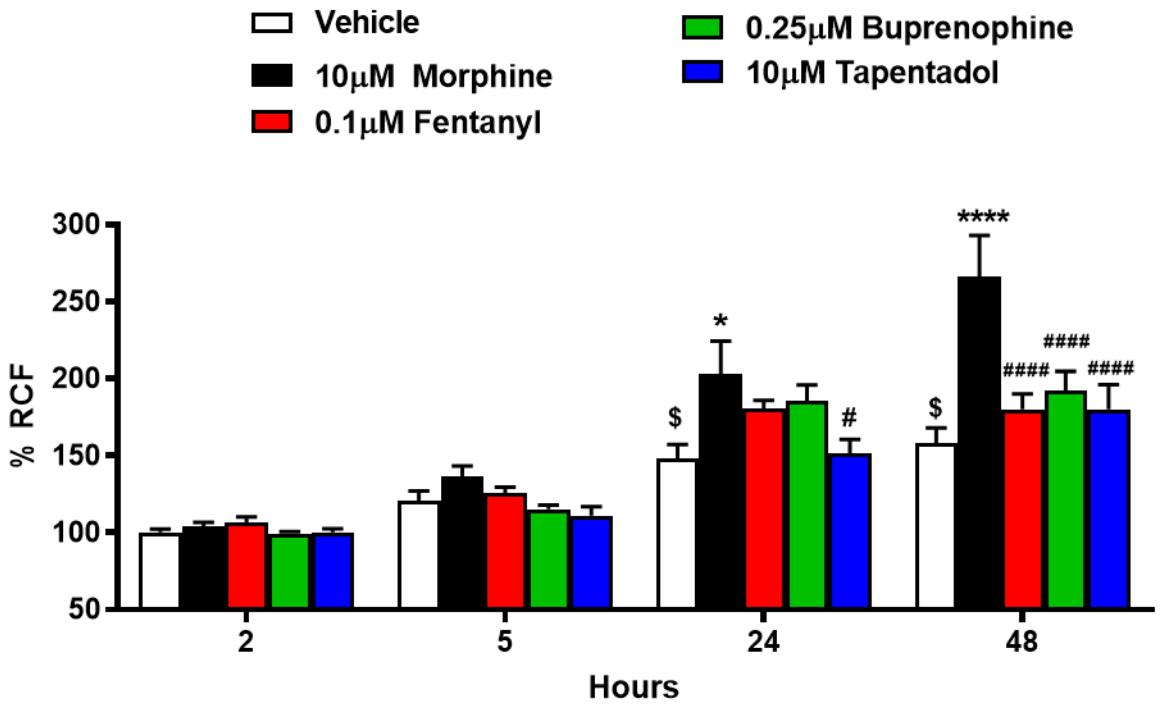

Figure 17. ROS production after treatment with Morphine, Fentanyl, Buprenorphine or Tapentadol in SH-SY5Y cells at different time point. Data are expressed as percentage of relative fluorescence (RFU) and are reported as mean ± SEM of five/six samples biological replicates per treatment (*$p < 0.05$; ****$p < 0.0001$ vs respective Vehicle group; $^{\$}p < 0.05$ vs 2h Vehicle group; $^{\#}p < 0.05$; $^{\#\#\#\#}p < 0.0001$ vs respective Morphine group) analyzed by Two-way ANOVA followed by Bonferroni's multiple comparisons test)

4.1.2 SOD activity after exposure to opioid analgesic drugs at different time points

A significant down-regulation of SOD activity was observed in all the groups of cells exposed to the selected opioids dugs at 2 hours (10µM Morphine 75.35 ± 4.40 vs Vehicle 100.00 ± 3.30, $p<0.01$; 0.1µM Fentanyl 54.71 ± 8.86 vs Vehicle 100.00±3.30, $p<0.01$; 0.25µM Buprenorphine 73.11 ± 6.34 vs Vehicle 100.00±3.30, $p<0.001$; 10µM Tapentadol 46.77 ± 10.21 vs Vehicle 100.00 ± 3.30, $p<0.01$) **(Figure 18a, b, c, d)**. Student's t-test revealed significantly lower levels of SOD activity at 5 hours in the group of cells treated with fentanyl (0.1µM Fentanyl 49.41 ± 12.33 vs Vehicle 100.00 ± 8.43, $p<0.05$) **(Figure 18b)**. While no changes were observed at longer exposure time intervals after treatment with all selected drugs **(Figure 18a, b, c, d)**.

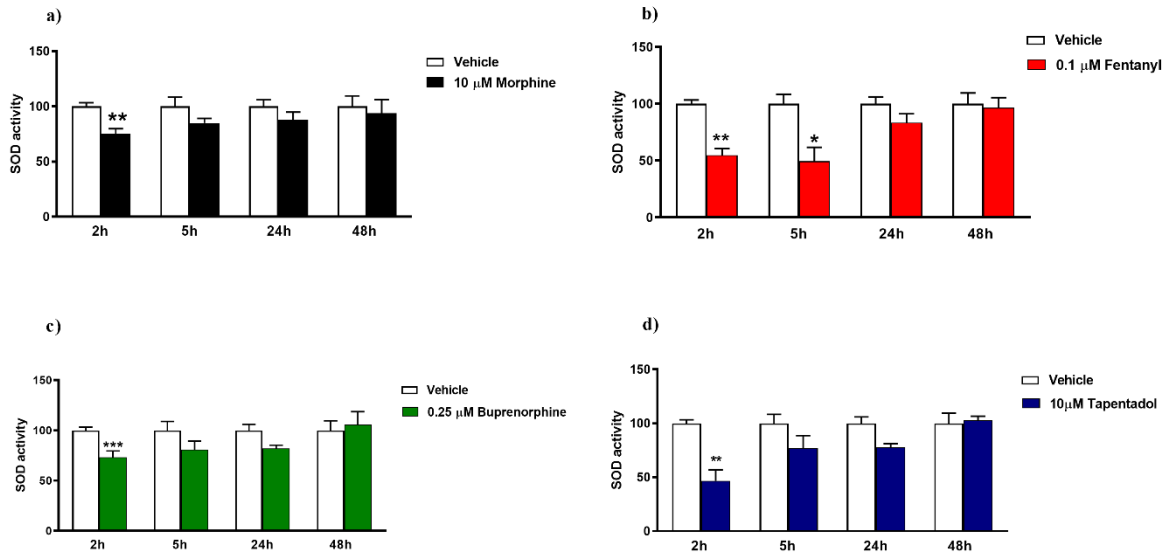

Figure 18. SOD activity after treatment with **a)** Morphine **b)** Fentanyl **c)** Buprenorphine or **d)** Tapentadol in SH-SY5Y cells at different time point. Data are expressed as percentage of Absorbance (Abs) and Data represent 2^{-DDCt} values calculated by DDCt method and are reported as mean ± SEM of four biological replicates per treatment (*p < 0.05; **p < 0.01; ***p < 0.001 vs respective Vehicle; analyzed by t-test)

4.1.3 SOD1 gene expression after exposure to opioid analgesic drugs at different time points

Statistical analisys revealed that both morphine and fentanyl were able to induce a significant up-regulation of SOD1 gene expression after a treatment of 5 hours (10μM Morphine 1.63 ± 0.17 vs Vehicle 1.00 ± 0.02, $p<0.05$; 0.1μM Fentanyl 1.53 ± 0.16 vs Vehicle 1.00 ± 0.02, $p<0.05$) **(Figure 19a, b)**. Moreover, the levels of mRNA of this gene appeared to be increased after the treatment with all the selected opioids at 24 hours (10μM Morphine 3.03 ± 0.22 vs Vehicle 1.00 ± 0.10 $p<0.001$; 0.1μM Fentanyl 3.63 ± 80.13 vs Vehicle 1.00 ± 0.10, $p<0.001$;0.25μM Buprenorphine2.77± 0.13 vs Vehicle 1.00±0.10, $p<0.001$; 10μM Tapentadol 1.49 ± 0.10 vs Vehicle 1.00±0.10, $p<0.05$) **(Figure 19a, b, c, d)**. These effects, except for the morphine, was also observed after a treatment of 48 hours (0.1μM Fentanyl 1.63 ± 0.09 vs Vehicle 1.00 ± 0.11 $p<0.05$; 0.25μM Buprenorphine 1.53± 0.10 vs Vehicle 1.00 ± 0.11, $p<0.05$; 10μM Tapentadol 1.49 ± 0.10 vs Vehicle 1.00 ± 0.11, $p<0.05$) **(Figure 19b, c and d)**.

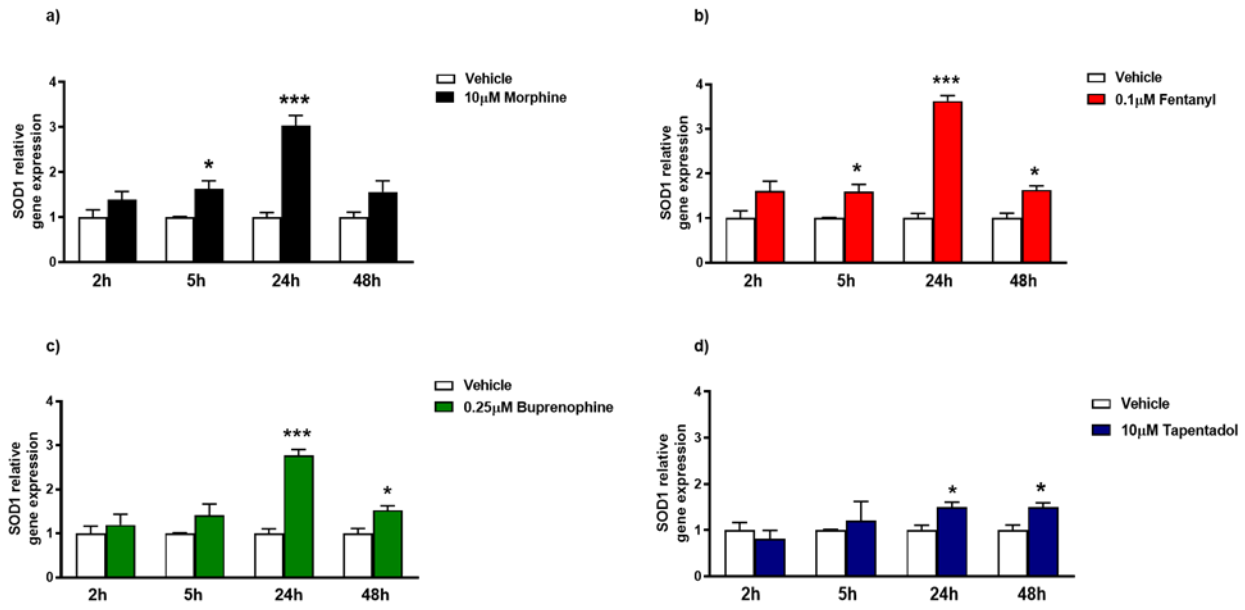

Figure 19. Relative gene expression of SOD1 after treatment with **a)** Morphine **b)** Fentanyl **c)** Buprenorphine or **d)** Tapentadol in SH-SY5Y cells at different time point. Data represent 2^{-DDCt} values calculated by DDCt method and are expressed as mean ± SEM of four biological replicates per treatment (*$p < 0.05$; ***$p < 0.001$ vs respective Vehicle; analyzed by t-tests)

