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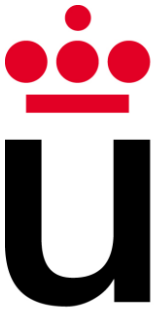

TESIS DOCTORAL

PRECLINICAL STUDY OF ACUTE OROFACIAL MUSCULAR PAIN IN ANIMAL AND HUMAN MODELS

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MADRID, 2012

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CERTIFICAN:

Que el trabajo de investigación titulado "Preclinical study of acute orofacial muscular pain in animal and human models" ha sido realizado por Dña. Ana Bagüés Arias en el Departamento de Farmacología y Nutrición de la Universidad Rey Juan Carlos y en el Laboratory of Orofacial Pain, Center for Sensory-Motor Interaction, University of Aalborg, bajo nuestra dirección como Tesis Doctoral Europea, y como tal autorizamos su lectura.

Y para que así conste donde proceda se firma este certificado en Alcorcón, a Octubre de 2012.

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A mi familia

Finalmente escribo los agradecimientos, la última parte que me queda de este manuscrito, y aunque sea la última no por ello la menos importante ya que sin la ayuda, profesional y/o personal, de tantas personas esta Tesis Doctoral no hubiera sido posible.

A la **Dra. Mª Isabel Martín**, Catedrática y Directora del Departamento de Farmacología y Nutrición, a mi Maestra durante estos últimos cinco años. Gracias por haberme dado la oportunidad de desarrollar mi Tesis Doctoral y por haberme acogido en tu grupo. Por enseñarme tanto durante estos años en el plano profesional, pero también en el personal. Por haber confiado y apostado en mí en estos momentos difíciles. Por haberme dado un empujoncito o una palmadita en la espalda cuando lo he necesitado. Por esto y mucho más te estoy sinceramente agradecida.

A la **Dra. Eva Mª Sánchez**, mi tutora y supervisora directa durante estos últimos años, con quien he compartido la mayor parte del tiempo. Gracias por haberme enseñado con paciencia, y siempre tener tiempo para resolver mis dudas. Por no solo haber sido una tutora, sino también una compañera y por tu búsqueda de perfección en todo lo que haces. Sin tu inestimable ayuda y enseñanza esta Tesis Doctoral no hubiera sido posible.

Al **Dr. Carlos Goicoechea**, mi primer contacto con este Departamento. Gracias por tener tu puerta siempre abierta, resolviendo mis dudas con una sonrisa.

A las **Dras. Visitación López-Miranda y Raquel Abalo**, a pesar de no haber trabajado directamente con vosotras, aprendemos también del ejemplo de otros. Recordaré siempre vuestra capacidad de trabajo y valor humano.

A los **Dres. David Pascual, Rocío Girón, Esperanza Herradón y Gema Vera**, por los buenos momentos compartidos, que hacen que el laboratorio sea un sitio de trabajo tan agradable.

A mis "compis de doctorado": **Cristina González, Nancy Paniagua** por el apoyo contínuo durante estos años. Por las palabras de ánimo en los momentos malos y risas en los buenos. Ánimo que ya queda menos.

A **Miguel Martínez y Ana Rodríguez,** por los ratos compartidos.

A **Maica Merino,** mi "confidente" en el laboratorio, por su gran y desinteresada ayuda en la realización de los experimentos, siempre atenta y dispuesta a ayudar en lo posible.

A **Guadalupe Pablo e Iván Álvarez**, técnicos y alma del laboratorio, las horas de trabajo sin vosotros no serían iguales.

A mis **padres**, si tuviera que escribir todo por lo que estoy agradecida no tendría suficiente espacio en este pequeño apartado. Por vuestro amor incondicional, por los esfuerzos que habéis hecho para que obtuviese la mejor educación, por vuestras enseñanzas de constancia y valores y por vuestro continuo apoyo. Aunque no lo diga muy a menudo como debiera: gracias.

A mis hermanas y cuñado, **Esther, Marta y Antonio**, por sus palabras de ánimo y ayuda constante y por los ratos de "juerga", que también son necesarios.

A mi marido **Juan**…"Porque los pilares sostienen el templo, pero están separados. Y ni el roble crece bajo la sombra del ciprés ni el ciprés bajo la del roble" ¿Te acuerdas? Gracias por hacer de esta lectura nuestra filosofía de vida. Por tu apoyo incondicional a pesar de los momentos duros y por estar siempre a mi lado, en lo bueno pero también lo malo. Gracias.

A los **Dres. Lars Arendt-Nielsen y Kelun Wang** por haberme acogido en su laboratorio y enseñarme a hacer investigación preclínica en humanos.

Finalmente al **Ministerio de Educación y Ciencia** por haber permitido que disfrutara de una beca predoctoral FPI. La investigación de esta Tesis Doctoral ha sido financiada por los siguientes proyectos: SAF-2006-13391-C03-01 y SAF-2009-12422-C02.

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ABBREVIATIONS

2-AG: 2-arachidonoyl glycerol

AC: adenylyl cyclase

ASIC: acid-sensing ion channels

ATP: adenosine-5'-triphosphate

CNS: central nervous system

DAG: diacylglycerol

DNIC: Diffuse Noxious Inhibitory Controls

DRG: dorsal root ganglia

DRN: dorsal raphe nucleus

FAAH: fatty acid amide hydrolase

GPCR: G protein coupled receptors

HS: hypertonic saline

i.m.: intramuscular

i.p.: intraperitoneal

IASP: International Association for the study of Pain

IP_3: inositol 1,4,5-trisphosphate

IS: isotonic saline

MAGL: monoacylglycerol lipase

MAPK: MAP kinases

MAPK: mitogen-activated protein kinase

MC: mechanical control

MO: mustard oil

MP: Mechanical pain

NADA: N-arachidonoyl dopamine

NArPE: N-arachidonoyl-phosphatidylethanolamine

NRM: nucleus raphe magnus

ORL: “opioid receptor-like”

PAG: periaqueductal gray matter

PIP_2: phosphatidylinositol 4,5-bisphosphate

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

PPT: Pressure pain thresholds

PPTol: pressure pain tolerance

PTX: pertussis toxin

QST: Quantitative sensory testing

RVM: rostroventrolateral medulla

SIA: stress induced analgesia

TCS: tocrisolve

TE: ethanol and Tween 80 (1:2)

TG: trigeminal ganglia

TPT: thermal pain thresholds

$TRPV_1$: transient receptor potential vanilloid receptor-1

VAS: visual analogue scale

VBSC: trigeminal brainstem sensory nuclear complex

Vc: subnucleus caudalis

Vi: subnucleus interpolaris

Vo: subnucleus oralis

WDR: wide dynamic range neurons

INTRODUCTION

1. PHYSIOLOGY OF PAIN

1.1 INTRODUCTION

The first documented attempt to study pain was written by René Descartes, with the theory that the transmission of pain is through a single channel from the skin to the brain. This simplified scheme of the reflex was published, in 1664, in the "Treatise of Man" and was the beginning of the development of the modern doctrine of reflexes. In this document he stated: "The small rapidly moving particle of fire moves the skin of the affected spot causing a thin thread to be pulled. This opens a small valve in the brain and through it animal spirits are sent down to the muscles which withdraw the foot." This theory suggests the existence of a single pathway that leads the painful stimulus from the skin receptor to a pain center in the brain (Fig. 1).

Figure 1. Principle of pain transmission as described by Descartes in Tractatus De Homine (Treatise of Man), the definitive French version of what was first published in 1664.

The Gate Control Theory proposed in 1965 by Ronald Melzack and Patrick Wall, disputed Descartes theory and led to further investigation into the phenomenon of spinal sensitization and central nervous plasticity. In a conference, in 1995, Wall stated that the classic picture of a single pain mechanism is being swept away in favor of a more dynamic interlocking series of biological reactive mechanisms.

The processing of pain takes place in an integrated matrix throughout the neuroaxis and occurs on at least three levels: periphery, spinal and supraspinal sites. Basic strategies of pain control use this concept of integration by:

1. attenuation or blockade of pain acting at the periphery
2. activation of inhibitory processes that gate pain at the spinal cord and brain
3. interference with the perception of pain (DeLeo, 2006).

Nowadays The International Association for the study of Pain (IASP) defines pain as "an unpleasant sensory an emotional experience associated with actual or potential tissue damage, or described in terms of such damage." It is important to

distinguish this concept from nociception which, is described by the IASP as “the neural processes of encoding and processing noxious stimuli.”

The difference between acute and chronic pain has traditionally been established by the duration of pain, generally it is considered as acute when it lasts 3-6 months and chronic when longer. These distinctions are arbitrary. It is important to distinguish these two types of pain because they have different pathophysiological mechanisms:

- Acute pain: it is due to the activation of the nociceptive system when somatic or visceral lesions occur, it is considered as an alarm so it has a protective role. Usually pain disappears once the underlying cause is resolved.
- Chronic pain: it usually lasts longer periods of time and cannot be explained by the underlying pathology.

1.2 NOCICEPTIVE TRANSMISSION

The translation of the nociceptive input into pain sensation involves several processing steps, which are illustrated in Figure 2 (Kitahata, 1993):

1. prior to perception, noxious threats must be converted into electrochemical signals (**Transduction**)
2. travel from the site of peripheral or visceral stimulation to the central nervous system (CNS) (**Transmission**)
3. modification of the transmission of the nociceptive impulse, specially an inhibition in dorsal root ganglia (DRG) but can also be in other sites of the CNS (**Modulation**)
4. Finally, these three processes interacting with individual factors create the subjective and emotional experience called pain (**Perception**).

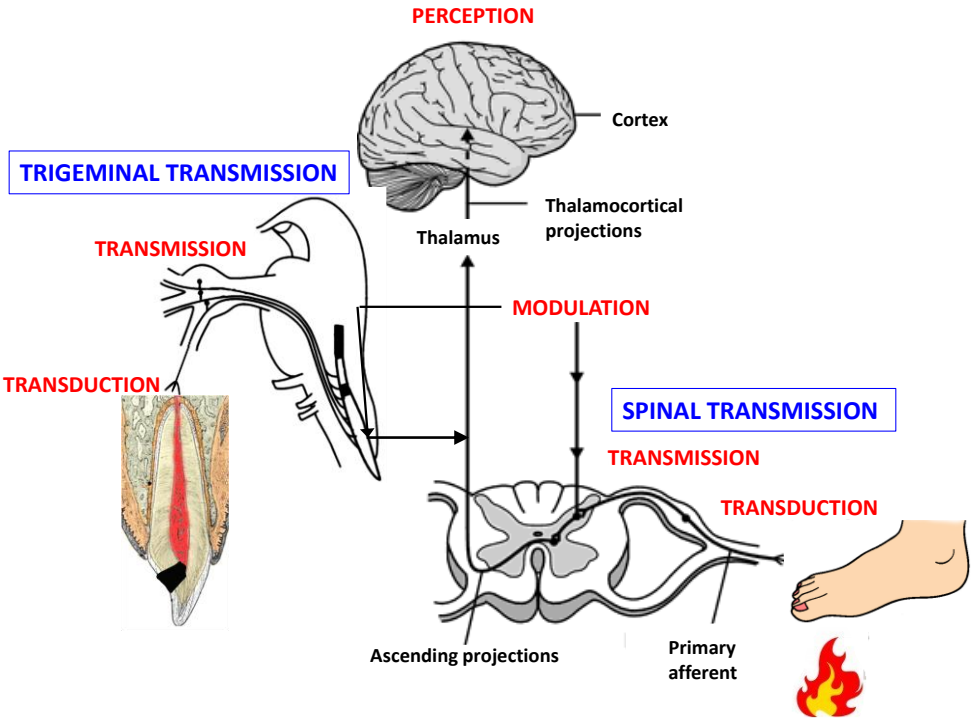

Figure 2. Schematic representation of the nociceptive impulse from the periphery to the CNS in the trigeminal and spinal systems.

1.2.1 Transduction

All peripheral sensory neurons are formed by a cell body which is located in the DRG or trigeminal ganglion (TG), a single axon which travels to the periphery (primary afferent fiber) and bifurcates at the end to innervate a peripheral tissue, and dendrites that synapse with second order neurons in the dorsal horn of the spinal cord or trigeminal brainstem sensory nuclear complex (VBSC). So the painful stimulus is transmitted from the periphery to the CNS through these first order neurons.

The specialized nerve endings of the primary sensory afferents are named nociceptors and their function is to warn the individual when a stimulus approaches tissue damaging intensity. Depending on their characteristics and localization they can be classified in cutaneous, muscular, joint or visceral (Cerveró and Laird, 2002; Basbaum et al., 2008). The primary fibers which transport the nociceptive signal in normal conditions can be thinly myelinated (Aδ fibers), or unmyelinated fibers (C fibers).

1.2.2 Transmission

In the organism, nociceptive stimuli are transmitted to different structures of the CNS depending on the location of the origin of the stimulus. In the orofacial area the main sensory nerve is the trigeminal nerve, it projects to the VBSNC located in the brainstem. In the rest of the body, sensory nerves project to the dorsal horn of the spinal cord.

1.2.2.1 Spinal transmission of nociception

It is in the spinal cord where first order neurons synapse with second order neurons. In a transverse section of an adult spinal cord there is white matter in the periphery and gray matter in the central part. The white matter is formed by the ascending and descending nerve fibers and the gray matter by the cell bodies of neurons and glia.

The gray matter has an H form, with 2 ventral and 2 dorsal horns. The dorsal horns receive the input of the sensory information coming from the periphery. Rexed classified the gray matter of the spinal cord in 10 different laminae depending on the cytoarchitecture of these (laminae I-X) (Rexed, 1952). The neurons which process the nociceptive stimuli are localized in the laminae of the dorsal part of the gray matter (Basbaum et al., 2008):

Laminae I and II: receive direct primary afferent input only from small diameter fibers. But, whilst many axons of neurons from lamina I contribute to the spinothalamic tract or project to the parabrachial nucleus, cells in lamina II (also named substantia gelatinosa) modulate signals from cells in lamina I and V.

Laminae III and IV: cells in these laminae respond to innocuous stimuli.

Lamina V: cells respond to noxious and non noxious stimuli, they are wide dynamic range neurons (WDR).

Lamina VI: cells respond to joint movement and also cutaneous stimulation.

In the dorsal horn of the spinal cord the transmission and modulation of pain is accomplished through the release of several excitatory and inhibitory neurotransmitters, the receptors for these substances can be located pre or postsynaptically (Yaksh and Malmberg, 1994). The primary afferents release these

neurotransmitters, i.e. substance P, calcitonin gene related peptide and glutamate, and activate (postsynaptically) second order neurons, which leads to the transmission of the nociceptive information to higher structures of the CNS. On the other hand inhibitory neurotransmitters reduce the release of the excitatory neurotransmitters through their union to their respective receptors presynaptically. Some of these inhibitory systems are the opioid, cannabinoid, γ-amino-butyric acid (GABA) and glycine; they play an important role in pain modulation (Dickenson, 1999).