4.1.4 β2 trypsin-like proteasome activity induced by exposure to opioid analgesic drugs at different time points

The statistical analysis, except for tapentadol, showed no changes in β2 trypsin-like activity after the cell's treatment with opioids at the shortest exposure time. Indeed, tapentadol was the only drug able to induce a significant increase of the analyzed subunits at 2 hours (10μM Tapentadol 114.21 ± 1.87 vs Vehicle 100.00 ± 2.42, $p<0.05$). Moreover, as revealed by Student's t-test β2 trypsin-like activity was significantly up-regulated by morphine both at 24 and 48 hours (24h: 10μM Morphine 122.97 ± 8.82 vs Vehicle 100.00 ± 4.25, $p<0.05$; 48h: 10μM Morphine 119.44 ± 4.24 vs Vehicle 100.00 ± 0.94, $p<0.05$) **(Figure 20a)** and by fentanyl at 24 hours (0.1μM Fentanyl 113.96 ± 3.13 vs Vehicle 100.00 ± 2.24, $p<0.05$) **(Figure 20b)**. Contrarily, buprenorphine determined a decrease of this proteasome subunits at 48 hours only (0.25μM Buprenorphine 64.46 ± 1.75 vs Vehicle 100.00 ± 5.20, $p<0.001$). No changes were observed at the longest exposure time in the cells treated with fentanyl or taentadol **(Figure 20b, d)**.

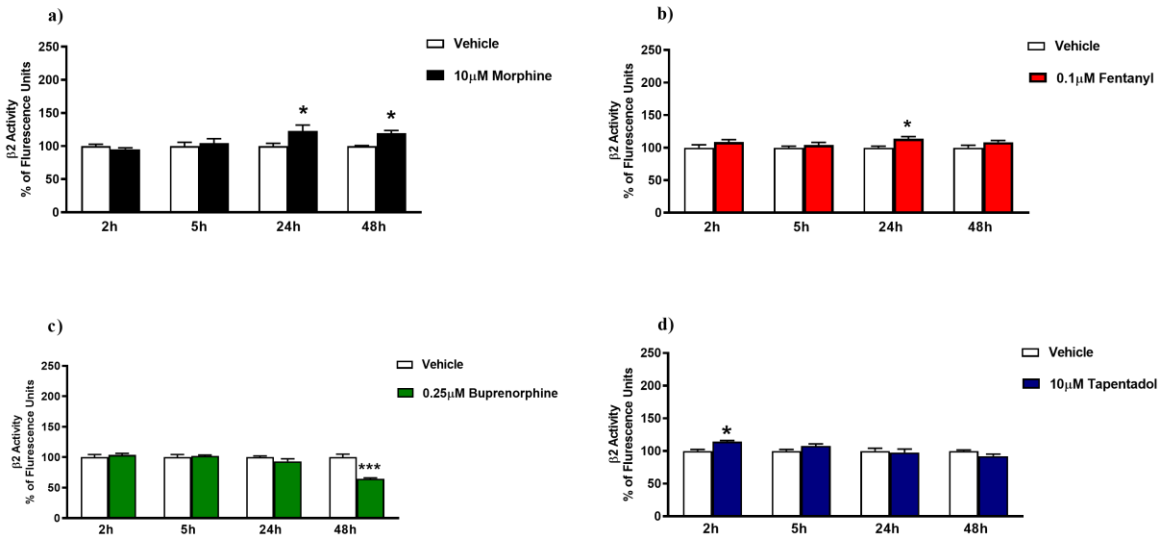

Figure 20. β2 trypsin-like activity after treatment with **a)** Morphine **b)** Fentanyl **c)** Buprenorphine or **d)** Tapentadol in SH-SY5Y cells at different time point. Data are expressed as percentage of relative fluorescence (RFU) and are reported as mean ± SEM of four biological replicates per treatment (*$p < 0.05$; ***$p < 0.001$ vs respective Vehicle group analyzed by t-test)

4.1.5 β5 chymotrypsin-like proteasome activity induced by exposure to opioid analgesic drugs at different time points

The statistical analysis showed that morphine was able to induce an increase of β5 chymotrypsin-like activity in SH-SY5Y treated cells both at 24 and 48 hours (24h: 10μM Morphine 120.03 ± 4.75 vs Vehicle 100 ± 0.65, $p<0.01$; 48h: 10μM Morphine 143.83 ± 4.59 vs Vehicle 100.00 ± 5.89, $p<0.01$) **(Figure 21a)**. Similarly, Student's t-test displayed a significant up-regulation of this proteolytic subunit after Fentanyl treatment already starting from the shortest exposition time (2h: 0.1μM Fentanyl 117.90 ± 4.38 vs Vehicle 100.00 ± 0.77, $p<0.01$; 5h: 0.1μM Fentanyl 119.50 ± 3.01 vs Vehicle 100.00 ± 2.71, $p<0.01$; 24h: 0.1μM Fentanyl 133.19 ± 3.35 vs Vehicle 100.00 ± 0.83, $p<0.01$; 48h: 0.1μM Fentanyl 112.29±1.93 vs Vehicle 100.00 ± 1.50, $p<0.01$) **(Figure 21b)**. For what concern Buprenorphine a significant increase of β5 activity was revealed at 2h (0.25μM Buprenorphine 128.62 ± 2.51 vs Vehicle 100.00±2.68, $p<0.01$). Contrarily, this subunit activity was down-regulated after a treatment of 24 hours (0.25μM Buprenorphine 68.25 ± 3.90 vs Vehicle 100.00 ± 4.31, $p<0.01$) **(Figure 21c)**. No changes were instead observed after treatment with tapentadol at all-time points **(Figure 21d)**.

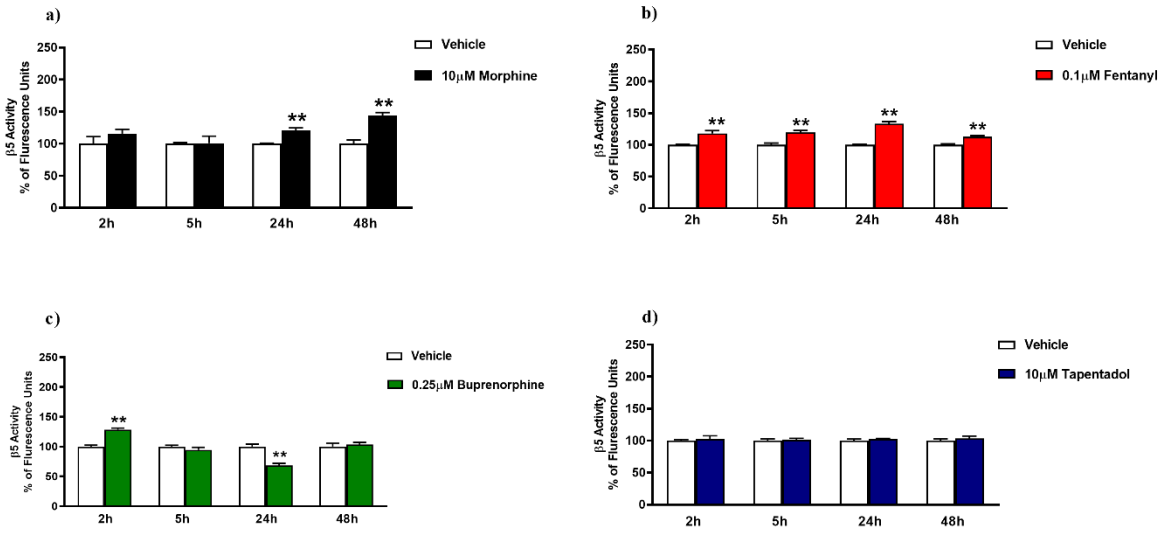

Figure 21. β5chymotrypsin-like activity after treatment with **a)** Morphine**, b)** Fentanyl **c)** Buprenorphine or **d)** Tapentadol in SH-SY5Y cells at different time point. Data are expressed as percentage of relative fluorescence (RFU) and are reported as mean ± SEM of four biological replicates per treatment (**p < 0.01 vs respective Vehicle group analyzed by t-test)

4.1.6 β2 subunit gene expression induced by exposure to opioid analgesic drugs at different time points

Statistical analysis showed that morphine induced a significant gene expression decrease of β2 subunits, both at 24 and 48 hours, in SH-SY5Y cell line (24h: 10μM Morphine 0.72 ± 0.04vs Vehicle 1.00 ± 0.08, $p<0.05$; 48h: 10μM Morphine 0.45 ± 0.07 vs Vehicle 1.00 ± 0.10, $p<0.01$) **(Figure 22a)**. Contrarily, Student's t-test comparison displayed a significant up-regulation of mRNA levels of this proteolytic subunit at 48 hours (0.1μM Fentanyl 0.78 ± 0.14 vs Vehicle 1.00 ± 0.06, $p<0.05$) **(Figure 22b)**. Moreover, results showed that both buprenorphine and tapentadol are able to induce a down-regulation of the coding gene for β2 subunits after a treatment of 24 hours (0.25μM Buprenorphine 0.54 ± 0.03 vs Vehicle 1.00 ± 0.06, $p<0.001$; 10μM Tapentadol 0.38 ± 0.09 vs Vehicle 1.00 ± 0.06, $p<0.01$) **(Figure 22c, d)**.