1.2.2.2 Trigeminal transmission of nociception

The primary afferent cell bodies of most trigeminal primary afferents innervating cutaneous, intraoral, deep (joint, muscle) and cerebrovascular tissues are in the TG, and synapse with second order neurons in the VBSC, which can be subdivided into (Fig.3):

- principal or main sensory nucleus
- spinal tract nucleus: which comprises three different subnuclei:
    - subnucleus oralis (Vo)
    - subnucleus interpolaris (Vi)
    - subnucleus caudalis (Vc)

The Vc is the principal brainstem relay site for the trigeminal nociceptive information, it is a laminated structure that resembles the spinal dorsal horn. Some of the similarities of these two structures are:

- The great majority of Aδ and C fibers carrying nociceptive information from the different orofacial tissues

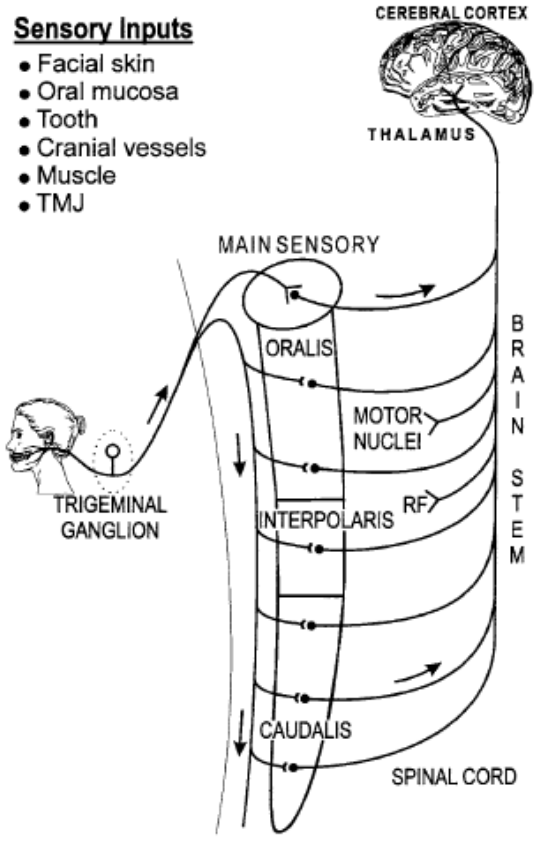

Figure 3. Major somatosensory pathways from the face and mouth. Trigeminal primary afferents project via the trigeminal ganglion to second-order neurons in the trigeminal brainstem sensory nuclear complex (Sessle, 2005)

terminate here, mainly in the laminae I, II, V and VI, whereas the larger afferents (non nociceptive afferents) end primarily in laminae III-VI.

- The laminated structure of the Vc includes lamina II, the substantia gelatinosa, which is an intrinsic neuronal circuit intimately involved in the modulation of nociceptive transmission. The other three nuclei have a more uniform structure (Sessle, 2005).

Both, nociceptive specific and WDR neurons predominate in the superficial and deep laminae of the Vc and process and relay nociceptive information to higher brain and local centers such as those involved in facial, jaw, tongue, skeletal muscle reflexes and also autonomic reflex responses evoked by noxious orofacial stimulation (Sessle, 2011).

Some caudalis nociceptive neurons respond only to the stimulation of cutaneous or mucosal mechanoreceptive field so, as a consequence, they are thought to play an important role in the ability to localize, detect and discriminate, superficial noxious stimuli. However most of them can also be activated by peripheral inputs from other tissues (tooth pult, temporomandibular joint). Such features are thought to be the cause of the very common clinical findings of poor localization and referred pain from deep tissues or teeth (Sessle, 2011).

Although it seems clear that this subnucleus is important for the nociceptive transmission and modulation, more rostral structures seem to be also involved in nociceptive transmission: lesions to some of these areas disrupt some orofacial nociceptive behaviors, and are involved in the ascending and reflex nociceptive pathways. But they lack of the substantia gelatinosa and receive few direct inputs from small diameter afferents. Future research is needed to determine the specific functional roles of the different components of the VBSNC in orofacial nociceptive mechanisms (Sessle, 2005).

1.2.2.3 Differences between trigeminal and spinal sensory systems

Besides the anatomical differences, previously addressed, there are emerging data that point to the existence of physiological differences which affect the transmission of nociception. The characteristics of the trigeminal system that differ from the spinal one have been revised by Sessle and are listed below (Sessle, 2005):

Peripheral Tissues and Innervation:

- There are tissues unique to the craniofacial region (as tooth pulp and cornea)
- There is higher innervation density in many craniofacial tissues than in most spinally innervated ones.
- There is shorter conduction distance in the trigeminal system of peripheral nerve pathways.
- Trigeminal afferents have slower conducting velocities.
- There is a higher ratio of myelinated:non myelinated fibers.
- Lower proportion of sympathetic efferents.
- Certain craniofacial receptors have their primary afferent cell bodies within the CNS (some periodontal mechanoreceptors, and jaw muscle spindles).

Central Nervous System:

- Face and mouth are represented completely at most rostrocaudal levels of the trigeminal brainstem sensory nuclear complex and a dual representation of some tissues occurs in the subnucleus caudalis.
- Distinctive brainstem termination patterns of some nociceptive afferents.
- Transitional regions between subnucleus caudalis and interpolaris and between subnucleus caudalis and upper cervical dorsal horn with distinctive properties (e.g., bilateral afferent inputs to Vc/Vi).
- Deep bundle fiber system is especially prominent in Vc (connects caudal and rostral levels of VBSNC) whereas Lissauer´s tract is absent.
- Significant ipsilateral and contralateral projections from VBSNC to the thalamus.

Pain Conditions Specific to the Craniofacial Region:

- Headaches.
- Toothaches.
- Trigeminal neuralgia.
- Miscellaneous (e.g., atypical facial pain, burning mouth syndrome, atypical odontalgia).

These anatomical and physiologic differences make it impossible to directly extrapolate findings from one system to the other.

1.2.3 Modulation of the nociceptive stimuli

After the interaction of first and second order neurons in the dorsal horn and VBSC, the axons of the second order neurons transmit the nociceptive information to structures of the brainstem and diencephalon including: the thalamus, periaqueductal substance, parabrachial region, reticular formation of the medulla, amygdaloid complex, septal nucleus and hypothalamus among others.

The different ascending bundles form two phylogenetic different systems. The first and older one runs through the medial region of the brainstem and is formed by the paleospinothalamic, spinoreticular, spinomesencephalic, spinoparabrachio-amygdaloid, spinoparabrachio-hypothalamic, and spinohypothalamic bundles. The other system, more recent, occupies the lateral region of the brainstem and consists on the neospinothalamic bundle, spinocervical bundle, and postsynaptic beam of the dorsal horn (Almeida et al., 2004).

The supraspinal regions that modulate pain sensation include the brain cortex, somatosensory cortex, hypothalamus, midbrain, nucleus raphe magnus (NRM), periaqueductal gray matter (PAG) and rostroventrolateral medulla (RVM). It is in these areas where the discriminative, sensory and affective components of pain integrate and finally lead to the sensation of pain. However these structures are also the origin of the descending pathways that, through the dorsolateral bundle, end in the dorsal horn. The aim of this pathway is to modulate the nociceptive information that enters the CNS. Part of this is performed by a group of neurons named “on” and “off”, and their nuclei are located in the RVM. The “on” cells allow the nociceptive information to enter the CNS or even facilitate it and can be inhibited by opioids. On the other hand the “off” cells, which normally are inhibited, are indirectly activated by opioids and inhibit nociceptive transmission.

1.2.3.1 Conditioned pain modulation

Most multireceptive and some nociceptive-specific neurons can be strongly inhibited by noxious inputs applied outside their receptive field, this phenomenon is

translated in the clinic as a reduction in pain perception when a second, conditioning, painful stimulus is applied. Such effects do not appear to be organised somatotopically but apply to the whole body; they apply to neurons in the dorsal horn of various segments of the spinal cord as well as to the Vc and Vo. The neurons affected include some which project to the thalamus and others which are involved in spinal reflexes, and is specifically triggered by the activation of Aδ and C fibers (Le Bars, 2002). It is presumed that the extent of pain inhibition, while the conditioning painful stimulus is applied, reflects the efficacy of the Diffuse Noxious Inhibitory Control (DNIC) system.

The DNIC system described for the first time by Le Bars et al. (Lebars et al., 1979) is a spinal-bulbo-spinal pathway; this effect is not observed in animals in which the spinal cord has been transected at the cervical level. Lesion in the mesencephalon, as to the PAG and RVM (including the nucleus raphe magnus), do not modify DNIC, whilst lesions of the subnucleus reticularis dorsalis in the caudal medulla strongly reduce DNIC. The ascending and descending limbs of this loop travel through the ventrolateral and dorsolateral funiculi, respectively (Villanueva L, Le Bars D., 1995). There is emerging data that supports that brainstem structures are influenced by cortical ones, such as the prefrontal cortex and anterior cingulated cortex, and this would explain why DNIC is influenced by psychological factors (Goffaux et al., 2007).

Some studies have shown that this system is altered in certain chronic pain conditions in humans (Pielsticker et al., 2005; Leonard et al., 2009) and plays a role in maintaining central sensitization and widespread hyperalgesia in animal models of pain (Porreca et al., 2002; Tillu et al., 2008)

Several studies have investigated the role of neurotransmitters and receptor systems in DNIC. DNIC seems to be modulated by serotonergic pathways (Chitour et al., 1982) and are probably opioid dependent (Lebars et al., 1992; Bie and Pan, 2007), though the mechanisms by which opioids affect DNIC are still unknown.

Most human studies have demonstrated that DNIC is triggered by a painful stimulus applied to a remote area of the body and induces inhibition of pain sensation to another part of the body but not an homotopic one (Graven-Nielsen et al., 1998; Svensson et al., 1999), though there have been previous reports on pain reduction after stimulation of ipsilateral adjacent regions. (Pud et al., 2005; Sowman et al., 2011).

1.3 MUSCLE PAIN

Already by the 18th century articular and muscular forms of rheumatism were differentiated and were defined as pain and stiffness of the muscle and soft tissue. In the 1930s the first approach to study muscle hyperalgesia and referred pain in humans was performed by Lewis and Kellgren, they introduced the concept of experimentally induced muscle pain (Kellgren, 1938). Since then, numerous are the studies on the physiology and treatment of this type of pain.

In the past two decades, research on muscle pain has increased enormously, the reasons for this is the need to develop new pharmacological targets for chronic musculoskeletal pain, as it is one of the main causes of disability, health problems and health care utilisation in the world (Badley et al., 1994).

1.3.1 Physiology of muscle pain

Muscle nociceptors are free nerve endings surrounded by a single layer of Schwann cell covering, except for the receptive areas which are free of Schwann cell processes and have direct contact with the interstitial fluid, around vessels and muscle fibers. The muscle fibers are not contacted by these free nerve endings, but are present mainly near small blood vessels. In these areas there are vesicles which contain neuropeptides and other endogenous substances joined by thin portions of axons. There is not any neuropeptide, or combination of these, that can be considered specific for muscle. Every time the ending is excited the peptides are released and influence the microcirculation of the tissue around the receptor (Mense, 2008).

As in the skin, muscle pain is transmitted to the spinal dorsal horn through thinly myelinated or group III fibers (which correspond to Aδ cutaneous afferents) or not myelinated, group IV fibers (which correspond to C cutaneous afferents) (Lloyd, 1943). Of all group III and IV fibers it is thought that 40% of them are nociceptors, the rest participate in cardiopulmonary adjustment that occurs during exercise, low threshold mechanical signalling and thermoregulation (Mense and Simons, 2000a).

As in skin, when injury occurs, a series of inflammatory mediators are released into the extracellular space by the damaged tissue, recruited immune cells and affected sensory fibers, some of these inflammatory mediators are bradykinine,

serotonine, substance P, adenosine-5'-triphosphate (ATP), prostaglandins, growth factors, proteases, protons, nitric oxide, cytokines, chemokines. Of all these proinflammatory substances, the release of ATP and protons is relatively specific for muscle pain, whereas in cutaneous pain they play a less important role (Mense, 2009). Though the study of the implication of ATP and protons in muscle pain is not an aim in this thesis, a small description will be done here because of their importance in this type of pain and difference with cutaneous pain.

Though it is known protons and ATP play an important role in the aetiology of muscle pain the exact mechanism has yet not been completely elucidated. Acid-induced nociceptor excitation is mediated by the activation of ion channels, particularly by acid-sensing ion channels (ASIC) and of transient receptor potential vanilloid receptor-1 ($TRPV_1$).

Of all ASIC receptors, $ASIC_3$ has shown to play an important role in the development of muscle hyperalgesia (Sluka et al., 2004; Sluka et al., 2007; Walder et al., 2010). A finding that supports these previous results is that higher proportion of DRG cells with afferent fibers from muscles express $ASIC_3$ receptors than neurons with afferents from the skin (50% *vs* 28%) (Molliver et al., 2005). Other receptors have been found to be involved as the purinergic receptors: P2X5 (Birdsong et al., 2010) or P2X3 (Dessem et al., 2010).

The nociceptive information from muscle is encoded in action potentials that enter the spinal cord or brainstem when cranial nerves are excited, and is transmitted to second order neurons through neurotransmitters to activate them. Electrophysiological studies have shown that inflammation of the gastrocnemius muscle increases the cell population in the DRG that can be excited by the input from that muscle (Hoheisel et al., 2005). A possible explanation for this is that previously ineffective synaptic connections between muscle afferents and neurons in these segments become effective (Li and Zhuo, 1998). This transformation from ineffective to effective can occur within a few minutes when a strong noxious stimulus acts on nociceptors in the periphery, which leads to an increase in dorsal horn convergence (Mense and Simons, 2000b). In the activation of these silent synapses AMPA and neurokinin-1 receptors seem to be involved (Liu and Sandkuhler, 1998; Mense, 2008).

This has been proposed as the explanation of why pain in the muscle usually refers to other deep somatic structures (Mense and Simons, 2000a).

After this synapse the nociceptive information from muscle is largely mixed with information from other tissues. There is no evidence for the existence of an ascending tract or cortical centre that exclusively mediates muscle pain.

Myofascial pain syndrome in any body part is characterized by the existence of trigger points. When applying pressure on a trigger point this will activate intense pain and can induce pain referral patterns to characteristic sites. The muscle around a trigger point usually appears as a taught band. The aetiology of trigger points is thought to be due to a higher release of acetylcholine in several adjacent neuromuscular junctions resulting in a localized contraction (Gerwin et al., 2004). Continued contractions in the areas of trigger points lead to localized hypoxia, lowered pH and the accumulation of local proinflammatory mediators.

In the orofacial area myofascial pain is classified under the temporomandibular disorders (TMD) which also include temporomandibular joint alterations. The aetiology of TMD is not clear; the multifactorial and biopsychosocial theories are the most accepted ones, where a complex interaction between environmental, emotional, behavioural and physical factors is proposed. The masseter muscle is the most frequently affected (>60%) followed by the medial pterigoid and temporalis (40-50%).

Because the exact aetiology is not known, the treatment of myofascial pain is symptomatic. Therapy for TMD is multidisciplinary and can be classified into: physical therapy, pharmacological, psychological and trigger point injection. Treatment with conservative methods (physical therapy, use of splints and NSAIDs) results in 75-90% of success rates (Benoliel and Sharav, 2008a). There are patients who do not respond to this treatment, in which pharmacologic alternatives are necessary, these include: tricyclic antidepressants, antiepileptic drugs, benzodiazepines and muscle relaxants (for review see (Benoliel and Sharav, 2008b). Although there is vast range of drugs for muscle pain, none of the drugs previously cited are free of side effects or interaction with other groups of drugs.

Repeated local anaesthetic injections are used to inactivate the trigger points, as these tend to reform after inactivation. The benefit of this treatment is that the drug is peripherally administered and lack of central side effects. However, in some

cases, trigger points tend to recur despite repeated trigger inactivation; several authors propose the injection of botulinum toxin as an alternative, as it has a longer duration of its action (Gerwin, 2012). Botulinum toxin has proven beneficial is some studies (De Andres et al., 2003; von Lindern et al., 2003) though other studies do not find clinical benefits in its use (Ernberg et al., 2011). The injection of botulinum toxin, actively being studied nowadays, is not free of side effects some of these are severe headaches, muscle atrophy or paralysis in areas adjacent to the injection site (Laskin, 2012).

New peripherally acting drugs would be interesting alternatives for the treatment of muscle pain. This would give a new option to those patients resistant to the treatments which exist nowadays, and possibly improve the pharmacologic options used up to date avoiding their important side effects.

1.3.2 Differences between muscle and cutaneous pain

Up to date, skin is the best studied organ for nociceptive transmission, but findings in this organ cannot be extrapolated to other organs of the body, due to the differences in innervation and physiology. Previous studies have found several differences in the nociceptive characteristics arising from muscle and skin. These differences have been summarized by Walder and Sluka (Walder and Sluka, 2008) and are detailed below:

Quality of pain

- Injury to the skin evokes well localized, sharp, stabbing or burning pain whilst Injury to deep structures results diffuse, difficult to localize aching pain.
- Muscle pain is rated as more unpleasant than skin pain.
- Intramuscular capsaicin induced pain is longer lasting than when induced in the skin and referred pain is more frequent.

Pathways and biochemical mediators:

- DRG neurons innervating muscle have less isolectin B4 and somatostatin and more CGRP and substance P than those innervating skin.
- Substance P, when injected in the skin causes spontaneous pain; in muscle it causes a decrease in pressure pain thresholds but not spontaneous pain.

- Small diameter primary afferent neurons innervating muscle are more likely to express acid-sensing ion channel-3 ($ASIC_3$) than those innervating skin.
- Central projections from neurons innervating muscle are predominantly to lamina I and the deeper dorsal horn but those from cutaneous tissue have a dense projection to lamina II.
- Formalin injection to the skin of the lower back causes c-Fos expression throughout lamina I-V whereas when injected in lower back muscles there is no labelling in lamina II.
- C fiber stimulation of a muscle nerve induces a longer lasting increase in the flexion reflex when compared to C fiber stimulation of a cutaneous nerve.

Though differences are not only restricted to muscle an skin, differences have also been found between different muscles in the organism as: $TRPV_1$ has been reported to be expressed in 5 to 25% of gastrocnemius muscle afferents in the DRG (Hoheisel et al., 2004; Fujii et al., 2008) and 24 to 37.5% of masseter afferents in TG of the rat (Tanimoto et al., 2005; Ro et al., 2009). Injection of capsaicin directly in the masseter of rats and humans evokes nociceptive behavior, pain and mechanical muscular hyperalgesia (Arima et al., 2009; Ro et al., 2009) and when injected in the gastrocnemius muscle it also reduces mechanical pain thresholds (Loram et al., 2007; Fujii et al., 2008).

1.3.3 Nociceptive models and tests in basic and preclinical research

To study pain in animals and healthy volunteers, different models and tests can be used. Next those who have interest for this thesis are described and then specific models of muscle pain are reviewed. Studies in animals or healthy volunteers cannot be translated directly into clinical applications or act as proxies for clinical conditions, but they can provide mechanistic knowledge to enhance the fundamental understanding of clinical signs and symptoms. Such translational steps can provide the theoretical basis for developing better diagnostic tools and targeted drugs to improve the treatment of disabling pain conditions (Arendt-Nielsen and Graven-Nielsen, 2008).

Muscle nociception is normally evoked by overuse, trauma and inflammation. It was in 1938 that Kellgren introduced intramuscular (i.m.) injection of hypertonic saline (HS) to study deep tissue pain in humans (Kellgren, 1938). In the past years i.m.

injections have become a widely used model to study acute and chronic pain in animals and humans. The i.m. injections of algesic substances induce a quantifiable pain which reproduces the characteristics of clinical muscle pain, hyperalgesia and altered motor function in humans. This method also reliably activates nociceptive pathways and produces nocifensive behaviors in animals. Though many algogens used in animal studies cannot be tested in human subjects (Capra and Ro, 2004), because the injection of these substances has important side effects.

1.3.3.1 Nociceptive tests in basic research

In animals there is an absence of verbal communication, so nociception can be studied by their reaction to different noxious stimuli. These reactions can be classified as:

1. Responses which are relatively low within the hierarchy of the CNS.
2. More complex responses organized by higher centers of the CNS.

The first group include basic motor responses, neurovegetative reactions, an increase in the sympathetic tone and vocalization. Depending on the stimuli two different responses can be observed: brief and sharp pains elicit phasic motor responses (withdrawal, startle reactions), whilst lasting pains may be associated with contractures to immobilize the painful region.

The second group includes more complex reactions as conditioned motor responses which result from a period of learning.

For the study of acute pain (short duration stimuli) different stimuli can be used: thermal, chemical, mechanical and electrical.

- **Thermal:** using heat stimulus one of the most used tests is the tail flick test, which consists on analysing the time a rat takes to withdraw its tail from a heat source. This test is considered to elicit a spinal reflex; it is maintained after section or cold block of upper parts of the spinal cord, although it is subject to control by supraspinal structures when the heating slope is slower.
- **Mechanical**: the most used mechanical stimulus is pressure applied to a punctiform area of the hindpaw. Devices used for this stimulus normally allow the application of increased measurable pressures and the interruption of the test when the nociceptive threshold is reached. Different behavioral responses can be seen: the

reflex of withdrawal of the paw, which is a spinal reflex and more complex movements as the animal tries to release its trapped paw, a sort of struggle and finally a vocalization. These are more complex responses that involve supraspinal sites (Le Bars et al., 2001).

- **Chemical:** the behaviors that are induced with this stimulation varies but are relatively stereotyped in rodents. Tests using chemical stimulation differ from the above in that it is not the threshold that is measured but a behavioral score. These experimental models are the closest in nature to clinical pain (Le Bars et al., 2001). Depending on the type of pain to study (acute or chronic) different chemicals can be used:

For acute pain HS 4-6% is one of the most used chemical stimuli. The quality of the pain is comparable to clinical muscle pain with localized and referred pain, and does not cause toxicity (Svendsen et al., 2005).

When injected in skeletal muscle it causes group III and IV fiber activation (Kumazawa and Mizumura, 1977; Mense, 1977). The mechanisms by which HS elicits noxious stimulation is still not clear, it has been hypothesized that the osmotic strength of the solution shrinks the terminal endings of the sensory fibers, excites nociceptors by opening mechanosensitive cation channels or causes the release of other excitants such as glutamate (Hamill and Martinac, 2001; Ro et al., 2007).

Neuronal and behavioral responses following HS injection in animals range from 2-4 min, which is similar to the time course of local pain ratings in humans (Capra and Ro, 2004). Formalin is also extensively used, the nociceptive response to formalin injection consists of two distinct phases, the first one (early phase) corresponds to acute pain and the second one (late phase) has an important inflammatory component (Clavelou et al., 1989)

For the study of more persistent pain different substances have been used. In animals, continuous infusion with HS has been scarcely used, though it has been tested in the masseter muscle (Ro et al., 2007). The advantage it presents is that findings on the effects of HS using animal models can be directly applied and translated to human experimental and clinical studies, as other more potent inflammatory substances cannot be used in humans. Other chemicals used for the study of inflammation in animal pain models have been: capsaicin, acidic saline, mustard oil, Freund's Complete

Adjuvant or carrageenan. Also the injection of endogenous substances has been used as substance P, bradykinin, serotonin and adenosine triphosphate (Capra and Ro, 2004), though they will not be revised in more detail because they have not been used in the experiments of this thesis.

1.3.3.2 Nociceptive tests in human preclinical research

In contrast to animal studies, verbal assessment of the subjective characteristics of pain (such as its intensity) can be recorded in humans and are necessary in all clinical and experimental muscle pain studies. Pain intensity is usually scored through a visual analogue scale (VAS). Also to assess the perceived pain quality and location, pain drawings, verbal descriptor scales, the McGill Pain questionnaire and similar scales and questionnaires may be useful.

As in animals, phasic pain can be also studied by thermal, electrical and chemical stimuli. Quantitative sensory testing (QST) is a term used to describe different forms of psychophysical testing of skin, mucosa or muscle tissue that assess sensory and pain perception pathways. It consists on a battery of tests which assess thermal, vibration and pressure detection and pain thresholds, as well as suprathreshold and pain tolerance thresholds.

The study of muscle pain can be induced by natural stimuli (as with exercise), ischemia, application of exogenous stimuli (as vibration, mechanical compression) or injection of some chemical substances.

The injection of intramuscular HS model has been widely used to induce experimental muscle pain. No side effects or muscle toxicity have been reported and therefore is considered appropriate for muscle experimentation in humans. Two different methodologies have been used: a single bolus (for the study of phasic pain) or a continuous injection (tonic pain) of HS. Other substances which have been used to induce pain are capsaicin, glutamate, acidic buffers or endogenous inflammatory mediators such as bradykinin and serotonin (Graven-Nielsen and Arendt-Nielsen, 2008).

2. OPIOIDS

Opium (poppy tears, lachryma papaveris) is the dried latex obtained from the opium poppy (Papaver somniferum). Opium was named after “opos”, the Greek word for juice. It is thought that, before people discovered how to prepare mekonium from the leaves and fruits of the plant, it may have been grown for its seed to obtain the liquid that appears on the unripe seed capsule when it is notched.

The use of records to decipher the early use of opium and abuse is difficult because the description of drugs, by ancient authors, is often ambiguous. Despite this, there is a general agreement among the authors that the Sumerians isolated opium from the seed capsules at the end of the third millennium B.C. From here opium spread to the rest of the world.

In 1806 Sertürner isolated the active ingredient in opium and named it morphine, after the god of dreams, Morpheus, after this pure morphine was synthesized (Sertürner, 1806). In the 1850s morphine began to be used for minor surgical procedures, for postoperative and chronic pain and as an adjunct to general anaesthetics with the invention of the hypodermic syringe and hollow needle. In the 19 hundreds new opiates were extracted from the plant or synthesized such as meperidine (Eisleb and Schaumann, 1939), methadone (Scott and Chen, 1946) and nalorphine, the first opioid antagonist (Unna, 1943).

In 1954 Beckett and Casy stated the hypothesis that these opioid drugs induced their effects due to their union to specific receptors (Beckett and Casy, 1954). But it wasn’t until 1973 that three laboratories succeeded, almost simultaneously, in demonstrating that there are specific binding sites of these drugs in the CNS (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). So, if these specific receptors existed, it was logical to think they might be the targets of neurotransmitters or endogenous opioids. The discovery of the first endogenous opioids did not take long (Hughes et al., 1975), which led to the acceptance of an endogenous opioid system with its receptors, ligands (Lord et al., 1977) and numerous physiologic and pharmacologic functions (Brownstein, 1993; Dhawan et al., 1996)

2.1 ENDOGENOUS OPIOID SYSTEM

The endogenous opioid system is formed by the endogenous opioid peptides, the receptors to which these peptides bind to induce a physiologic response and the enzymes in charge to metabolize these peptides.

2.1.1 Endogenous opioids

The endogenous opioid peptides are generally classified by their precursor. In the mammalian system there are four known precursor peptides: pro-opiomelanocortin, proenkephalin, prodynorphin and pronociceptin/orphanin FQ (N/OFQ),

- pro-opiomelanocortin: β endorphin
- proenkephalin: methionine and leucine-enkephalin
- pro-dynorphin: dynorphins A and B and neo-endorphin
- pronociceptin/orphanin FQ: nociceptin/orphanin, although it shares some structural similarities with pro-dynorphin derived peptides it has no action on opioid receptors.

Besides these previous four peptides, there are two endogenous peptides, endomorphin 1 and 2, which are not derived from the above precursors but through a via that has not been completely clarified yet.

The precursor peptides are synthesized in the body of the neuronal cell and afterwards transported to the axonal terminal where they are enzymatically cleaved by proteases to the active peptide and stored in vesicles (Janecka et al., 2010).

Most of the peptide degradating enzymes are membrane bound exo and endopeptidases, which have active sites also facing the extracellular space. So, most of the degradation processes of opioid peptides takes place in the extracellular space, although an intracellular cleavage of opioid peptides by cytosolic peptidases is also possible. According to the sequence of aminoacids in the N-terminus: typical (Tyr-Gly-Gly-Phe), or atypical (Tyr-Pro-Phe-Pro), these peptides are metabolized through different routes.

2.1.2 Opioid receptors

The initial classification of opioid receptors was based on the action of morphine and ketocylcazocine on a spinal dog model. So, in this manner, the first idea of μ (greek "m" for morphine; MOR) and κ (for ketocyclazocine; KOR) opioid receptor was drafted (Martin et al., 1976). Just a year later Kosterlitz et al., (Lord et al., 1977) proposed a δ receptor (for deferens; DOR) while working with the recently discovered enkephalin peptides on the vas deferens of mouse.

Opioid receptors belong to the rhodopsin-like subfamily of G protein coupled receptors (GPCR) seven transmembrane domain metabothropic receptors. The three receptors interact preferentially with the α subunit of the pertussis toxin (PTX) sensitive GPCR of the Gi and Go family (Gi_{1-3} and $G0_{1-2}$) as well as two PTX insensitive subunits (Gz and G_{16}) (Connor and Christie, 1999). The union of the ligand with the receptor facilitates coupling to the inhibitory Gi/o which inhibits adenylyl cyclase (AC). This enzyme catalyzes the conversion of ATP into cAMP, so its inhibition decreases the levels of intracellular cAMP. The reduction of intracellular levels of cAMP inhibits the enzyme protein kinase A (PKA) that will induce several changes in the cellular activity such as: activation of A type potassium channels.

Opioids have also shown to activate MAP kinases (MAPK) and phospholipase C (PLC). PLC is an enzyme located in the citoplasmatic part of the cellular membrane and cleaves the phospholipid localized in the membrane: phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG remains bound to the membrane, and IP_3 is released into the cytosol, and then diffuses through the cytosol to bind to IP_3 receptors, which causes the cytosolic concentration of calcium to increase, and this induces a cascade of intracellular changes and activity as the release of neurotransmitters. In addition, calcium and DAG together work to activate protein kinase C (PKC), which goes on to phosphorylate other molecules, leading to altered cellular activity (Gutstein and Akil, 2001) (Fig. 4).