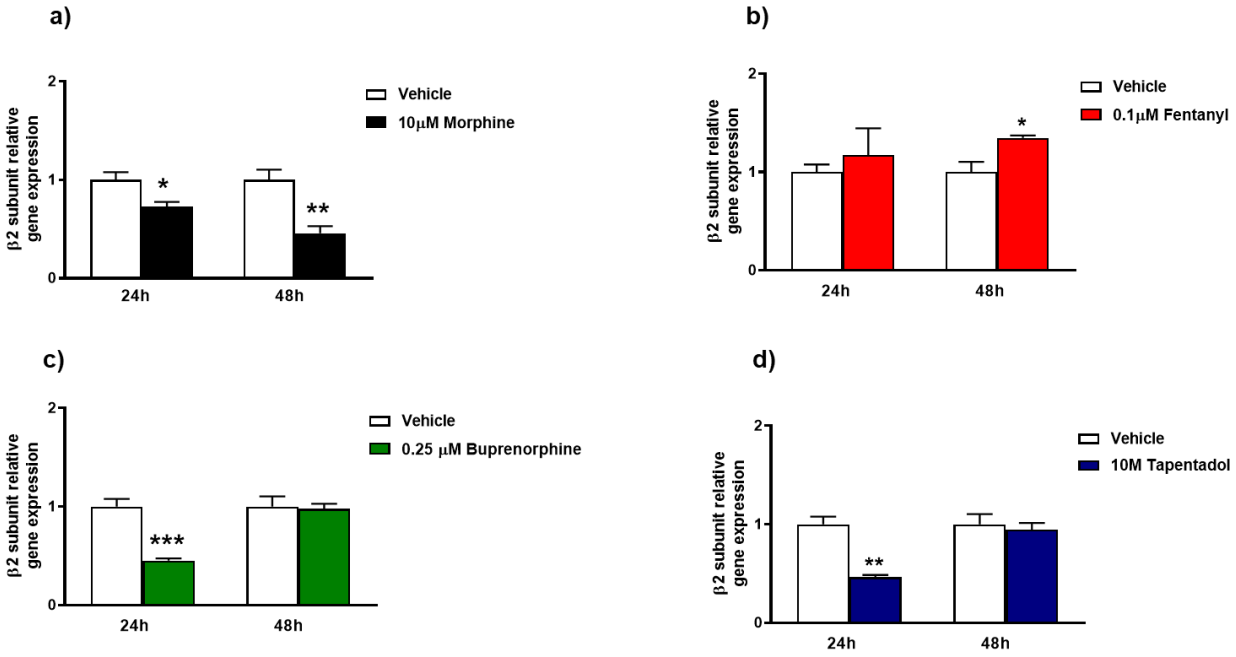

Figure 22. Relative gene expression of β2trypsin-like subunit after treatment with **a)** Morphine **b)** Fentanyl **c)** Buprenorphine or **d)** Tapentadol in SH-SY5Y cells at different time point. Data represent 2^{-DDCt} values calculated by DDCt method and are expressed as mean ± SEM of four biological replicates per treatment (*p < 0.05; **p < 0.01; ***p < 0.001 vs respective Vehicle; analyzed by t-test)

4.1.7 β5 subunit gene expression induced by exposure to opioid analgesic drugs at different time points

Statistical analysis showed no significant gene expression alteration of β5 subunits after either morphine or fentanyl treatment at 24 and 48 hours **(Figure 23a, b)**. Differently, Student's t-test displayed a significant down-regulation of mRNA levels of this proteolytic subunit after buprenorphine treatment only at 24 hours (0.25μM Buprenorphine 0.54 ± 0.03 vs Vehicle 1.00 ± 0.06, $p<0.001$) **(Figure 23b)**. In addition, results showed that the levels of coding gene for β5 subunits were decreased by tapentadol either after 24 or 48 hours (24h 10μM Tapentadol 0.38 ± 0.09 vs Vehicle 1.00± 0.06, $p<0.01$; 10μM Tapentadol 0.51± 0.04 vs Vehicle 1.00 ± 0.10, $p<0.01$) **(Figure 23c, d)**.

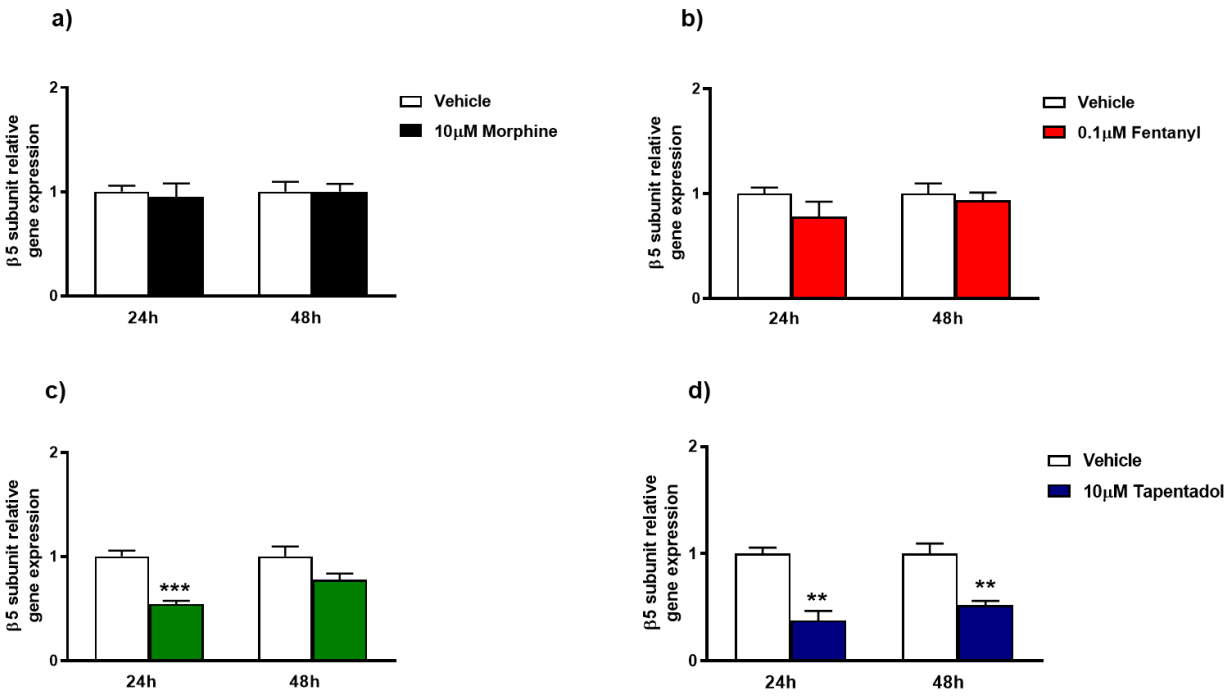

Figure 23. Relative gene expression of β5 chymotrypsin-like subunit after treatment with **a)** Morphine **b)** Fentanyl **c)** Buprenorphine or **d)** Tapentadol in SH-SY5Y cells at different time point. Data represent 2^{-DDCt} values calculated by DDCt method and are expressed as mean ± SEM of four biological replicates per treatment (**p < 0.01; ***p < 0.001 vs respective Vehicle; analyzed by t- test)

4.1.8 β2 trypsin-like and β5 chymotrypsin-like activities induced by the exposure to different concentrations of buprenorphine at different time points

Statistical analysis showed that lower doses of buprenorphine(>MOR agonist) induced a significant decrease of β2 proteasome activity at 48 hours (0.02μM Buprenorphine 83.67 ± 2.50 vs Vehicle 100.00 ± 3.62, $p<0.01$) while it promoted in cells an up-regulation of β5 proteolytic activity either after 5, 24 or 48 hours (5h: 0.02μM Buprenorphine 126.79 ± 2.24 vs Vehicle 100.00 ± 3.26, $p<0.01$; 24h: 0.02μM Buprenorphine 121.73 ± 0.99 vs Vehicle 100.00 ± 1.23 $p<0.001$; 48h: 0.02μM Buprenorphine 126.58 ± 2.07 vs Vehicle 100.00 ± 4.39, $p<0.01$) **(Figure 24a,c)**.

Differently, the exposure of SH-SH5Y cells to higher doses of Buprenorphine (>NOP agonist) was able to induce a reduction of β2 trypsin-like activity either at 24 or 48 hours (24h: 2.5μM Buprenorphine 69.01 ± 2.11 vs Vehicle 100.92 ± 1.60, $p<0.001$; 48h: 2.5μM Buprenorphine 59.10 ± 2.54 vs Vehicle 100.00 ±1.52, $p<0.001$) **(Figure 24b)** and a reduction of β5 chymotrypsin-like activity at 24 hours (24h: 2.5μM Buprenorphine 80.02 ± 2.05 vs Vehicle 100.00 ± 3.41, $p<0.01$) **(Figure 24c)**.