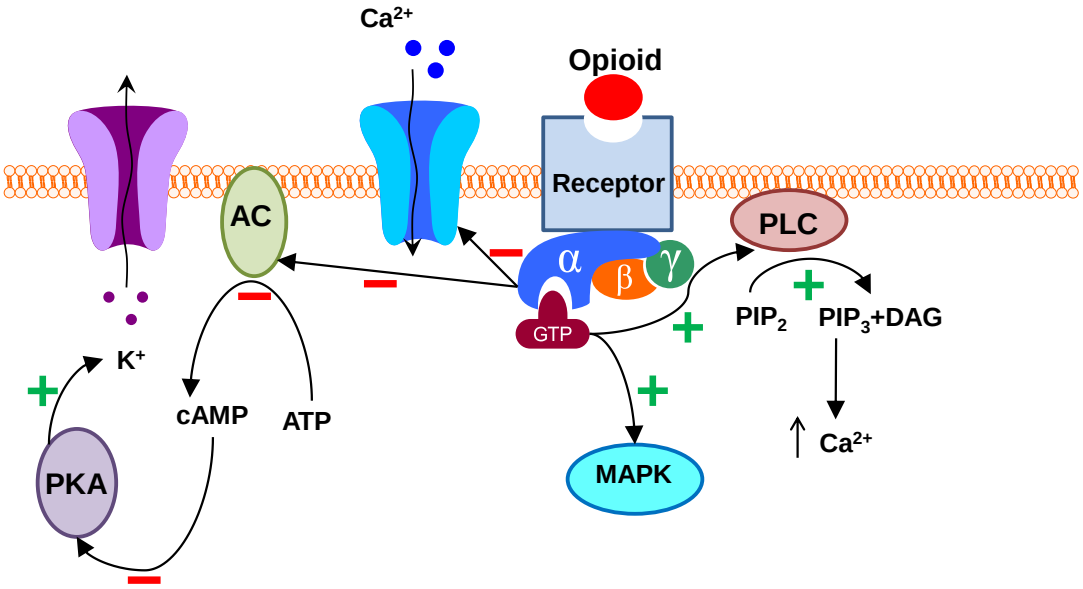

Figure 4. Mechanisms of signal transduction described for opioid receptors.

The genes for the three types of receptors were cloned in the early nineties: δ (Evans et al., 1992), μ (Chen et al., 1993; Thompson et al., 1993) and κ (Li et al., 1993; Meng et al., 1993; Yasuda et al., 1993). Although the three types of opioid receptor are encoded by a different gene they share high homology but can also exist in several splice variants due to a differential mRNA processing. These receptors are about 60% identical to each other, with the greatest identity in the transmembrane domains (73–76%) and intracellular loops (86–100%) and the greatest divergent areas in the N terminus (9–10%), extracellular loops (14–72%), and C terminus (14–20%) (Law et al., 2000).

The location of opioid receptor expression has been reviewed by Lesniak and Lipkowski and is shown in Table 1 (Lesniak and Lipkowski, 2011)

The first direct suggestion for the existence of opioid receptor subtypes started with the μ receptor, with the observation that different ligands for this receptor could differently affect the analgesic response and one of the unwanted side effects of opioids: the respiratory depression.

Dietis et al., 2011 describe a proposed distribution, pharmacology and function of putative receptor subtypes for the μ, δ and κ receptors. The authors conclude that there is no doubt that there are pharmacological differences between ligands but do not believe that based on these differences receptor subtype status can be ascribed.

But these pharmacological subtypes need to be reconciled with molecular data (Dietis et al., 2011).

	Expression		
Opioid receptor type	Central Nervous System	Peripheral Nervous System	Non-neural tissues
μ	Noecortex, caudate-putamen, nucleus accumbens, thalamus, hippocampus, amygdala, nucleus tractus solitarius	Peripheral sensory neuron, DRG, stomach, duodenum, jejunum, oleum, proximal and distal colon	Vascular endothelium, cardiac epithelium, keratynocytes, vas deferens, Sertoli cells
δ	Olfactory related areas, neocortex, caudate-putamen, nucleus accumbens, amygdala	Peripheral sensory neuron, DRG	
κ	Caudate-putamen, nucleus accumbens, amygdala, neural lobe of the pituitary gland.	Sensory neuron, DRG, stomach, duodenum, jejunum, oleum, proximal and distal colon.	

Table 1. Expression of opioid receptors in the central and peripheral nervous system and non-neural tissues.

Soon after the cloning of the opioid receptor genes, several laboratories revealed the existence of another "opioid receptor-like" (ORL) sequence in humans (Mollereau et al., 1994), rat (Bunzow et al., 1994; Chen et al., 1994; Fukuda et al., 1994; Lachowicz et al., 1995) and mouse (Nishi et al., 1994) that was similar to that of the already known opioid receptor. In 1995 the endogenous ligand of this receptor was identified, simultaneously, by two different laboratories and was named nociceptin (Meunier et al., 1995) and orphanin FQ (Reinscheid et al., 1995). However the results of this endogenous ligand is controversial, some behavioral studies show hyperalgesia,

analgesia and sometimes no effect. Plus most opioids do not bind to ORL receptors (Mogil and Pasternak, 2001).

2.1.2.1 Interaction of endogenous opioids and their receptors

Not all endogenous opioids bind to all opioid receptors with the same affinity:

- Methionine-enkephalin and leucine-enkephalin have high affinity for δ opioid receptor, moderate for μ and very little for κ opioid receptor.
- Dynorphins display high affinity for κ and little for μ and δ opioid receptor whilst β endorphins display μ and δ recognition and low for κ opioid receptor.
- Endomorphins have very high and selective affinity for μ opioid receptor.

The differences in opioid binding affinity explains the differences in the physiological actions of the different endogenous opioids, as endorphins and enkephalins are postulated to play a leading role in endogenous analgesia (Lesniak and Lipkowski, 2011).

2.2 CLASSIFICATION OF LIGANDS THAT BIND TO OPIOID RECEPTORS

As endogenous opioids, exogenous ones act upon these specific opioid receptors. Opioid drugs can be roughly divided into two groups: agonists and antagonists.

- Agonists are those ligands which bind to a receptor and activate it to induce an effect similar to that of the physiological signal molecule.
- Antagonists are ligands which bind to the receptor and prevent the action of the agonist on the receptor or the subsequent response, but do not have any effect of their own.

In research, agonists and antagonists are useful tools for multiple purposes as, for example, to elucidate the mechanisms of nociceptive signalling (Ro et al., 2007), characterize an antinociceptive drug *in vitro* and *in vivo* (Giron et al., 2002), or study interactions of a drug with different types of receptors and their effect *in vivo* (Sevostianova et al., 2005)

In this thesis different opioid agonists and antagonists have been used to determine the implication of peripheral and central receptors in the antinociceptive effects of opioids. Because the pharmacokinetic and pharmacodynamic properties of each drug can be consulted in a book of pharmacology (Gutstein and Akil, 2001), here I will mention the characteristics of the opioids used in this thesis.

2.2.1 Opioid receptor agonists

Morphine: this compound binds mostly to the μ opioid receptor, though it can bind also to δ and κ opioid receptors. It is considered as the reference when studying new antinociceptive drugs.

Morphine exerts its analgesic effect by acting at peripheral sites, but because it crosses blood-brain barrier it also acts upon spinal and supraspinal sites. So, morphine not only inhibits the nociceptive transmission of pain at the three levels, but also its perception and emotional component, so pain is not felt as distressing or unpleasant.

Loperamide: it is an opiate analogue with major peripheral μ opioid receptor binding. Although it is well absorbed from the gastrointestinal tract, loperamide is almost completely extracted and metabolized in the liver and the metabolites are excreted in the bile. Because of this very little loperamide reaches the systemic circulation.

Its main clinical use is as an antidiarrheal agent, it decreases propulsive activity and increases non-propulsive activity through its binding to μ opioid receptor in the myenteric plexus in the longitudinal muscle layer. It also increases the anal sphincter tone. Loperamide has been investigated as a possible topical analgesic for painful ulcers in the skin or mouth; there are preliminary reports of the successful use of orodispersible tablets as an adjuvant analgesic for oral pain arising from mucositis or cancer (Regnard et al., 2011).

In basic research it is frequently used to study the peripheral effects of opioid receptors, many previous studies have used it to study the peripheral contribution of opioid receptors in antinociception (Guan et al., 2008).

2.2.2 Opioid receptor antagonists

Naloxone: it is mainly a μ opioid receptor antagonist though at high concentrations it can bind to all three receptors. It crosses the blood brain barrier, so it antagonises the central and peripheral effects of opioids. Clinically it can be used to treat opioid intoxication and in the diagnosis of opioid dependence.

Naloxone methiodide: it is a quaternary derivative of naloxone. It is mainly a μ opioid receptor antagonist which does not cross the blood–brain barrier. It is not used in the clinic but it is mainly used as a research tool to distinguish between central and peripheral sites of action for drugs acting on opioid receptors (Brown and Goldberg, 1985; Goicoechea et al., 2008).

2.3 PHARMACOLOGICAL EFFECTS OF OPIOIDS

Morphine and most of the clinically used opioid agonists exert their effects through the μ opioid receptor, so these drugs affect many physiologic functions:

- **Analgesia**: opioids have shown to have an antinociceptive effect at the peripheral and central nervous system. Since the study of the antinociceptive effect of opioids is one of the aims of this thesis this section will be exposed in more detail below.
- **Psicomometic effects**: these effects are probably related to with dopaminergic pathways and structures. Opioid receptors are present in the main structures related with the reward circuits, when μ and δ agonists are administered dopamine is released and the reward circuits are activated; on the contrary κ agonists inhibit the release of this neurotransmitter.
- **Respiratory effects**: the origin is due to peripheral and central receptors. When acting upon the CNS opioids induce respiratory depression that is mainly due to the diminished sensitivity of the bulbar respiratory center to carbon dioxide, peripherally they can cause muscle stiffness.
- **Cardiovascular effects:** opioids do not alter cardiovascular parameters though when administered at high doses or to patients with higher risk they can inhibit the baropresor reflexes and induce peripheral vasodilatation.

- **Gastrointestinal effects**: opioids alter gastrointestinal activity by binding directly to receptors in this system and CNS. Opioids increase the tone and segmentation but decrease the propulsive movements, spasm of the sphincters. By the activation of μ receptors in the area postrema they can cause nauseas and vomit.
- **Genitourinary effects**: when opioids bind to μ and δ receptors they can cause urinary retention.
- **Effects on the immune system**: the mechanisms by which opioids affect it is not quite elucidated though it is thought to be due to μ and δ receptors and though in basic research it has been well established in humans their immune effects are not so evident.
- **Tolerance and dependence**: addiction is the compulsive desire to consume a substance. Opioids have a high capacity to create addiction due to their high implication in the dopaminergic reward circuits, though when administered for pain treatment addiction is rare.

Tolerance appears as a reduction in the analgesic effect which makes necessary an increase in the dose. It is related to interactions of the opioid system with other neurotransmitters such as glutamate and nitric oxide (Martin and Goicoechea, 2005).

2.4 OPIOID SIDE EFFECTS

Unfortunately, binding of opioids to their receptors does not only elicit a wanted analgesic effect but also several side effects that can hamper their use. Most of these side effects (Table 2) are due to the binding of opioids to receptors within the CNS. So, peripheral opioid receptor agonists can have important advantages such as decreasing these side effects and reducing the onset of tolerance and addiction while maintaining the analgesic effect.

Frequent	Sporadic	Infrequent
Acute Nausea and vomits Drowsiness Lack of stability Confusion	Dry mouth Restlessness Pruritus Hallucinations Myoclonus	Respiratory depression Opioid dependency
Chronic Constipation Nausea and vomits		

Table 2. Side effects of opioids listed by their frequency (Martin and Goicoechea, 2005).

2.5 OPIOIDS AND ANALGESIA

The analgesic effect of opioids is due to the presence of the opioid receptors in the pathways that transmit pain. These receptors can be found in structures located in the CNS but also in the periphery.

2.5.1 Central mechanisms of opioid analgesia

The activation of opioid receptor at the supraspinal level can relieve pain through three different mechanisms:

- Activation of descending inhibitory projections to the spinal cord.
- Modulation of the affective and emotional component of pain.
- Inhibition of the ascending transmission of pain.

Going back to the chapter of physiology of pain, nociceptive signals entering the spinal cord are not only modulated by interneurons but also by inhibitory descending projections from supraspinal sites which are activated by opioid receptors. The best characterized circuit is a descending pathway linking the dorsal raphe nucleus (DRN), the PAG, NRM and the dorsal horn of the spinal cord. The PAG activates the NRM and the adjacent nucleus reticularis paragigantocellularis through neurons releasing excitatory amino acids. The NRM has two characteristic neuronal populations, the “on” and “off” cells, which project to the spinal cord and modulate pain. Nociceptive transmission is inhibited by “off” cells in the dorsal horn and facilitated by “on” cells. This, although a simplistic classification, is thought to mediate the analgesic effect of opioids in the brainstem. Opioids as morphine cause an

activation of a population of cells in the PAG that in turn excites the RVM. Microinjections of μ opioids within the PAG and RVM suppress nociceptive reflexes organized within the spinal cord similar microinjections have shown to influence the activity of nociceptive dorsal horn neurons in electrophysiological experiments.

Besides the PAG and RVM other supraspinal sites have shown to express opioid receptors as the frontal cortex, nucleus accumbens, hippocampus, thalamus and hypothalamus, though if these structures inhibit ascending pain pathways or activate descending ones is still not well known.

Although the exact mechanisms by which cortical and subcortical structures mediate the analgesic effect is not completely elucidated, some structures, as the anterior cingulate cortex or thalamus, intervene in the emotional aspect of pain perception, so pain is not felt as distressing.

An aspect that has been the aim of some studies is the stress induced analgesia (SIA) which consists on suppression in pain response that occurs during or following exposure to a stressful or fearful stimulus. Conceptually, this definition is similar to the one described before for DNIC, and in both phenomenon serotoninergic and opioid systems are involved. But there is a neuroanatomical difference, while DNIC is mediated by a spinal-medulla-spinal loop SIA involves supraspinal structures, as the cortex, hippocampus or amygdala.

Spinal opioid analgesia is directly binded to the supraspinal one as described previously, and they cannot act in an independent way. The three major opioid receptors are present in the dorsal horn. Studies performed with autoradiographs have shown that both μ and κ receptors are primarily concentrated in the outer laminae, although especially in lamina II, where small afferent fibers mainly project. In contrast, δ receptors have been found to be diffusely distributed through the dorsal and ventral horn (Gouarderes et al., 1993).

Autoradiographic studies suggest that the great majority of spinal μ opioid receptors (60%) probably reside in the central terminals of afferent neurons (Besse et al., 1990; Besse et al., 1991); the remaining receptors are believed to reside on either interneurons or on cell bodies of second order neurons that transmit nociceptive inputs to supraspinal sites that process nociceptive signals (Ossipov et al., 2004).

In different experimental studies, systemically administered morphine has shown to reduce the nociceptive behavior in multiple pain models as for example the paw formalin test during the early and late phase (Sevostianova et al., 2005; Burgos et al., 2010) and neuropathic pain (Erichsen et al., 2005).

The effect of opioids in muscle pain has barely been studied; intrathecal administration of morphine has shown to increase mechanical withdrawal thresholds after repeated injection of acidic saline into the gastrocnemius muscle (Sluka et al., 2002) and levorphanol reverts the mechanical hyperalgesia after carrageenan injection in the forelimb muscle (Kehl et al., 2000).