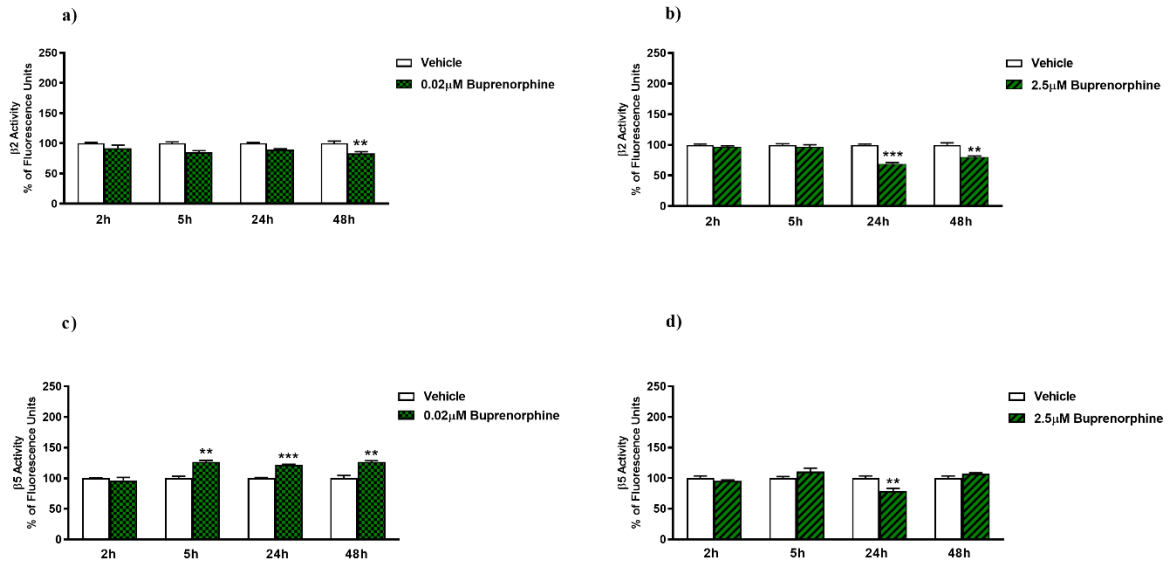

Figure 24. β2 trypsin-like and β5chymotrypsin-like activities after treatment with 0.02μM Buprenorphine **(a, c)** 2.5μM Buprenorphine **(b, d)** in SH-SY5Y cells at different time point. Data are expressed as percentage of relative fluorescence (RFU) and are reported as mean ± SEM of four biological replicates per treatment (**p < 0.01; ***p < 0.001 vs respective Vehicle group analyzed by t-test)

4.1.8 β2 and β5 subunits gene expression induced by the exposure to different concentrations of buprenorphine at different time points

Statistical analysis did not show significantly changes of β2 subunit gene expression either at the lower and higher concentrations of buprenorphine **(Figure 25a)**.

Differently, the exposure of SH-SH5Y cells to these two selected concentrations of drug was able to induce a reduction of β5chymotrypsin-like activity at 24 and 48 hours (24h: 0.02μM Buprenorphine 0.68 ± 0.11 vs Vehicle 1.00 ± 0.01, $p<0.05$;2.5 μM Buprenorphine 0.53 ± 0.09 vs Vehicle 1.00 ± 0.01, $p<0.01$; 48h: 0.02μM Buprenorphine 0.56 ± 0.07 vs Vehicle 1.00 ± 0.04, $p<0.01$; 2.5 μM Buprenorphine 0.42± 0.08 vs Vehicle 1.00 ± 0.04, $p<0.01$) **(Figure 25b)**.

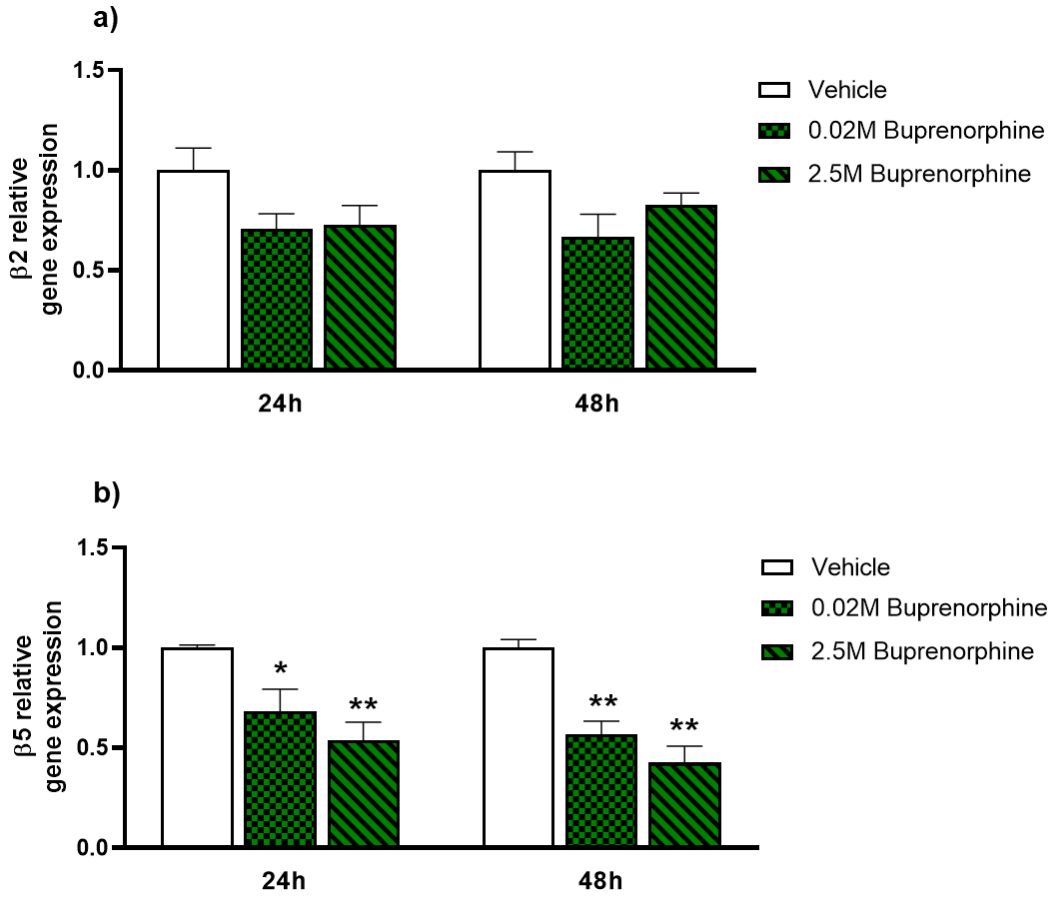

Figure 25. Relative gene expression of **a)** β2 trypsin-like or **b)** β5 chymotrypsin-like subunits after treatment after treatment with 0.02μM or 2.5μM Buprenorphine in SH-SY5Y cells at different time point. Data represent 2^{-DDCt} values calculated by DDCt method and are expressed as mean ± SEM of four biological replicates per treatment (*p < 0.05; **p < 0.01 vs respective Vehicle; analyzed by One-way ANOVA followed by Bonferroni's multiple comparisons test)

4.2 In vivo results

4.2.1 Assessment of oxaliplatin-induced neuropathic signs

Two weeks of oxaliplatin repeated administrations significantly decreased the pain threshold to non-noxious (allodynia-like) and noxious (hyperalgesia-like) stimuli, evaluated on day 15 **(Figure 26)**. The licking latency in response to a thermal non-noxious stimulus (cold plate test) decreased from 19.3 ± 0.7 s of the control group to 9.5 ± 1.1 s in oxaliplatin-treated rats **(Figure 26a)**. The withdrawal threshold to a mechanical non-noxious stimulus (von Frey test) was significantly reduced in oxaliplatin-treated group (13.1 ± 0.7 g) with respect to control (20.7 ±1.2 g) **(Fig. 26b)**. Similarly, the weight tolerated by the animals on the posterior paws (mechanical noxious stimulus, paw pressure test) decreased from 65.0 ± 0.5 g of control groups to 45.3 ± 1.0 g in oxaliplatin-treated animals **(Figure 26c)**. Moving to the evaluation of oxaliplatin impact on motor coordination and physical endurance, the Rotarod test showed only a slight reduction of the time spent on the rotating rod with respect to the control group **(Figure 26d)**.

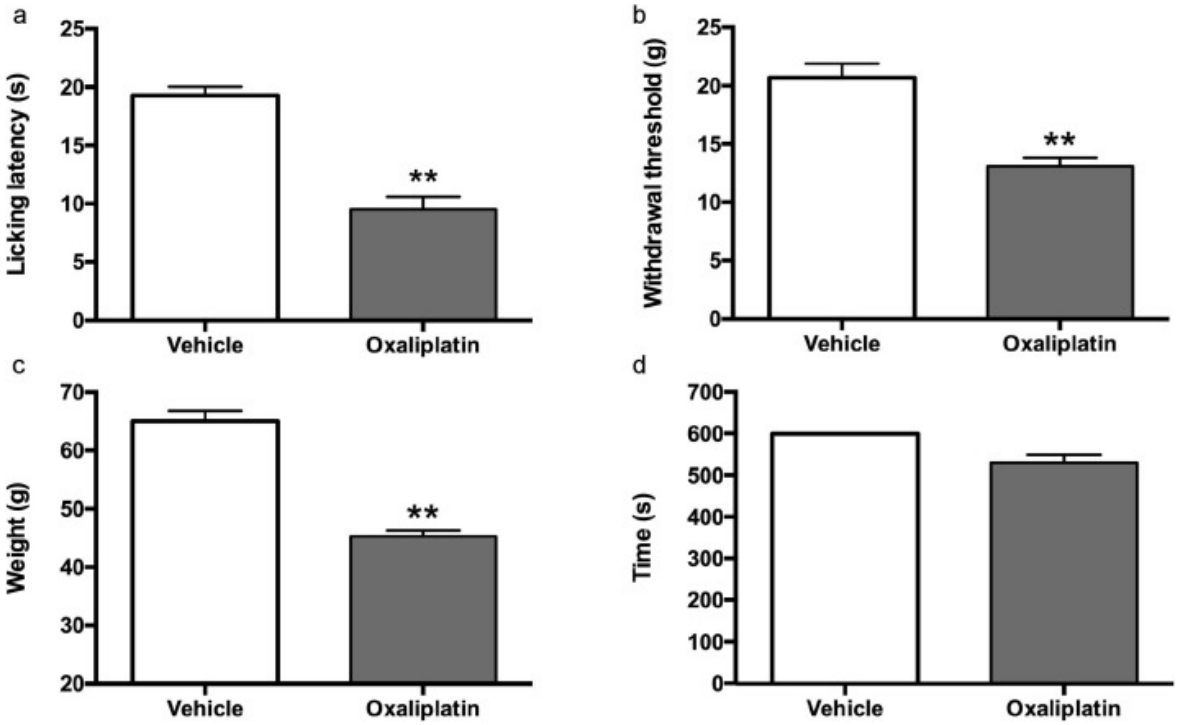

Figure 26. Behavioral measurements. Pain: non-noxious stimuli. **(a)** The response to a thermal stimulus was evaluated by the cold plate test measuring the latency (s) to pain-related behaviors (licking or lifting of the paw); **(b)** The von Frey test was used to measure the pain threshold as a response to a mechanical stimulus. Pain: noxious stimulus: **(c)** the paw pressure test was used to measure the sensitivity to a mechanical stimulus. Motor coordination. **(d)** The integrity of the animals' motor coordination was assessed using the Rota rod apparatus measuring the time spent to keep their balance. Animals were daily treated with 2.4 mg kg^{-1} oxaliplatin intraperitoneally (i.p.), over two weeks for a total of 10 injections. Behavioral evaluations were performed on day 15 when neuropathy was well established. Control animals were treated with vehicle. Each value represents the mean ± SEM of five rats per group. Statistical analysis is one-way ANOVA followed by Bonferroni's post-hoc comparison test. **$p < 0.01$ vs vehicle group

4.2.2Proteasome activities modulation in neuropathic rats

Spinal cord: Intracellular trypsin- (β2) and chymotrypsin- (β5) like activities of the constitutive proteasome were assessed after oxaliplatin treatment in three different portions of spinal cord. Results indicated that oxaliplatin-treated rats did not show significant alteration at β2 subunit activity level in the three different portions of spinal cord examined (lumbar 91.44 ± 2.82 vs 100.00 ± 2.76; thoracic: 91.58 ± 3.78 vs 100.00 ± 2.41; cervical: 100.25 ± 3.49 vs 100.00 ± 1.68, n.s.) **(Figure 27a, b and c)**. Similarly, the analysis of the β5 subunit activity did not show changes in the spinal cord of OXAIN-suffering rats (lumbar 95.93 ± 2.12 vs 100.00 ± 1.57; thoracic: 93.65 ± 6.96 vs 100.00 ± 3.28; cervical: 100.29 ± 5.75 vs 100.00 ± 5.38, n.s.) **(Figure 27d, e and f)**.

Supraspinal areas: In the TH and in the SSCx, neuropathic rats exhibited no alteration of β2 subunit cleavage activity (TH: 105.78 ± 5.68 vs 100.00 ± 8.78; SSCx: 106.13 ± 3.76 vs 100.00 ± 2.81, n.s.) **(Figure 28a and b)**. In contrast, the analysis of the β5 subunit showed a significant increase of the chymotrypsin-like activity in both TH and SSCx areas (TH: 115.10 ± 3.75 vs 100.00 ± 4.48, $p < 0.05$; SSCx: 137.61 ± 5.73 vs 100.00 ± 2.76, $p < 0.01$) **(Figure 3d and e)**. The analysis of β2 and β5 subunit cleavage activities showed no alteration in the PAG of oxaliplatin-treated rats compared to controls (β2 activity: 108.27 ± 5.39 vs 100.00 ± 4.85; β5 activity: 93.93 ± 9.73 vs 100.00 ± 8.01, n.s.) **(Figure 28c and f)**.

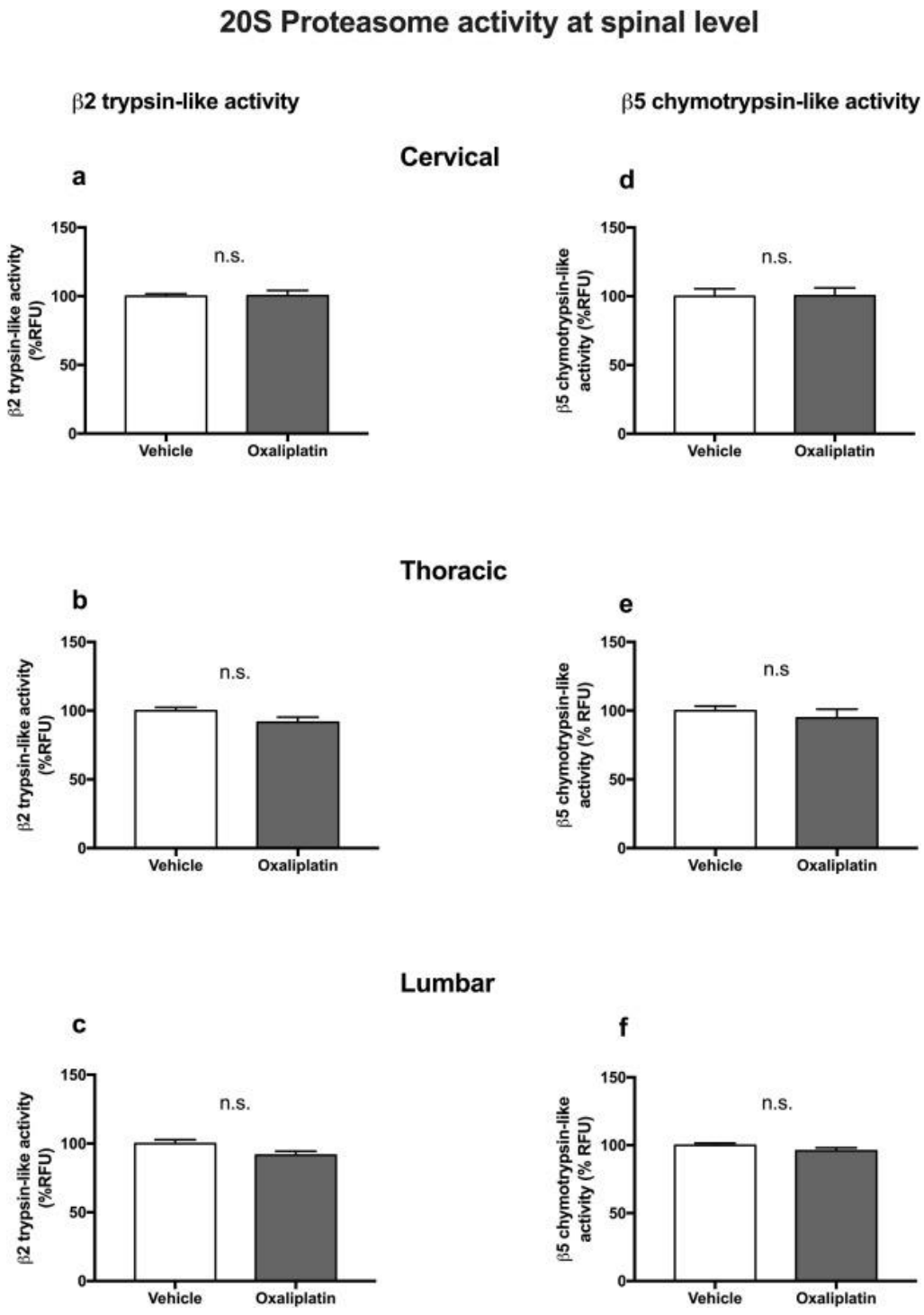

Figure 27. β2 trypsin-like activity **(left panels: a, b and c)** and β5 chymotrypsin-like activity **(right panels: d, e and f)** of 20S Proteasome in the cervical, thoracic and lumbar portion of rat spinal cord after vehicle or oxaliplatin (2.4 mg kg−1, i.p., daily for 10 days) treatment. Data are expressed as percentage of relative fluorescence (RFU) and are reported as mean ± SEM of five rats per group (n.s. $p > 0.05$ vs vehicle, t-test)