2.5.2 Peripheral mechanisms of opioid analgesia

In contrast to the traditional view that opioid antinociception is mediated exclusively within the CNS, peripheral opioid receptors have been discovered and shown to mediate analgesic effects when activated by locally applied exogenous opioid agonists. Such effects are particularly prominent in painful inflammatory conditions and have been demonstrated both in animals and humans (Barber and Gottschlich, 1992; Stein and Lang, 2009)

All three receptors have been found to be expressed in C and Aδ fibers of the DRG cells, as well as in small diameter primary afferent nerve fibers (Dado et al., 1993; Arvidsson et al., 1995; Ji et al., 1995). The activation of these receptors is through the inhibition of Ca^{2+} channels of small diameter nociceptors and not of large diameter cells and this receptor activation inhibits the activity of C fibers (Taddese et al., 1995). In line with these findings, there are *in vivo* studies indicating that capsaicin-sensitive primary afferents indeed mediate the peripheral antinociceptive effects of morphine and of μ, δ, and κ selective agonists, although the comparison of agonists with different affinities for the three types of receptors has shown that ligands with a preference for μ receptors are generally the most potent (Bartho et al., 1990).

Peripheral opioid receptors are synthesized in cell bodies of primary afferent neurons and intraxonally transported to peripheral sensory nerve endings where they can interact both with endogenous and exogenous opioid agonists (Rau et al., 2005; Wang et al., 2010a). However the upregulation of opioid receptors cannot account for the increase in opioid efficacy following inflammation since the time course of opioid

receptors synthesis does not always correlate with opioid effects that could appear within minutes (Nunez et al., 2007). The role of peripheral opioid receptors is more pronounced during peripheral inflammation due to a number of inflammation-driven processes:

1. Under normal conditions nerve fibers are encapsulated by a perineural barrier preventing the diffusion of high molecular weight particles and hydrophilic opioid receptor ligands. In pathological conditions, such as inflammation, easier access to opioid agonists through the perineurium of sprouting peripheral nerve fibers is observed (Olsson, 1990; Antonijevik et al., 1995).
2. The expression of peripheral opioid receptors and their axonal transport to the sensory nerve terminals is enhanced in the course of inflammation (Hassan et al., 1993).
3. Low pH which can increase agonist efficacy by altering the interaction of opioid receptors with G-protein (Vetter et al., 2006).

Opioids increase potassium and decrease calcium and sodium currents in the soma of dorsal root ganglion sensory neurons through the interaction with G proteins. Provided that these events are similar throughout the neuron, they may underlie the following observations:

1. Opioids attenuate the excitability of the peripheral nociceptive terminal and the propagation of action potentials.
2. Similar to their effect at the soma and central terminals, opioids inhibit the calcium dependent release of excitatory proinflammatory compounds (for example, substance P) from peripheral sensory endings.
3. In addition morphine has shown to inhibit the neurotransmitter release from sympathetic varicosities and the antidromic vasodilatation evoked by C fibers (Stein et al., 1999).

To avoid the unwanted side effects of opioids, studies in human subjects have aimed to target peripheral opioid receptors. As a result, systemically administered opioid agonists, that don't cross the blood brain barrier, are beneficial in patients with neuropathic (Wallace et al., 2006) and visceral pain (Mangel et al., 2008). Another approach has been the local administration, as for example intrarticular administration of morphine after knee surgery (Heine et al., 1994).

In animal studies locally administered morphine has also shown to prevent the secondary mechanical allodynia induced after formalin injection in the paw (Ambriz-Tututi et al., 2009). Loperamide has shown to reduce the mechanical allodynia and heat hyperalgesia in a neuropathic pain model (Guan et al., 2008).

2.5.3 Opioids and orofacial pain

Most studies have been performed on spinally innervated structures, there are fewer data on the effect of opioid drugs on orofacial pain.

The first authors to report the presence of opioid receptors in the trigeminal system were Jessell and Iversen in 1977 in which they found that substance P release from the body of primary afferent fibers was inhibited by treatment with opioids (Jessell and Iversen, 1977). After that, mRNA for the three types of receptors was found in the spinal trigeminal nucleus (Mansour et al., 1994). In 1998 Li and coworkers found trigeminal neurons coexpressing μ opioid receptors with substance P or CGRP which implicates these receptors in the modulation of nociceptive inputs from orofacial regions (Li et al., 1998). A few years later δ opioid receptors were found to also be colocalized in neurons with substance P or CGRP (Ichikawa et al., 2005).

In behavioral studies opioids have been studied for their peripheral and systemic antinociceptive effect. When administered systemically, morphine reduces the nociceptive behavior in the orofacial and temporomandibular joint formalin test during both phases (Eisenberg et al., 1996; Burgos et al., 2010), in muscle pain models (Ro et al., 2003) and the mechanical allodynia (Deseure et al., 2002) and heat hyperalgesia (Chichorro et al., 2009) in neuropathic pain models. Locally administered morphine reduces the nociceptive behavior in a muscle pain model (Han et al., 2008), and in both phases of the orofacial formalin test (Eisenberg et al., 1996).

Data on the role of δ and κ receptors in orofacial pain modulation is very limited, δ receptors have been implicated in the nociceptive transmission in muscle orofacial pain and mRNA has been detected in TG of rats (Saloman et al., 2011) and κ receptor agonists have shown to reduce the nociceptive behavior in the TMJ formalin test (Clemente et al., 2004).

3. CANNABINOIDS

Cannabis sativa, commonly known as “hemp” or “marijuana”, is a plant that belongs to the family of the Cannabaceae. Cannabis has two main varieties: *Indica* and *Sativa*. Some authors note a third variety, the *Rudelaris*, but most only recognize the first two.

All through history cannabis has been used by mankind for several purposes: religious, medical (used as preventive and for treatment of different diseases) and industrial (because of its fibre, resin, and oil).

Although the first crops cultivated found are dated on the year 8000 B.C. it is not known when it first was used with medical purposes. The first written references of the use of marijuana with this purpose are found in the Chinese pharmacopoeia of Shen Nung in 2600 BC and Shen Nung in 2700 BC.

The first scientific studies on the therapeutic effect of cannabis were performed by Sir William B. O´Shaughnessy, who published in 1839 the first article on the analgesic, antispasmodic, and muscle-relaxing properties of cannabis. O'Shaughnessy's pronouncements roused the interest of doctors all over the world, and as a result, over the next sixty years, more than a hundred scientific studies of cannabis were published.

During the first decades of the 20^{th} century the use of cannabis with therapeutic aims lost interest because of its psychoactive effects, the introduction of new analgesic options (morphine was purified) and the synthesis of new drugs considered safer. In 1932 its use was abolished from the English pharmacopoeia and ten years later from the American one.

The first component of the cannabis to be isolated was cannabinol (Wood et al., 1899). Adams and coworkers completed the full chemical elucidation of this compound (Adams et al., 1940a), after this, cannabidiol was also purified (Adams et al., 1940b) and characterized (Mechoulam and Shvo, 1963) and in the sixties Δ^9-Tetrahydrocannabinol (THC), which is the main responsible for the psychoactive effects of cannabis, was also characterized (Gaoni and Mechoulam, 1964). Nowadays it is known that the *Cannabis sativa* plant has around 400 compounds and more than 60 cannabinoids have been identified.

Depending on their origin cannabinoids can be classified as:

- molecules which can be found in the plant (phytocannabinoids)
- endogenous substances found in the animal organisms which are going to form part of the endocannabinoid system (endocannabinoids)
- molecules which are synthesized in the laboratory (synthetic cannabinoids).

3.1 ENDOCANNABINOID SYSTEM

The endocannabinoid system is formed by several elements: at least two specific receptors named cannabinoid receptors, the endogenous ligands, also called endocannabinoids, that bind to these receptors and several enzymes and proteins which regulate ligand concentration.

3.1.1 Endocannabinoids

Although cannabinoid receptors will be exposed in the next section, the first component of the endocannabinoid system to be discovered was the CB_1 receptor. So, Mechoulam and coworkers followed the reasoning that had led to the discovery of endogenous opioids: evolution could not have maintained in the organism a receptor only for a plant to stimulate, this led to the identification of the first endocannabinoid: N-arachidonoylethanolamine (anandamide) (Devane et al., 1992). A few years later a second endocannabinoid was identified: 2-arachidonoyl glycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995). Since then other compounds have been isolated such as: 2-Arachidonyl glyceryl ether (noladin ether) (Hanus et al., 2001), *O*-arachidonoyl ethanolamine (virodhamine) (Porter et al., 2002) or N-arachidonoyl dopamine (NADA) (Bisogno et al., 2000; Huang et al., 2002). However the endogenous functions in physiological processes for all these compounds have not yet been established in detail (Pagotto et al., 2006).

Unlike other neurotransmitters, endocannabinoids cannot be stored in vesicles because they are very lipophilic, so they are synthesized on demand. Several different stimuli lead to the cleavage of membrane phospholipids and to the synthesis of endocannabinoids; these stimuli include membrane depolarization, increase of

intracellular calcium and/or $G_{q/11}$ protein coupled receptor stimulation (Pagotto et al., 2006).

N-arachidonoyl-phosphatidylethanolamine (NArPE) has been identified as the precursor molecule of anandamide and diacylglycerols with arachidonic acid on the 2-position as the most likely compounds from which 2-AG is generated. Both, anandamide and 2-AG are inactivated by enzymatic hydrolysis of the amide and ester bonds, respectively, and the major enzymes responsible for these reactions were cloned and named fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively (De Petrocellis and Di Marzo, 2009).

3.1.2 Cannabinoid receptors

Up to date only two cannabinoid receptors have been well characterized: CB_1 and CB_2. Both receptor types are members of the seven transmembrane-domain, G protein-coupled family. They share 44 % homology in their amino acid sequences or 68 % when only the transmembrane region is taken into account (Munro et al., 1993) (Fig 5). The main differences between these receptors are: the way the signals are transduced to the interior of the cell and their distribution in the different tissues (Howlett et al., 2002).

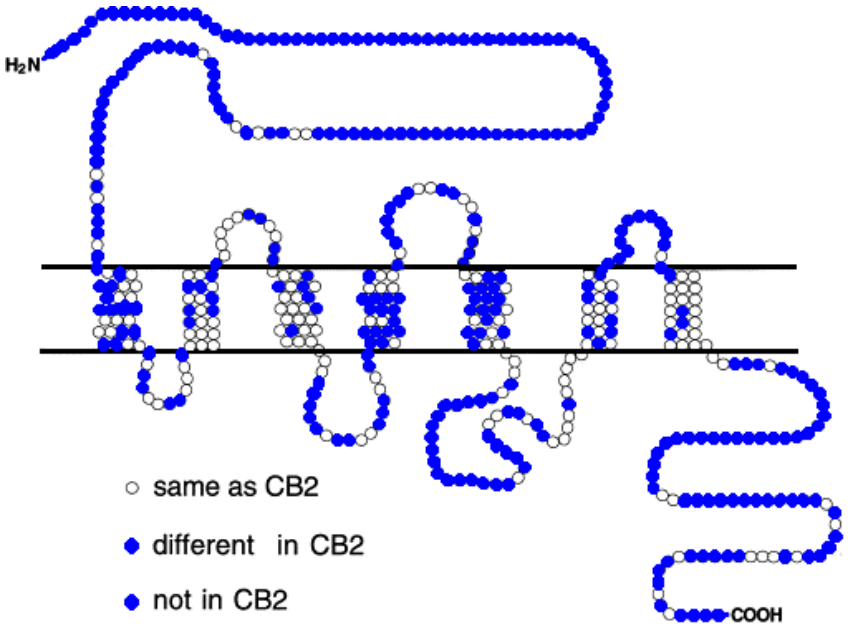

Figure 5. Schematic representation of the primary structure of CB_1 and CB_2 receptors. The structure shown is that of CB_1. The aminoacids that are identical to CB_2 are shown as open circles, those that are different or do not appear are shown as filled circles (Childers and Breivogel, 1998).

3.1.2.1 CB_1 Receptor*:*

This receptor was the first to be cloned from the brain of the rat (Matsuda et al., 1990), sometime after in humans (Gerard et al., 1991) and five years later in the mouse (Chakrabarti et al., 1995). Overall these receptors have 97-99% sequence identity throughout the species (Howlett et al., 2002). All species have a similar distribution of this receptor, although there are certain differences, like a higher expression in the amygdala and cingulated cortex in humans when comparing them to rat or monkey (Herkenham et al., 1990).

The first mechanism of signal transduction described for this receptor was the inhibition of the enzyme AC (Howlett and Fleming, 1984), the final effect of this will be changes in the cellular activity such as: activation of A type potassium channels (Hampson et al., 1995; Mu et al., 2000), and gene expression regulation (Demuth and Molleman, 2006).

Also the interaction of CB_1 receptor with Gs has also been demonstrated, this would lead to an increase in the levels of cAMP instead of a decrease, the results of previous studies seem to suggest that the resulting effect of cannabinoid agonists on AC activity could be attributed to specific isoforms present in different cellular preparations (Demuth and Molleman, 2006).

CB_1 receptor has been found to inhibit the L-type, N-type and P/Q-type calcium channels although the mechanism of this response is not clear if it is due to Gi/o or Gq regulation of phospholipase C (Sugiura et al., 1996; Lauckner et al., 2005), which causes the cytosolic concentration of calcium to increase, and this induces a cascade of intracellular changes and activity as the release of neurotransmitters. In addition, calcium and DAG together work to activate PKC, which goes on to phosphorylate other molecules, leading to altered cellular activity. There have been found cell type specific differences in the mechanisms of CB_1 mediated Ca^{2+} signal generation which could be due to the different G-protein subunit composition of various cell types or may be caused by different dimerization/interaction of CB_1 with other receptors in these cell types. (Turu and Hunyady, 2010)

Activation of mitogen-activated protein kinase (MAPK) pathways are often activated by G protein coupled receptors, these pathways regulate numerous cell

functions as cellular growth, differentiation, movement and apoptosis. The mechanisms through which CB_1 receptors activate this pathway is not completely elucidated, one possible mechanism could be indirectly through its effects on cAMP accumulation and consequently on protein PKA (Derkinderen et al., 2003), although others have also been reported (del Pulgar et al., 2000).

Finally, some cannabinoid agonists have shown to inhibit voltage dependent sodium channels (Nicholson et al., 2003).

The CB_1 receptor is mainly expressed within the CNS and PNS to a lesser extent. In peripheral nonneural tissues it has been described to be in: adrenal gland, adipose tissue, heart, liver, lung, prostate, uterus, ovary, testis, bone marrow, thymus and tonsils (Galiegue et al., 1995). This receptor is the most abundant G-protein coupled receptor in the brain with especially high levels in the striatum, cerebellum, basal ganglia, cerebral cortex and hippocampus (Herkenham et al., 1990; Herkenham et al., 1991).

The widespread distribution of the CB_1 receptor is consistent with the multiplicity of effects of cannabinoid agonists, including hypomotility, increased food intake, disruption of short term memory consolidation, antinociception, deficits of executive function, anxiety/anxiolysis and psychotropic effects. CB_1 receptor density is moderate to high in areas related to pain transmission and modulation such as DRG, spinal cord, thalamus, PAG, amygdale and RVM (Tsou et al., 1998).

3.1.2.2 CB_2 receptor:

Three years after CB_1 receptor was cloned, a second cannabinoid receptor was identified from human promyelocytic cells and was named CB_2 (Munro et al., 1993). It presents a slight lower homology in the sequence of aminoacids between humans and rats and mice, 81 and 82 %, respectively (Shire et al., 1996; Griffin et al., 2000).