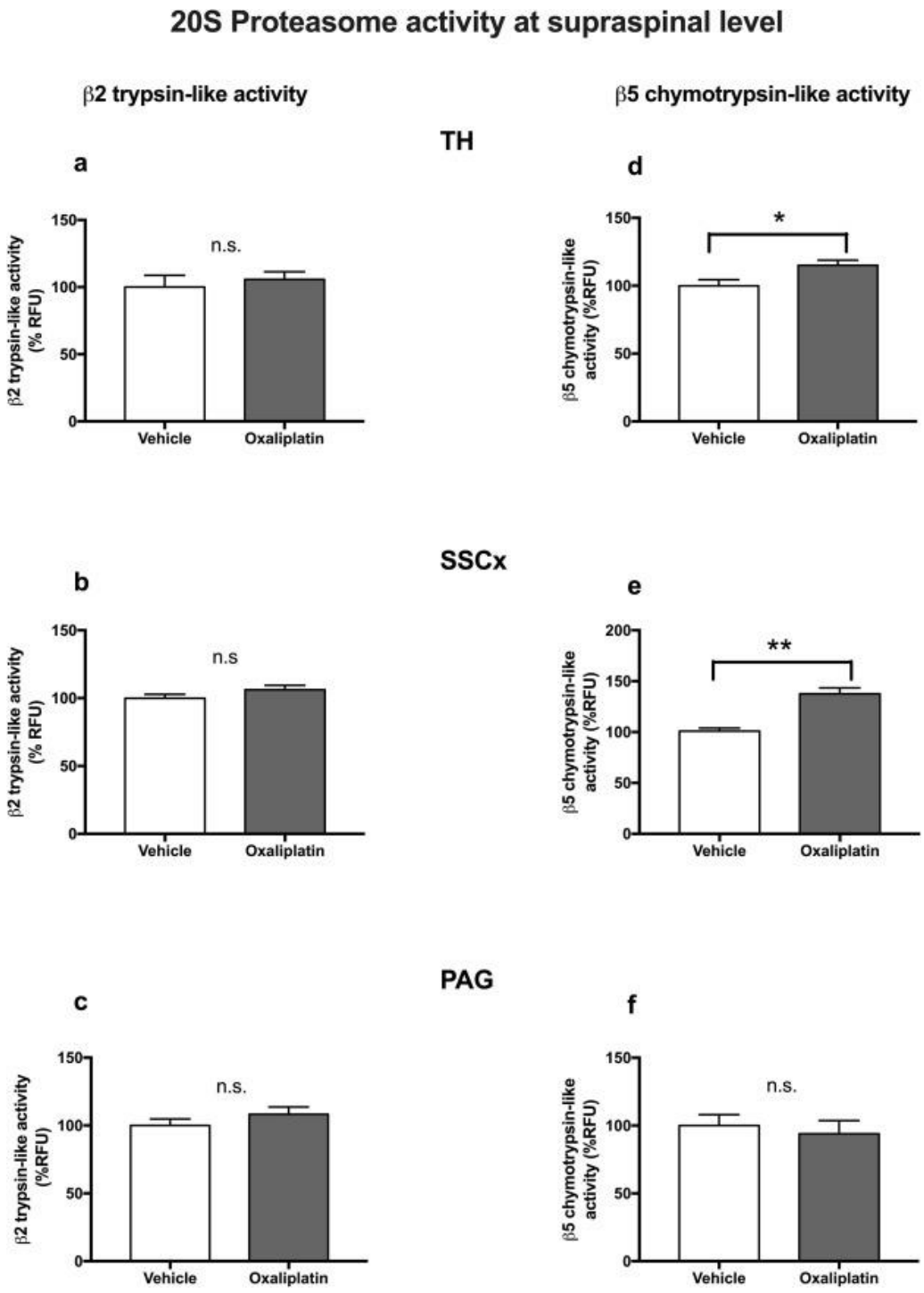

Figure 28. β2 trypsin-like activity **(left panels: a, b and c)** and β5 chymotrypsin-like activity **(right panels: d, e and f)** of 20S Proteasome in the TH, SSCx and PAG of rat after vehicle or oxaliplatin (2.4 mg kg−1, i.p., daily for 10 days) treatment. Data are expressed as percentage of relative fluorescence (RFU) and are reported as mean ± SEM of five rats per group (n.s. $p > 0.05$; *$p < 0.05$; **$p < 0.01$ vs vehicle; t-test)

4.2.3 Gene expression analysis of β5 and LMP7 (or β5i) proteasome subunits

Since the chymotrypsin-like activity assay does not allow to discriminate between the constitutive and immunoproteasome activation and based on the proteasome activity alterations detected in the TH and SSCx, we further evaluated the gene expression analysis of β5 and LMP7 proteasome subunits in the supraspinal areas.

Oxaliplatin treatment induced a significant gene expression increase of β5 and LMP7 proteasome subunits in the SSCx (β5 mRNA levels: 1.78 ± 0.08 vs 1.00 ± 0.08, $p < 0.001$; LMP7 (or β5*i*) mRNA levels: 1.32 ± 0.09 vs 1.00 ± 0.06, $p < 0.05$) **(Figure 29c and d)**, which is more pronounced for the constitutive β5 subunit. On the contrary, gene expression analysis conducted in the TH did not show significant alterations in the gene codifying for both β5 and LMP7 proteasome subunits (β5 mRNA levels: 1.07 ± 0.04 vs 1.00 ± 0.07; LMP7 (or β5*i*) mRNA levels: 1.03 ± 0.06 vs 1.00 ± 0.15, n.s.) **(Figure 29a and b)**.

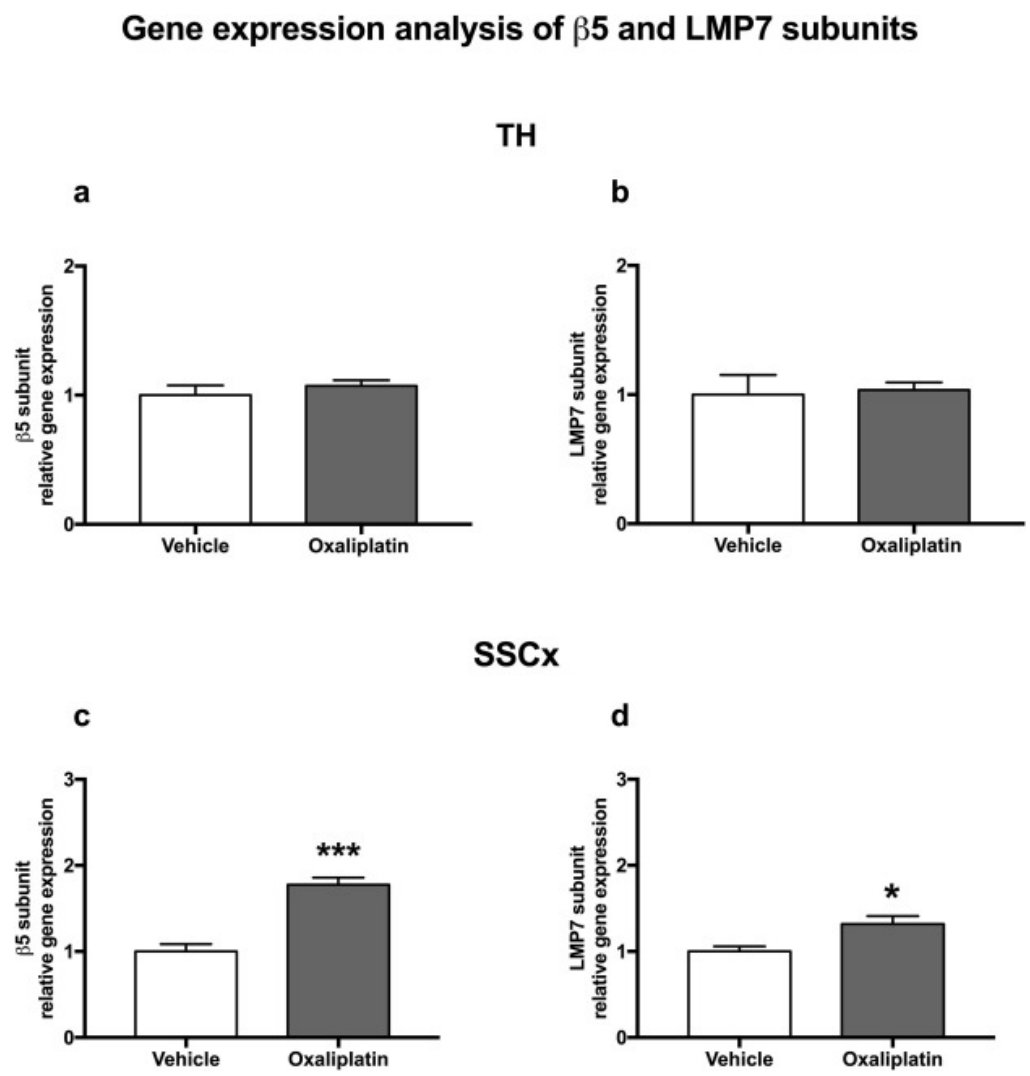

Figure 29. Relative gene expression of β5 subunit of constitutive proteasome **(left panels: a,c)** and LMP7 (β5i) subunit of immunoproteasome **(right panels: b,d)** in the TH and SSCx of rat after Vehicle or Oxaliplatin (2.4 mgkg−1i.p., daily for 10 days) treatment. Data represent 2−DDCt values calculated by DDCt method and are expressed as mean ± SEM of five rats per group (n.s. $p > 0.05$; *$p < 0.05$; ***$p < 0.001$ vs Vehicle; analyzed by t-test)

4.2.5 Acute oprozomib abolishes oxaliplatin-induced increase of spinal prodynorphin gene expression

Results indicated that oxaliplatin-treated rats exhibit significant alterations in spinal pDYN gene expression. In fact, in all the three different portions of spinal cord examined, a significant increase in pDYN mRNA levels was ascertained (cervical: 1.44 ± 0.08 vs 0.96 ± 0.14, $p < 0.05$; thoracic: 2.42 ± 0.19 vs 1.01 ± 0.09, $p < 0.001$; lumbar 1.69 ± 0.12 vs 1.01 ± 0.09, $p < 0.001$; in oxaliplatin- or vehicle-treated groups, respectively) **(Figure 29a, b and c)**. Differently, pDYN mRNA levels measured in a separate group of oxaliplatin-exposed rats that were acutely treated with 30 mg kg^{-1} i.p. of the proteasome inhibitor oprozomib on day 15 were significantly reduced compared with oxaliplatin-exposed animals in all spinal cord segments (cervical: 0.53 ± 0.12 vs 1.44 ± 0.08, $p < 0.001$; thoracic: 0.48 ± 0.14 vs 2.42 ± 0.18, $p < 0.001$; lumbar 0,60 ± 0.07 vs 1.69 ± 0.12, $p < 0.001$; in oxaliplatin-oprozomib or oxaliplatin-vehicle treated groups, respectively) **(Figure 29a, b and c).**

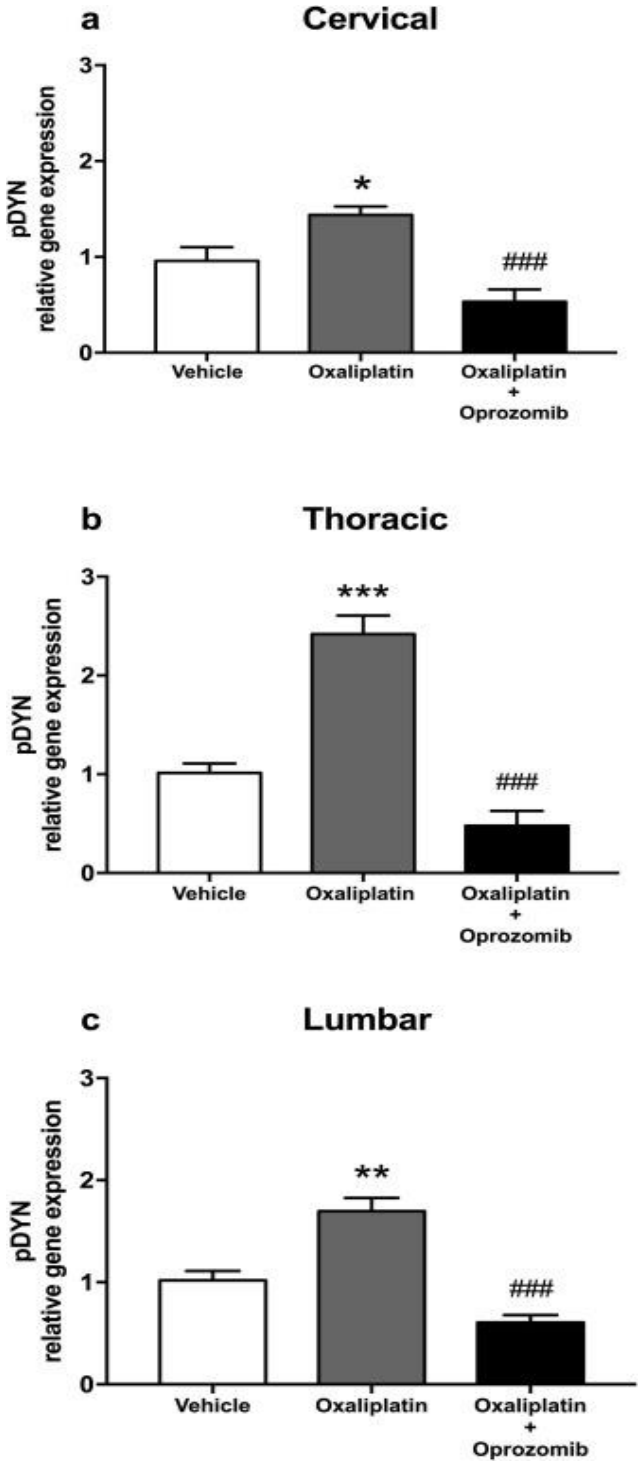

Figure 29. Relative gene expression of pDYN in the **a)** cervical **b)** thoracic and **c)** lumbar portion of rat spinal cord. Data represent 2−DDCt values calculated by DDCt method and are expressed as mean ± SEM of five rats per group (*$p < 0.05$; ***$p < 0.001$ vs Vehicle; ###$p < 0.001$ vs Oxaliplatin-treated group; analyzed by One-way ANOVA followed by Bonferroni multiple comparisons test)

4.2.6Acute oprozomib administration reverts oxaliplatin-induced hyperalgesia

Oprozomib was tested on day 15 of oxaliplatin protocol. After a single p.o. administration, oprozomib exerted an anti-hypersensitivity effect in a dose-dependent manner as depicted in Figure. 30. In particular, oprozomib 100 mg kg−1, completely counteracted the thermal allodynia 30 min after treatment, lasting up to 60 min. The lower dose (30 mg kg−1) significantly increased the licking latency time between 30 min and 60 min (Cold plate test, **Figure 30a)**. Comparable results were obtained with the von Frey test, the response to a non-noxious mechanical stimulus was significantly enhanced by oprozomib administration in a dose dependent-manner **(Figure 30b)**. The higher dose was effective from15 min after administration up to 60 min while the dose of 30 mg kg−1 was active from 30 min to 60 min. The anti-hyperalgesic effect of oprozomib against oxaliplatin neuropathy was evaluated by the Paw pressure test where the compound was able to revert the hypersensitivity to a noxious mechanical stimulus in a dose dependent-manner **(Figure 30c).**

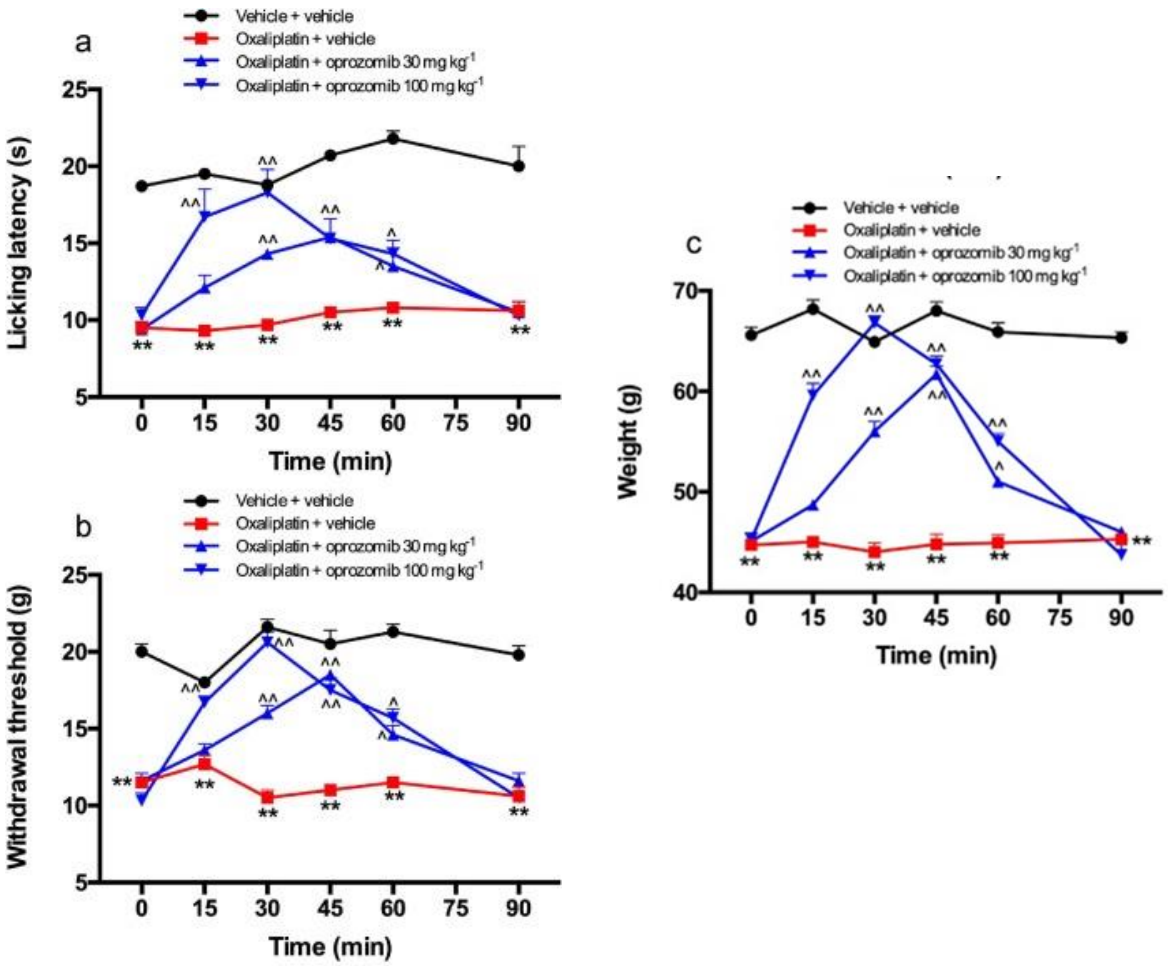

Figure 30. Behavioral measurements. Pain: non-noxious stimuli. **(a)** The response to a thermal stimulus was evaluated by the cold plate test measuring the latency (s) to pain-related behaviors (licking or lifting of the paw); **(b)** The von Frey test was used to measure the pain threshold as a response to a mechanical stimulus. Pain: noxious stimulus: **(c)** the paw pressure test was used to measure the sensitivity to a mechanical stimulus. Animals were daily treated with oxaliplatin 2.4 mg kg−1 intraperitoneally (i.p.), oprozomib (30–100 mg kg−1, p.o.) administration and behavioral evaluations were performed on day 15, before and 15, 30, 45, 60 and 90 min after oprozomib administration. Control animals were treated with vehicle. Each value represents the mean ± SEM of five rats per group. Statistical analysis is one-way ANOVA followed by Bonferroni's post-hoc comparison. **p < 0.01 vs Vehicle + vehicle group; ^p < 0.05 and ^^p < 0.01 vs Oxaliplatin + vehicle group

5. Discussion

In the last years, several mechanisms have been suggested to be involved in the pathogenesis of chronic pain as well as in the appearance of the most important side effects related to the use of opioids, which to this day still represent the gold standard analgesics to treat acute and chronic pain (Skrabalova et al.,2013; Du Pen et al.,2007; Rockel et al.,2016).

Among the plethora of molecular mechanism proposed, particular attention has been devoted to oxidative stress phenomena and proteasome function (Skrabalova et al., 2013; Salvemini et al.,2009; Wang etal.,2008; Muscoli et al.,2007) both involved in the control of cellular homeostasis and signaling as well as in neuroinflammatory response (Salvemini et al.,1998; Chaturvedi et al.,2006; Moulédous et al.,2005).

In order to add useful information to elucidate these aspects, the aims of this PhD thesis were to investigate:

1) the effects elicited by a series of opioid drugs (morphine, fentanyl, buprenorphine and tapentadol) on cell oxidative stress, antioxidant enzymatic machinery as well as on proteasome expression and activity, in vitro.

2) the involvement of the proteasome complex in the development of chronic pain conditions in a model of oxaliplatin-induced neuropathy (OXAIN*), in vivo*.

Data here reported, showed that the selected drugs differently alter ROS production level. In this respect, some studies already suggested the ability of morphine to induce a time-dependent oxidative stress in SH-SY5Y cells, at concentrations similar to those produced at CNS level by human therapeutic doses (Ma et al., 2015; Lin et al.,2009).
However, our results indicate that this ROS increasing effect of morphine is not shared by the other opioid drugs here investigated, thus suggesting that the different drug pharmacological profile possibly influences this parameter.
Regarding the effects of the selected opioids on the antioxidant machinery, our results showed that SOD activity was significantly decreased by all molecules at two hours while the activity of this enzyme family seems to be restored at longer exposure times. In line with this finding, gene expression analysis displayed an increase of SOD1 mRNA levels in SH-SY5Y cells

already after 5 hours of exposure to morphine or fentanyl and at later intervals for buprenorphine or tapentadol. It is conceivable that the increase of the SOD1 gene expression might represent a cellular adaptive mechanism aimed to raise enzyme availability and to counteract ROS production and the consequent oxidative damage. Taken together, these results suggest that morphine is probably able to produce a ROS-mediated neurotoxic effect, higher than the other investigated compounds. In this context, it is interesting to note that the oxidative processes could be related to the development of tolerance phenomena since some studies showed that the administration of molecules acting as antioxidant or SOD-mimetics prevent or counteract morphine tolerance in different animal models (Zhou et al.2011; Janes et al., 2012; Ghavimi et al., 2015; Caputi et al., 2019).