CB_2 (Fig. 6), as CB_1 receptors, have shown to inhibit AC activity in a concentration dependent manner and activates MAPK coupling to Gi/o proteins (Bayewitch et al., 1995; Slipetz et al., 1995; Kobayashi et al., 2001), though CB_2 does not couple to G_s (Kaminski, 1996). This would be translated in less stimulating effects, though coupling to Gi can also inhibit inhibitory systems.

CB_2 cannabinoid receptor has also been found to increase calcium concentrations through activation of PLC (Zoratti et al., 2003).

CB_2 receptors are widely expressed in peripheral tissues and especially in immune tissues. These receptors are mainly expressed in spleen, tonsils and thymus, tissues responsible for immune cell production and regulation. These immune cells include mast cells, B cells, T_4 and T_8 cells, microglial cells, macrophages natural killer cells and to a lesser extent monocytes and polymorphonuclear neutrophils (Galiegue et al., 1995; Howlett et al., 2004).

The first studies indicated that CB_2 receptors were not present in neurons of the CNS, although recently they have been localized in several structures of the CNS: mRNA has been found in cortex, hippocampus, cerebellum and brainstem (Van Sickle et al., 2005a). A more detailed review on the distribution of CB_1 and CB_2 receptors in the CNS has been performed by Svizenska and coworkers (Svizenska et al., 2008).

3.1.2.3 Non CB_1 non CB_2 receptors for endocannabinoids

Though there are some physiologic responses that are regulated by cannabinoids which cannot be explained through their union to CB1 or CB2 receptors. So, this led to the thought that if other receptors existed for endocannabinoids these must already have been discovered for other mediators or have very little homology to CB_1 and CB_2. Both hypotheses have gained experimental support (De Petrocellis and Di Marzo, 2009).

The TRPV1 has shown to mediate some of the *in vivo* effects of anandamide. Other receptors belonging to the TRP family which could be activated through endocannabinoids are TRPM8 and TRPA1.

The orphan GPCR, GPR55 has also been proposed to mediate some of the effects of endocannabinoids and THC. This protein is highly expressed in the brain and peripheral tissues, though it presents little homology with both cannabinoid receptors already cloned. The results are contradicting and future experiments are needed.

Another possible explanation for the multiple endocannabinoid actions and targets might be represented by the finding of heterodimers between CB_1 receptors and several other GPCR, including adenosine $A_{2A,}$ dopamine D_2, and $orexin_1$ receptors (De Petrocellis and Di Marzo, 2009).

3.2 CLASSIFICATION OF LIGANDS THAT BIND TO CANNABINOID RECEPTORS

The next classification of cannabinoid ligands has been summarized from the one by Howlett et al. (Howlett et al., 2002).

3.2.1 Cannabinoid receptor agonists

- Classical cannabinoids:

In this group are included those compounds found naturally in the plant *Cannabis sativa* or synthetic analogues of these. The most studied in this group are Δ^9-THC, Δ^8-THC and HU-210. The first two are found in the plant whilst HU-210 is a synthetic analogue. All have selectivity for both cannabinoid receptors.

- Nonclassical cannabinoids:

They are bicyclic and tricyclic analogues of the THC molecule that lack the dihydropyran ring. In this group the most studied is the bicyclic compound CP 55,940, which is less lipophilic and binds to both receptors.

- Aminoalkyndoles:

These compounds are structurally analogues of pravadoline, with a reduced ability to act as nonsteroidal anti-inflammatory agents but increased ability to bind to cannabinoid receptors. The most studied compound is WIN 55,212-2 which displays high affinity for both cannabinoid receptors but moderate selectivity for CB_2.

- Eicosanoids:

This group is formed by those compounds that structurally derive from the araquidonic acid. Anandamide is the prototypic member of this group and its structural modification has led to the development of the first generation of CB_1 selective agonists, in which arachydonyl-2´-chloroethylamide (ACEA) is included. ACEA has a decreased susceptibility to FAAH-mediated hydrolysis but a reduced CB_1 affinity.

3.2.2 Cannabinoid receptor antagonists

- Diarylpyrazoles:

The prototypic members of this group are the SR141716A, a potent CB_1 selective ligand and SR144528, a potent CB_2 ligand. Two analogues of the SR141716A compound are AM251 and AM281.

- Other chemical series:

The most important compounds of this series are LY320135 which binds with higher affinity to CB_1 and the aminoalkyndole 6-iodopravadoline or AM630 which is a CB_2 selective antagonists/inverse agonist.

3.3 PHARMACOLOGICAL EFFECTS OF CANNABINOIDS

Due to the broad localization of cannabinoid receptors in numerous organs they play an important modulating role in the physiology of the organism, so cannabinoids can affect numerous processes and cause:

- Analgesia: this section will be revised in more detail below.
- Mood changes, feeling of euphoria, sedation and relaxation
- Alteration of the perception of time and short term memory
- Orexigenic and antiemetic activity
- Effects on muscular tone and motor coordination
- Reduction of the intraocular pressure
- Hypothermia
- Bronchodilation
- Cardiovascular effects: hypotension and tachycardia
- Neuroendocrine effects: inhibition of the release of sex related hormones and increased release of hormones related to stress
- Immunomodulatory effects: immunostimulation at low doses and immunostimulation at higher doses
- Antiproliferative effects

Both cannabinoid receptors have been related to these effects. Though, because of the different distribution of cannabinoid receptors, the side effects in the

CNS have been attributed mostly to CB_1 receptors whilst those occurring at the peripheral level are due mainly to CB_2.

The importance of the great variety of physiological effects of cannabinoids makes them a possible target for the treatment of numerous pathologies. Up to date there have been few cannabinoid drugs approved for clinical use in Spain: Dronabinol and Nabilona which are structural analogues of the THC molecule as antiemetic in cancer therapy, and very recently Sativex® for the treatment of pain and spasticity related to multiple sclerosis (Sastre-Garriga et al., 2011).

3.4 CANNABINOIDS AND ANALGESIA

To understand the possible implications of cannabinoids in pain treatment it is necessary to analyze the relation between these compounds and the pathways of pain transmission. For this, it is necessary to know:

- The localization of endocannabinoid system in the structures which transmit and process pain
- Its ability to modulate the transmission of pain
- Its relation with other systems implicated in the modulation of pain (Martin and Goicoechea, 2009).

As stated before, cannabinoid receptors have been found to be expressed in the peripheral and central nervous system.

3.4.1 Central mechanisms of cannabinoid analgesia

In supraspinal sites CB_1 receptor has also been localized in several areas involved in pain processing such as the PAG, RVM and the A5 noradrenergic nucleus of the medulla participating in the descending pain modulation (Walker and Huang, 2002). Also cannabinoids exert an antinociceptive effect when acting upon the PAG (Lichtman et al., 1996; Vaughan et al., 2000) and RVM (Meng et al., 1998; Vaughan et al., 1999).

Recently CB_2 receptors and mRNA have been localized within the CNS, including the brainstem and cortex (Van Sickle et al., 2005b).

In the spinal cord most CB_1 receptors have been localized in the superficial layers of the dorsal horn. Here they have been found to be both in presynaptic

terminals of the primary afferents and postsynaptically in the endings of the nerve fibers that project to the medulla or interneurons from supraspinal sites (Hohmann and Herkenham, 1999). Several studies have demonstrated that the antinociceptive effect of cannabinoids is partly due to the direct interaction of these with nociceptive spinal neurons (Lichtman and Martin, 1991; Welch and Stevens, 1992; Drew et al., 2000; Kelly and Chapman, 2001).

The participation of CB_2 cannabinoid receptors in antinociception is contradictory. The first studies failed to localize CB_2 receptors in the DRG, though recently CB_2 mRNA has been reported in the spinal cord and selective agonists reduced the release of calcitonin gene-related peptide in the rat spinal cord (Beltramo et al., 2006). Studies have demonstrated that spinal CB_2 receptor mediated analgesia is preferentially mediated in conditions of neuronal sensitization (Elmes et al., 2004; Nackley et al., 2004), this is in line with the finding that pathological pain states and injury are associated with upregulation CB_2 receptor, whereas expression levels are at the limit of threshold detection in naïve animals.

In animal studies, systemically administered cannabinoids have shown to have antinociceptive effects in multiple pain models; this has been reviewed by Guindon and Hohman (Guindon and Hohmann, 2009). The effect on cannabinoids on muscle pain has hardly been studied, as only one study has investigated the antinociceptive effect of cannabinoids, where a non-selective cannabinoid agonist reverted the grip force in an inflammatory (carrageenan) and tumor model (Kehl et al., 2003).

3.4.2 Peripheral mechanisms of cannabinoid analgesia

Cannabinoid receptors are not only localized in the spinal cord but also in the DRG. Cells from the DRG synthetize cannabinoid receptors and transport them towards the peripheral terminals of primary afferent neurons.

CB_1 and CB_2 receptors have been found to be expressed on nociceptive primary afferents neurons of the rat DRG (Hohmann and Herkenham, 1999; Ahluwalia et al., 2000; Salio et al., 2002) and also in large myelinated and small unmyelinated human cutaneous nerve fibers (Stander et al., 2005) and CB_1 receptor in the trigeminal ganglion (Price et al., 2003) and in human skeletal muscle (Eckardt et al., 2009; Guindon and Hohmann, 2009).

To avoid psychoactive effects locally administered CB_1 selective or non-selective CB_1/ CB_2 agonists have been tested and shown to have an antinociceptive effect in acute (Johanek and Simone, 2004), inflammatory (Richardson et al., 1998; Gutierrez et al., 2007) and neuropathic (Fox et al., 2001) pain models in animal. Recently, CB_1 selective agonists with very low CNS penetration have been synthetized and shown to induce antinociception in inflammatory, neuropathic (Yu et al., 2010) and muscle (Cumella et al., 2012) pain models.

Another aim to avoid the psychoactive effects of cannabinoids has been to target the CB_2 receptor. Some selective agonists, administered systemically or locally, have shown an antinociceptive effect in acute pain models (Malan et al., 2001), neuropathic (Kinsey et al., 2011) and inflammation (Nackley et al., 2003; Gutierrez et al., 2007; Kinsey et al., 2011). These CB_2 selective agonists have shown to lack centrally mediated side effects associated with the activation of CB_1 receptors (Malan et al., 2001; Kinsey et al., 2011). The absence of CNS side effects is consistent with the relative existence of CB_2 receptors in brain of naïve animals as they are predominantly (though not exclusively) located in the periphery.

3.4.3 Cannabinoids and orofacial pain

The first research work to study the expression of cannabinoid receptors in the trigeminal ganglion was performed by Price et al. They found that about 30 % of the trigeminal neurons contain CB_1 mRNA, though most were found to be expressed mainly in neurons involved in light touch and vibration sensations (Aβ). An important difference between the trigeminal and spinal systems found in this study is that only 1.5 % of all trigeminal ganglion neurons contained both TRPV1 and CB_1 mRNA inmunoreactivity, whilst most cultured DRG neurons contain inmunoreactivity to both receptors. No evidence of CB_2 receptor transcripts were found in the trigeminal ganglion (Price et al., 2003). The exact mechanisms by which cannabinoid agonists exert their antinociceptive effect in the trigeminal ganglion are not well understood and several receptor subtypes could be implicated.

Few behavioral studies have been published in which they study the antinociceptive effect of cannabinoids in the orofacial region. These have shown that WIN 55,212-2 has an antinociceptive effect in neuropathic (Liang et al., 2007) and

orofacial formalin test (Burgos et al., 2010). In both studies this effect was reversed by the CB_1 selective antagonist but not by the CB_2 selective agonist. When starting the experiments of this thesis no data were published on the antinociceptive effect of cannabinoids on orofacial muscle pain models, recently a selective CB_1 and peripherally acting agonist has shown to have an antinociceptive effect in a model of masseter pain induced by HS (Cumella et al., 2012).

4. TO SUMMARIZE…

Pain, as described initially by Descartes, is not just an ascending tract from the periphery to the brain, but a complex interaction of different steps which, will not only transmit but also modulate the nociceptive information that will finally result in the subjective pain sensation.

Sensory information coming from the orofacial region is mostly transmitted through the trigeminal system. It differs anatomically from the rest of the body as the information is directly transmitted mostly to the Vc in the VBSC, whilst in the rest of the organism sensory information is transmitted to spinal cord. Besides this anatomical difference there is emerging data that point out certain differences between them in the physiology of nociceptive transmission.

Although results obtained in basic and preclinical studies cannot be translated directly to the clinical setting they do serve to study the physiology of pain transmission and as a result develop new drugs for its treatment. There are multiple and different animal and human pain models; the administration of algogen substances is a widespread method for the study of nociception, and HS (i.m. injection) is one of the most used algogen to study acute muscle pain in humans and animals.

The aetiology and physiology, although not fully understood, and clinic of muscle pain is quite different from pain arising from the skin. In the past decades research in muscle pain has increased because it is one of the main causes of disability, and, because of this, new therapeutic approaches are needed.

Opioids have been used since ancient history for the treatment of pain, but also for recreational use. The endogenous opioid system, which is formed by endogenous opioids, receptors for these opioids and enzymes, is involved in multiple physiological functions in the organism, among which is pain modulation. There are three main types of opioid receptors, μ, δ and κ.

In a similar manner, exogenous opiates bind to these opioid receptors, though clinically administered opioids are mainly μ opioid agonists or antagonists. All three receptors are distributed along the pain pathways in both the peripheral and central nervous system, so they can induce analgesia by binding to peripheral, spinal or supraspinal located receptors. Though opioids are very good analgesic options, they

have important side effects, mainly due to their binding to opioid receptors located in the CNS, which can hamper their use.

In the past decades a special interest in research has been the study of drugs which target the peripheral opioid receptors, as peripheral opioids have shown to be effective in pain modulation. To study the implication of these receptors two different approaches can be used:

1. The administration of opioids which do not cross the blood brain barrier, as loperamide.
2. The local administration of low doses of opioids which cross the blood brain barrier, as morphine.

The participation of peripheral opioid receptors in muscle pain has hardly been studied and, if the implication of these receptors in orofacial and spinal muscle pain differs, has yet not been analysed.

As opioids, cannabinoids have long been used for their analgesic properties, though one of their main disadvantages is their psychoactive effects. Exogenous administered cannabinoids bind to the cannabinoid receptors CB_1 and CB_2, although there are a number of other receptors which they can bind to too. CB_1 receptor is mainly expressed in the CNS whilst the CB_2 receptor is preferentially located in peripheral tissues. The psychoactive effects are attributed to the binding of cannabinoids to the CB_1 receptors in the CNS.

In line with opioid research, cannabinoid drugs which lack these CNS effects are being studied, this has can be performed following two approaches:

1. Targeting peripheral CB_1 receptors.
2. Targeting CB_2 receptors, as CB_2 agonists lack this psychoactive effects.

Following these approaches cannabinoids have proven to have an antinociceptive effect without the unwanted side effects in several pain models. But, when the cannabinoid experiments of this thesis were started, only one research paper studied the analgesic effect of cannabinoids in muscle pain and there was no published work on their effects in orofacial muscle pain.

AIMS

The aims of this thesis are divided into:

1. To study the antinociceptive effect of opioid and cannabinoid drugs in a model of orofacial muscular pain *vs* spinally innervated muscular pain

    a. To develop a spinally innervated muscle pain model that permits the comparison between orofacial and spinally innervated muscle pain.

    b. To study the participation of central and peripheral opioid and cannabinoid receptors in these pain models

2. To study the modulation of the perception of pain in two models of muscular orofacial pain in healthy volunteers

The experiments of the first aim were performed at the Department of Pharmacology and Nutrition of the Universidad Rey Juan Carlos, Madrid Spain. The experiments of the second aim were performed at The Center for Sensory-Motor Interaction, Aalborg, Denmark.