Our data also indicated that morphine, fentanyl, buprenorphine, and tapentadol produced different alterations of β2 trypsin- like and β5 chymotrypsin- like activities. The overall data analysis of the two proteasome subunits' activity showed that morphine was able to increase the proteolytic activity after prolonged exposure, according with previous studies (Yang et al.,2008). Interestingly, a similar and even earlier effect was observed after fentanyl exposure. Instead, a different picture was observed for buprenorphine and tapentadol, with buprenorphine reducing proteasome activity after prolonged exposure intervals and tapentadol not inducing significant alterations at any assessment interval.

However, with a separate experiment, we observed that buprenorphine can affect Proteasome activity in opposite directions, depending on its concentrations. In fact, and similarly to morphine and fentanyl, it decreased proteasomal activity at the lower concentration whereas an increase of the same parameter was observed at higher ones.

In the light of literature data (Gudin and Fudin, 2020; Khroyan et al., 2015) showing the peculiar binding profile of buprenorphine (MOR agonist profile at low concentrations and NOP agonist profile at high concentration), this picture might suggest that the level of MOR agonism could be strongly related with proteasome activation.

In this frame, additional studies will be useful to investigate if the selected opioid drugs could promote specific post-translational modifications (protein ubiquitination) which differently activates proteasomal machinery (Chaturvedi et al., 2001; Moulédous et al.,2005).

On the other hand, given the strong correlation between proteasome and oxidative stress (Grune et al., 2008), the increase of proteasome activity could be also related to the rise of oxidized proteins. In fact, it is known that an excessive ROS production and the consequent protein damage induce a rapid activation of proteasome degradation complex and the *de novo* synthesis of its proteolytic subunits (Grune et al., 2008).
In this frame, gene expression data overall showed that tested compound are able to differently affect β subunits biosynthesis. Interestingly, buprenorphine and tapentadol caused a reduction of β5subunit which represents the more active proteasome subunit and the more clearly induced during oxidative stress processes (Chondrogianni et al., 2005). This results could suggest that a lesser MOR activation can be related with a lower ROS production that do not request a *de novo* synthesis of proteolytic subunits.

As a whole, these results showed that the tested compounds affect cellular processes related to oxidative stress and proteasome complex function. Moreover, specific changes of the assessed parameters caused by different drugs appear related to the different pharmacological profile.
In light of literature data about the effect of antioxidants and proteasome inhibitors upon analgesic tolerance to morphine (Wang et al.,2008; Caputi et al.,2019; Raghavendra et al.,2000; Hemati et al.,2020; Lauro et al.,2016; Ndengele et al.,2009), our data obtained in SH-SY5Y cells support their involvement in the appearance of this phenomenon.

With respect to the *in vivo* study, the results revealed that repeated exposure to oxaliplatin evokes alterations of the proteasome degradation machinery, which are strictly dependent on the pain transmission–related region. In fact, the analysis of β2 and β5 enzymatic activities indicates that the repeated oxaliplatin exposure promotes a significant increase of intracellular chymotrypsin- (β5) like activity at supraspinal level only, in particular in the TH and SSCx, without changes in the three different portions of the spinal cord. On the other hand, the activity changes observed in the SSCx are accompanied by alterations in gene expression, whereas in the TH only enzymatic activity is increased without gene expression activation. These results are consistent with evidence demonstrating that antineoplastic agents may increase the pro-inflammatory mediator signaling (Melli et al., 2008) likely through the activation of 26S proteasome complex (Chen et al.,2013). In this view, the nuclear factor κ enhancer binding protein (NF-κB) transcription factor family activation requires the ubiquitination of inhibitory proteins of κB family (IκB) (Palombella et al.,1994). The 26S proteasome proteolytic activity allows IκB post-translational modification and its degradation and the subsequent NF-κB

translocation to the nucleus, where it regulates the expression of several genes including inflammatory mediators such as cytokines and chemokines (Hyden et al.,2008) In other words, it is likely that repeated oxaliplatin administration promotes the increase of proteasome activity inducing the NFκB transcription factor family activation as a consequence (Wang et AL.,2008). In this frame, the inhibition of NFκB activation through the use of proteasome inhibitor MG132 has been indicated as useful strategy to counteract the NF-κB induction of inflammatory pathways (Cusack et al.,2003), and to modulate inflammatory pain in a rheumatoid arthritis rat model (Ahmed et al.,2010). Therefore, the increase of proteasome activity reported in our experimental model corroborates the possibility that proteasome inhibitors could be effective in the treatment of painful states, including the peripheral neuropathy induced by oxaliplatin. Indeed, proteasome inhibitor administration seems to attenuate, prevent and revert pain behavioral signs in different neuropathic pain models (Ossipov et al.,2007; Moss et al.,2002).

The considerable increase of proteasome activity in the TH and SSCxand the selective up-regulation of β5 and LMP7 (or β5i) genes in the SSCxalso indicates a peculiar activation of this supraspinal region which is mainly involved in the discriminative aspect of pain and sensory coding (Apkarian et al.,2009). The protein degradation mediated by UPS is a crucial aspect in thesynaptic plasticity regulation and, particularly, in the long-term synapticmodifications typical for chronic abnormal pain (Caputi et al.,2019). The main involvement of β5 isoforms, belonging to the constitutive and immuneproteasomes respectively, suggests a relevant role played by these specific subunits in the oxidative stress processes induced by prolongedexposure to oxaliplatin. In this regard, β5 subunit represents the most important active site at the 20S-CP (Wolf et al.,2004; Heinemeyer et al.,2004), and it also participates in the increased response to oxidative stress carrying out a cytoprotectiveaction (Chondrogianni et al.,2005). Indeed, it has been demonstrated that the overexpression of the β5 subunit is able to increase the proteasome activityand to promote cell survival following H2O2 exposure (Chondrogianni etal.,2005; Liu et al.,2007). Therefore, it is reasonable to speculate that proteasome activity increase and the selective β5 and LMP7 (or β5i) gene up regulation at supraspinallevel could represent an adaptive response to the prolonged oxidativestress condition as that evoked by oxaliplatin also in the central nervoussystem (Di Cesare Mannelli et al.,2016). This hypothesis is supported by the efficacy of antioxidant compounds in reducing the proteasome activation (Roy et al.,2020). The same was reported by using the natural antioxidant melatonin (Rubio-Gonzalez et al.,2018). Parallel to the increase of proteasome activity, we also observed asignificant increase of pDYN gene expression in all three portions

of thespinal cord. This observation confirms the involvement of the dynorphinergic system in the development of OXAIN as expected, given its role in several chronic pain conditions (Dubner et al.,1992; Laughlin et al.,2001). In this regard, Malanand colleagues associated the increase of dynorphin peptide level atspinal level, from a spatial and temporal point of view, to the appearanceof mechanical allodynia (Malan et al.,2000). An interesting hypothesis suggests that the increase of DYN release induced by oxaliplatin treatment may lead to its interaction with NMDA receptors (Laughlin al.,2001) determining the presence of allodynia signs through the production of radical species and pro-inflammatory cytokines, which in turn contribute to the development of spinal neuronal damage (Laughlin et al.,2001; 2000). Indeed, it is known the DYN ability to modulate nociception through its action on immune function (Long et al., 1987). In particular, the increase of dynorphin levels could be associated with the activation of a signaling cascade which, starting from the NMDA receptor, activates the transcription factor NF-κB and increase the expression of pro-inflammatory cytokines, nitric oxide and also DYN itself, as a consequence (Laughlinet al.,2000). Therefore, the reduction of pDYN mRNA levels observed after oprozomib treatment could be associated to the ability of this second-generation proteasome inhibitor compound to inhibit NF-κB activation. Our data show a rapid DYN up-regulation at spinal level and a proteasome activity increase at supraspinal level that could be related to the central sensitization. Nevertheless, the lack of proteasome alteration at spinal level represents an unexpected result which may suggest that this defense complex may be subjected to a temporal activation according to the area or cell population like microglia cells. In addition, our data show that although oxaliplatin induces a significant increase of β5 subunit belonging to the constitutive proteasome, it is also able to promote the LMP7 (or β5i) subunit gene expression upregulation. In this regard, some evidence demonstrated a prominent role of immunoproteasome in the adaptive immune response implicated in several biological and pathological processes. In fact, the immunoproteasome is known to be crucial in the degradation of damaged proteins generated by oxidative stress (Seifert et al.,2010) and in inflammatory processes (Miller et al.,2013). Therefore, given the role played by oxidative stress in peripheral neuropathy induced by oxaliplatin (Di Cesere Mannelli et al.,2016), we tested the ability of an immunoproteasome inhibitor to reduce OXAIN signs. In our study, a singleoprozomib administration was able to counteract hyperalgesia and allodynia induced by oxaliplatin repeated injections that mimics, in an animal model, the clinical adverse effects of this antineoplastic drug (Zanardelli et al.,2015). The results obtained on the efficacy of oprozomib could help to achieve the effect of oxaliplatin therapy reducing its toxicity. In particular, the use of immunoproteasome inhibitors has proved to be a potential useful approach especially in

inflammatory and immune related conditions. Indeed, it seems that the use of selective inhibitors for immuno subunits may result in a reduction of pro-inflammatory cytokines production (Miller et al.,2013; Muchamuel et al., 2009). Moreover, our data suggest for the first time that oprozomibis able to revert the increased prodynorphin gene expression occurring in OXAIN, thus indicating one of the possible molecular mechanisms underlying this pathological condition and identifying dynorphin as a possible target of immunoproteasome inhibitors, for new therapeutic approaches. We are also aware of the difficulty to directly associate oprozomib effects upon OXAIN to the UPS inhibitory action of the drug.

6. Conclusions

Data here presented provide original evidence about the ability of the selected opioids to alter in different manner the activities of Proteasome as well as to determine a different degree of oxidative stress in cells. Moreover, the in vivo study also showed the involvement of proteasome in phenomena related to neuropathic pain development. Even though this degradation complex seems to be a promising pharmacological target, it is difficult to determine the precise cellular pathways that proteasome system is able to modulate. Hence, further studies are required to better clarify its role.

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