ANTINOCICEPTIVE EFFECT OF OPIOIDS AND CANNABINOIDS ON MUSCLE PAIN

METHODS

1. ANIMALS

For all experiments, male Wistar rats, weighing between 250 to 300 g, obtained from the Veterinary Unit of Rey Juan Carlos University were used. Animals were supplied with food and water "ad libitum" and were housed in a temperature-controlled room at 23 ± 1°C under standard 12/12-h light/dark cycle (08:00-20:00 h). All animals were housed in the test room for at least two days before experimentation. The spontaneous behavior of the rats was observed before all experiments and those which presented an abnormal behavior were discarded.

Throughout all experimental procedure, the international ethics standards for pain-inducing experiments in laboratory animals (Zimmermamn, 1983) and the European Communities Council Directive of 24 November 1986 (86/609 EEC, Nov 24, 1986) were followed. All animal procedures were reviewed and approved by the Animal Care and Use Committee of Rey Juan Carlos University.

2. DRUGS

Next, the opioid and cannabinoid drugs used in the experimental procedures of this thesis are listed:

2.1 OPIOIDS

Opioid Agonists:

- Morphine (sulphate salt): a central and peripherally acting opioid (Alcaliber, Madrid, Spain).
- Loperamide Hydrochloride: a peripherally acting opioid (Sigma-Aldrich Química, Madrid, Spain).

Opioid antagonists

- Naloxone hydrochloride: an opioid receptor antagonist that reaches the central nervous system.
- Naloxone methiodide: an opioid receptor antagonist that does not cross the blood-brain barrier.

Both drugs were purchased from Sigma-Aldrich Química, Madrid, Spain.

2.2 CANNABINOIDS

Cannabinoid agonists

- WIN 55,212-2 (WIN): a non-selective CB_1 and CB_2 agonist (Sigma-Aldrich Madrid, Spain).
- ACEA: a selective CB_1 agonist (TOCRIS, Biogen Científica S.L. Madrid, Spain).
- JWH015: a selective CB_2 agonist (Sigma-Aldrich Madrid, Spain)

Cannabinoid antagonists

- AM251: a selective CB_1 antagonist.
- AM630: a selective CB_2 antagonist.

Both drugs were purchased from TOCRIS, Biogen Científica S.L. Madrid, Spain

Morphine, naloxone and naloxone methiodide were dissolved in saline solution (0.9%) and loperamide in 20% Cremophor EL (Sigma) (DeHaven-Hudkins et al., 1999).

Due to the lipophilic character of cannabinoids they were dissolved in ethanol 1 mg: 1 ml and subsequently in ethanol and Tween 80 (1:2) (TE), after which ethanol was evaporated and saline was added to reach the final concentration (Pertwee et al., 1992). To evaluate if the cannabinoid vehicle induced any effect on the HS nociceptive response, the amount required to dissolve the greatest dose of cannabinoid drug was chosen; the same process was followed but without adding the cannabinoid compounds. All cannabinoid drugs were dissolved in TE except for JWH015 (1-5 mg/kg) which was dissolved in Tocrisolve (TCS) (Tocris, S.A.), 1mg/50 µl, because, at these concentrations, JWH015 does not dissolve well in TE. Doses of JWH015, greater than 3 mg/kg, required vortex and sonnication to be dissolved.

3. ANIMAL PAIN MODELS

Most of the experimental procedures performed throughout this thesis have been done in two different muscle pain models. In all of the models pain was induced by HS (5% NaCl). In one of the models pain was induced in a trigeminally innervated muscle, whilst in the other, spinally innervated muscles were used (gastrocnemius and triceps).

3.1 MASSETER PAIN MODEL

The behavioral assessment of craniofacial muscle nociception was performed in lightly anesthetised rats as previously described (Ro et al., 2003; Ro, 2003). Animals

were anesthetized with Equitesin (3 ml/kg intraperitoneal (i.p.), chloral hydrate 2.1 g, sodium pentobarbital 0.46 g, $MgSO_4$ 1.06 g, propylene glycol 21.4 ml, ethanol (90%) 5.7 ml, H_2O 23 ml) and the hair over the masseter muscle was carefully shaved. The level of "light" anesthesia was determined by providing a noxious pinch to the tail or the hindpaw with a serrated forceps as previously described (Ro et al., 2003); when animals are maintained under this level of anesthesia they typically respond to the noxious pinch of the tail with an abdominal constriction and to the noxious pinch of the hindpaw, with a withdrawal reflex within 30 min after the initial anesthesia. Experiments were continued only after the animals showed reliable reflex responses to every noxious pinch. A constant level of anesthesia was maintained by the administration of approximately 0.2 ml every 30 min.

The ipsilateral hindpaw shaking behavior induced by HS stimulation of the masseter muscle is accepted as an index of muscle nociception (Ro et al., 2003). 50 and 100 µl of HS were administered into the mid-region of the right masseter muscle via a 30-gauge cannula (Fig. 6). To minimize the effects of the insertion of the cannula on the hindpaw shaking behavior, it was inserted into the masseter muscle 10 min prior to the injection of HS. The cannula consisted of a 30-gauge needle connected to a PE10 tube. HS was manually infused through the cannula with an insulin syringe over a 10 second period. The nociceptive behavior after intramuscular injection of HS was quantified by counting the total number of shakes over a 2.5 min period. Experiments were recorded on video and then played back in slow motion to count the number of shakes. This was performed by only one experimenter to maintain consistency.

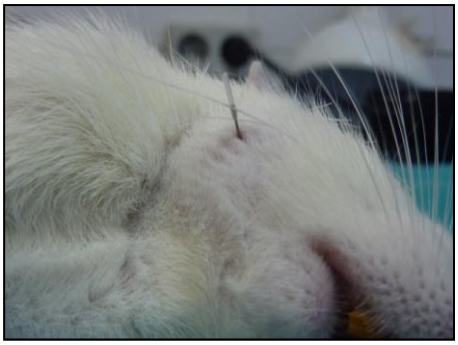

Figure 6. Cannula placed in the masseter muscle of the rat.

Then we tested if this behavioral response was reproducible, this was done by infusing of 100 µl of HS into the masseter 2 times separated each by an interval of 30 min.

3.2 GASTROCNEMIUS AND TRICEPS PAIN MODELS

To develop this model in the most similar way as the masseter pain one, HS was injected in the muscle of anesthetized rats, but as HS did not induce any visible changes, these pain models had to be performed on conscious rats. Injection of HS in the mid-region of the gastrocnemius or triceps, in conscious rats, evokes a nociceptive behavior which consists on withdrawal or flexing of the affected paw.

To inject HS the rat was immobilized by one person with a cloth while another injected HS with an insulin syringe in the mid-region of the muscle. To evaluate the nociceptive behavior the animal was positioned on an elevated acrylic board, covered by a plexiglas box and two mirrors were positioned underneath and behind it to permit unobstructed viewing of the paw. The time (s) that the animals remained with the paw flexed or withdrawn was measured for up to 5 min.

To validate this model, 3 different groups of rats were tested:

1. Sham group: received a pinch in the gastrocnemius or triceps with the insulin syringe.
2. Control group: 0.5 ml of saline solution were injected into the gastrocnemius or triceps.
3. Experimental groups: 3 different groups of rats received each a different volume of HS (0.1, 0.25 and 0.5 ml) into the gastrocnemius or triceps.

To test if this model could be reproduced, three injections of 0.5 ml of HS into the gastrocnemius were performed. All injections were separated by intervals of 30 min.

4. PROTOCOL

Each pharmacological group, throughout the whole thesis, was formed by at least six animals and each one was used only for one experiment.

Before any experiments with opioids or cannabinoids were started, control experiments were performed to be sure that the nociceptive behavior was due to HS injection and not to animal manipulation. A control group was done, injecting saline solution i.m. (masseter, triceps and gastrocnemius). Once this was evaluated, saline and vehicles (i.p. and i.m.) were tested to be sure they did not modify the nociceptive response induced by the injection of HS.

All drugs were administered i.p. with a volume of 1.5 ml/kg, except when studying the peripheral effects of cannabinoids: they were administered i.m. through the injection cannula with a volume of 30 µl (masseter) or 100 µl (gastrocnemius).

4.1 EVALUATION OF THE ANTINOCICEPTIVE EFFECT OF OPIOIDS.

4.1.1 Opioid antinociceptive effect on masseter

To evaluate the systemic effect of opioids, rats were anesthetised as previously described and then, morphine and loperamide were injected i.p. 30 min before HS infusion in separate groups of rats. To antagonise the effect of these drugs, naloxone and naloxone methiodide were injected, as described in Fig 7.

The effect of opioid agonists and antagonists can be seen on Table 3:

MASSETER			
OPIOID TREATMENT		**DOSES(mg/kg)**	**NUMBER OF ANIMALES PER GROUP**
Agonists	Morphine	0.6, 1.25, 2.5	N=7 per group
	Loperamide	0.6, 1.25, 2.5	N=7 per group
Antagonists	Naloxone	1	N=7 per group
	Naloxone methiodide	1	N=7 per group
Antagonists + Agonists	Naloxone + Morphine	0.5 + 1.25	N=10
		1 + 1.25	N=8
		2 + 1.25	N=10
	Naloxone + Loperamide	0.5 + 1.25	N=6
		1 + 1.25	N=7
	Naloxone methiodide + Morphine	0.5 + 1.25	N=6
		1 + 1.25	N=6
		2 + 1.25	N=6
	Naloxone methiodide + Loperamide	0.5 + 1.25	N=9
		1 + 1.25	N=9

Table 3. Opioid agonists and antagonists and their respective doses tested in the masseter muscle.

4.1.2 Opioid antinociceptive effect on Gastrocnemius and triceps

The protocol of opioid administration was the same as in the masseter pain model. A schematic figure of this protocol can be seen in Figure 7.

The effect of opioid agonists and antagonists were tested in the following experimental groups (Table 4):

OPIOID TREATMENT		GASTROCNEMIUS DOSES (mg/kg)	TRICEPS DOSES (mg/kg)	NUMBER OF ANIMALES PER GROUP
Agonists	Morphine	2.5, 5, 10	2.5, 5, 10	N=6 per group
	Loperamide	5, 10	5, 10	N=6 per group
Antagonists	Naloxone	1	1	N=6
	Naloxone methiodide	1	1	N=6
Antagonists + Agonists	Naloxone + Morphine	1 + 10	1 + 10	N=6
	Naloxone methiodide + Morphine	1 + 10	1 + 10	N=6

Table 4. Opioid agonists and antagonists and their respective doses tested in the gastrocnemius and triceps muscles

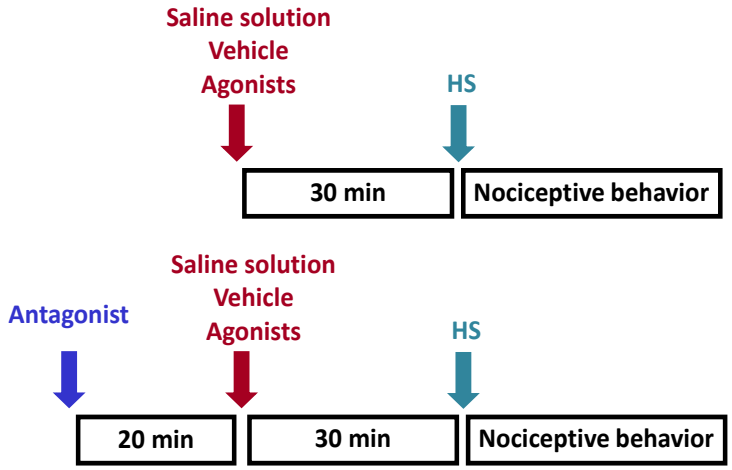

Figure 7. Timeline of systemically administered opioid drugs in the masseter, triceps and gastrocnemius.

4.1.3 Tail-flick procedures

The tail-flick test is widely used to evaluate the antinociceptive effect of pharmacological agents (D'amour and Smith, 1941). It is a test of acute nociception which consists on a high-intensity thermal stimulus directed to the tail of the rat; this provokes a simple spinally mediated nociceptive reflex: a flick/withdrawal of the tail away from the heat source. The tail flick latency was measured by recording the time that passed from the onset of the heat stimulus until withdrawal of the tail from the heat source using a standard tail-flick apparatus (Analgesic-Meter LI7106, Letica

Scientific Instruments). The intensity of the heat source was adjusted to induce tail-flick latencies of 2-4 s; to prevent tissue damage cut-off time for each measurement was set at 10 s. Radiant heat was focused approximately 5 cm from the tip of the tail.

Data from the tail-flick test are expressed as the percentage of maximum possible effect (% M.P.E.).

% M.P.E. = (test-baseline)/(cut-off-baseline) x 100,

where test is the latency to response after treatment, baseline is the latency to response prior to treatment, and cut-off is the preset time at which the test ends in the absence of a response.

This test was performed in two different groups of rats: conscious and anesthetized rats. Conscious rats had to be lightly restrained in a plexiglas tube, and were accustomed to the tube two days before the experiment. Rats were anesthetized with Equitesin as described for the masseter pain model.

The protocol of drug administration was the same as in the previous muscle pain models. The effect of opioid agonists and antagonists were tested in the following experimental groups (Table 5):

		CONSCIOUS RATS	**ANESTHETISED RATS**	
OPIOID-TREATMENT		**DOSES (mg/kg)**	**DOSES (mg/kg)**	**NUMBER OF ANIMALES PER GROUP**
Agonists	Morphine	2.5, 5, 10	2.5, 5, 10	N=6 per group
	Loperamide	5, 10	5, 10	N=7; N=8
Antagonists + Agonists	Naloxone + Morphine	0.5 + 10	1 + 10	N=6 per group
		1 + 10		
	Naloxone methiodide + Morphine	1 + 10	1 + 10	N=6 per group
		2 + 10		

Table 5. Opioid agonists and antagonists and their respective doses tested in the tail flick test.

4.2 EVALUATION OF THE CANNABINOID ANTINOCICEPTIVE EFFECT.

The antinociceptive effect of cannabinoid drugs was tested in the masseter and gastrocnemius muscles.

4.2.1 Antinociceptive effect of cannabinoids on masseter

4.2.1.1 Systemic administration of cannabinoids

To evaluate the systemic effect of cannabinoids all agonists were administered i.p. 30 min before HS infusion, except for ACEA which was administered 15 min before. To antagonise the effect of the agonists all antagonists were administered 20 min before agonists (Fig.9).

Systemically administered cannabinoids were tested in the following experimental groups (Table 6):

		MASSETER	
CANNABINOID TREATMENT		**DOSES (mg/kg)**	**NUMBER OF ANIMALES PER GROUP**
Agonists	WIN 55,212-2	0.25, 0.5, 1, 3	N=7, N=14, N=24, N=10
	ACEA	0.125, 0.5, 1, 3	N=7, N=9, N=9, N=14
	JWH015	1, 3, 5	N=6, N=12, N=11
Antagonists	AM251	0.5	N=7
	AM630	0.5	N=9
Antagonists + Agonists	AM251 + WIN 55,212-2	0.5 + 1	N=7
	AM251 + ACEA	0.5 + 1	N=7
	AM630 + WIN	0.5 + 1	N=7
	AM630 +JWH015	0.5 + 5	N=7

Table 6. Cannabinoid agonists and antagonists (i.p.) and their respective doses tested in the masseter muscle

4.2.1.2 Peripheral administration of cannabinoids

To determine the antinociceptive effect of cannabinoids, these drugs were administered locally (i.m.) through the cannula.

The antinociceptive effect of WIN was tested at two times: 30 and 5 min before HS. The rest of the cannabinoid agonists (ACEA and JWH015) were infused 5 min before HS injection. To antagonise the effect of the agonists, antagonists were administered 5 min before the agonists through the same cannula (Fig. 8).

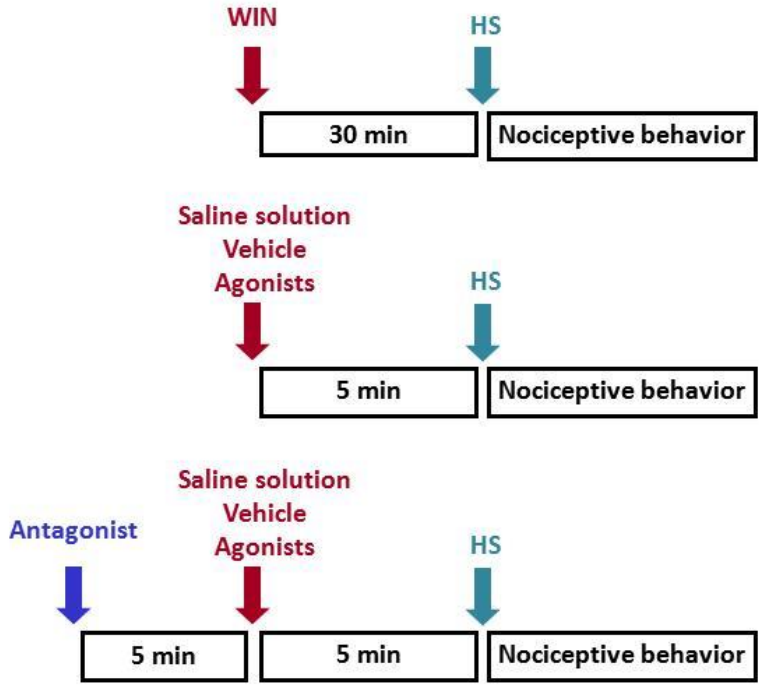

Figure 8. Timeline of peripherally administered cannabinoid drugs in the masseter.

Cannabinoid drugs were tested in the following experimental groups (Table 7):

MASSETER			
CANNABINOID TREATMENT		**DOSES (mg/kg)**	**NUMBER OF ANIMALES PER GROUP**
Agonists	WIN 55,212-2	0.05, 0.1, 0.2 (30 min)	N=7, N=15, N=11
		0.0125, 0.01285, 0.025, 0.05 (5 min)	N=6, N=6, N=7, N=6
	ACEA	0.00625, 0.0125, 0.025	N=7, N=7, N=6
	JWH015	0.025, 0.05	N=7, N=6
Antagonists	AM251	0.02	N=6
	AM630	0.02	N=6
Antagonists + Agonists	AM251 + WIN 55,212-2	0.01+0.0185	N=7
		0.02 + 0.0185	N=6
	AM251 + ACEA	0.02 + 0.0125	N=7
		0.04 + 0.0125	N=7
		0.5 (i.p.) + 0.0125	N=7
	AM630 + WIN	0.02 + 0.0185	N=6
	AM630 + JWH015	0.02 + 0.05	N=6

Table 7. Cannabinoid agonists and antagonists (i.m.) and their respective doses tested in the masseter muscle

4.2.2 Cannabinoid Antinociceptive effect on Gastrocnemius

4.2.2.1 Systemic administration of cannabinoids

The systemic administration of drugs was performed through the same route (i.p.), timing and volumes as in the masseter model (Fig. 9).

Systemically administered cannabinoids were tested in the following experimental groups (Table 8):

GASTROCNEMIUS			
CANNABINOID TREATMENT		**DOSES (mg/kg)**	**NUMBER OF ANIMALES PER GROUP**
Agonists	WIN 55,212-2	0.5, 1, 3	N=6 per group
	ACEA	0.5, 1	N=6, N=8
	JWH015	3, 5	N=6 per group
Antagonists	AM251	0.5	N=6
	AM630	0.5	N=6
Antagonists + Agonists	AM251 + WIN 55,212-2	0.5 + 1	N=6
	AM251 + ACEA	0.5 + 0.5	N=6
	AM630 + WIN 55,212-2	0.5 + 1	N=6

Table 8. Cannabinoid agonists and antagonists (i.p.) and their respective doses tested in the gastrocnemius.

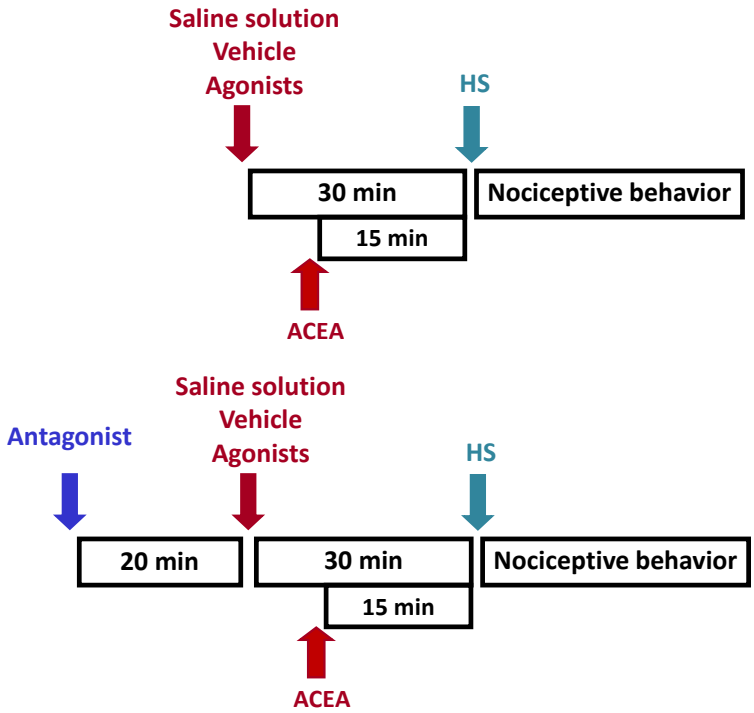

Figure 9. Timeline of systemically administered cannabinoid drugs in the masseter and gastrocnemius muscles

4.2.2.2 Peripheral administration of cannabinoids

To evaluate the antinociceptive effect of cannabinoids administered locally, they were administered with an insulin syringe i.m. 5 min before HS. The area where the drug was administered was marked with a marker so HS could be injected in the same place. Antagonists were administered 5 min before the agonists (Fig. 10).

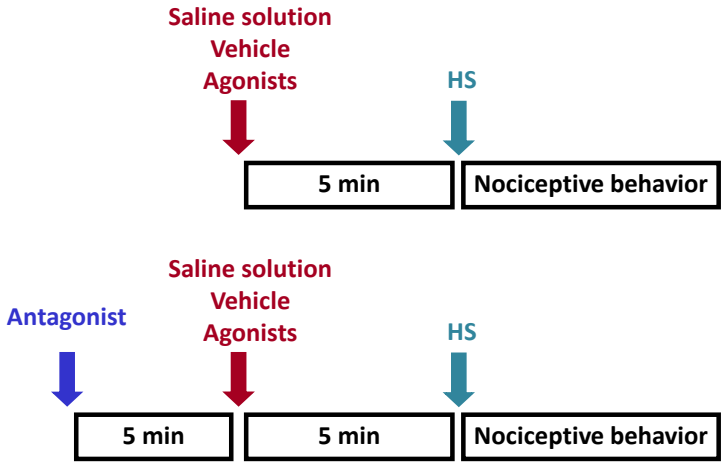

Figure 10. Timeline of peripherally administered cannabinoid drugs in the gastrocnemius muscle.

Cannabinoids were locally administered in the following group of rats:

GASTROCNEMIUS			
CANNABINOID TREATMENT		**DOSES (mg/kg)**	**NUMBER OF ANIMALES PER GROUP**
Agonists	WIN 55,212-2	0.0125, 0.05, 0.1	N=6 per group
	ACEA	0.0125, 0.025, 0.05	N=6 per group
	JWH015	0.0125, 0.05	N=6 per group
Antagonists	AM251	0.02	N=6
	AM630	0.04	N=6
Antagonists + Agonists	AM251 + WIN 55,212-2	0.02 + 0.05	N=6
	AM251 + ACEA	0.02 + 0.0125	N=6
	AM630 + WIN 55,212-2	0.02 + 0.05	N=6
		0.04 + 0.05	N=6
	AM630 + JWH015	0.02 + 0.0125	N=6
		0.04 + 0.0125	N=6

Table 9. Cannabinoid agonists and antagonists (i.m.) and their respective doses tested in the gastrocnemius muscle.

4.2.3 Cannabinoid tetrad

To evaluate possible psychoactive effects of cannabinoids, the cannabinoid tetrad was performed in rats treated with WIN (Pertwee, 1997). 20 min after WIN administration the following four parameters were measured (Fig. 11).

Body temperature: rectal temperatures in the rat were measured before and after cannabinoid treatment using a P6 thermometer and a lubricated rectal probe (Cibertec, Spain) inserted into the rectum at a constant depth of 5 cm.

Antinociception: pressure pain thresholds at the gastrocnemius muscle were measured as previously described (Schafers et al., 2003). Rats were restrained and the right hindpaw was positioned so that a constant increasing mechanical force was applied to the gastrocnemius muscle using a Randall-Selitto apparatus, (Analgesic-Meter LE7306, Letica Scientific Instruments) until the rat withdrew the hindpaw. The mean of three trials (with an interstimulus interval greater than 30 s) was calculated. Rats were accustomed to be restrained with the cloth for 3 days before behavioral experiments.

Catalepsy: was measured using a modification of the "ring test" (Fox et al., 2001) originally described by Pertwee (Pertwee, 1972). Rats were hung by their front paws from a rubber coated metal ring (12 cm diameter) fixed horizontally at a height allowing their hindpaws to just touch the bench, and the time taken for the rat to move off the ring was measured with a cut-off of 30 s. The mean of five trials was calculated.

Locomotor activity: Spontaneous locomotor activity was evaluated using individual photocell activity chambers (CIBERTEC, Spain). Rats were placed in the recording chambers (55×40 cm, spacing between beams 3 cm) after drug administration and starting 2 min later. The number of interruptions of photocell beams was recorded over a 30-min period.

As it had been done in the muscle pain models, first a control groups treated with saline solution and TE were tested, then the effect of WIN 0.5 and 1 mg/kg was evaluated and compared to the TE treated group.

All tests were performed following the same sequence: temperature, nociception, catalepsy and locomotor activity. The first three tests took about twenty min so the locomotor activity was evaluated 40 min after drug administration.

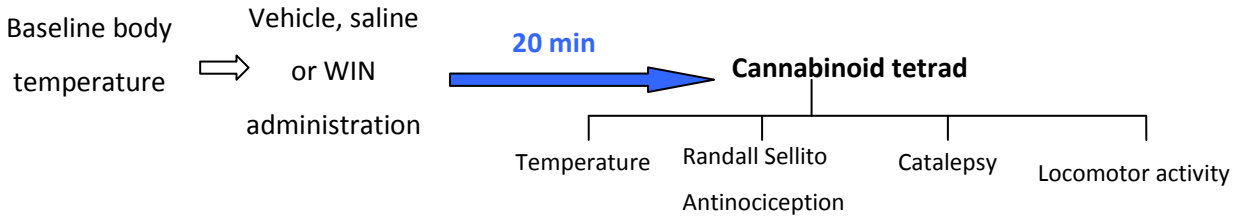

Figure 11. Timeline of the parameters evaluated in the cannabinoid tetrad.

5. STATISTICAL ANALYSIS

Statistical analyses of all data were carried out with One-way analysis of variance (ANOVA), followed by Newman-Keuls post hoc analysis using the Prism program (GraphPad software). In all statistical comparisons, $p < 0.05$ was used as the criterion for statistical significance. All data are expressed as the means ± SEM. Groups treated with agonists were compared to their respective vehicle treated group. The values obtained from the groups treated with antagonist and agonist, were compared to those of treated with the agonist alone.

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RESULTS

1. IMPLEMENTATION OF THE PAIN MODELS

1.1 MASSETER MUSCLE PAIN

Animals maintained on light anesthesia showed no significant hindpaw shaking behavior.

Different volumes of HS, 50 and 100 µl, were tested in separate groups of rats. Both volumes induced a similar pattern of nociceptive response and no statistical differences were found between them. Both volumes induced numerous fast hindpaw shakes that persisted for 2 min, with the peak number of shakes occurring during the initial 30 s after the injection (Fig. 12). For further experiments we chose the volume of 100 µl, as described by Ro et al (Ro et al., 2003).

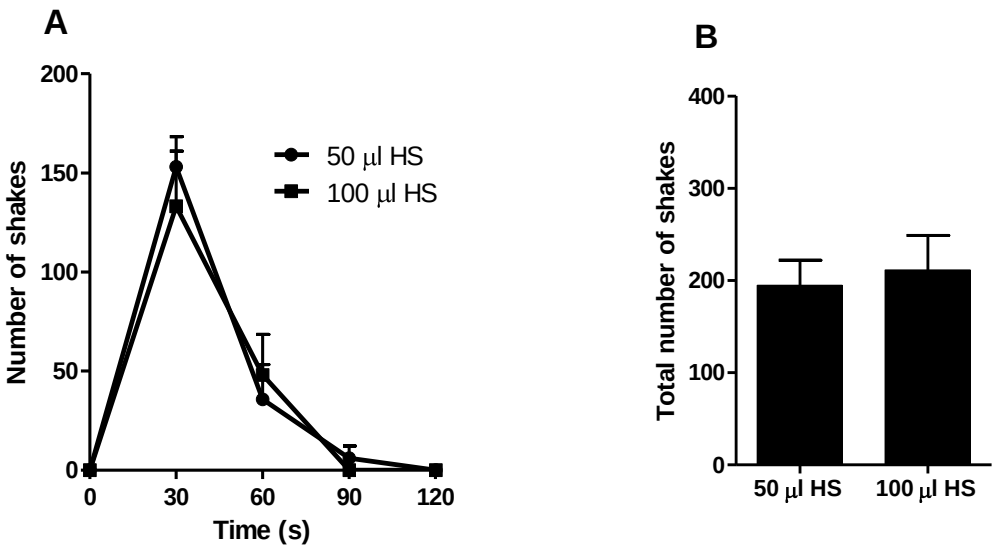


Figure 12. Time course of the hindpaw shaking behavior induced by i.m. injection of HS (A). HS was injected into the masseter muscle in different groups at different volumes (50 and 100 µl). The total number of hindpaw shakes was measured (B). Each point shows the mean ± SEM of paw shakes (n ≥ 6).

To test if this behavior was reproducible we injected 100 µl of HS 2 times, each administration 30 min apart, in the same group of rats and through the same cannula; the response to the second injection of HS was similar to the first one and no statistically significant differences were found (Fig. 13).

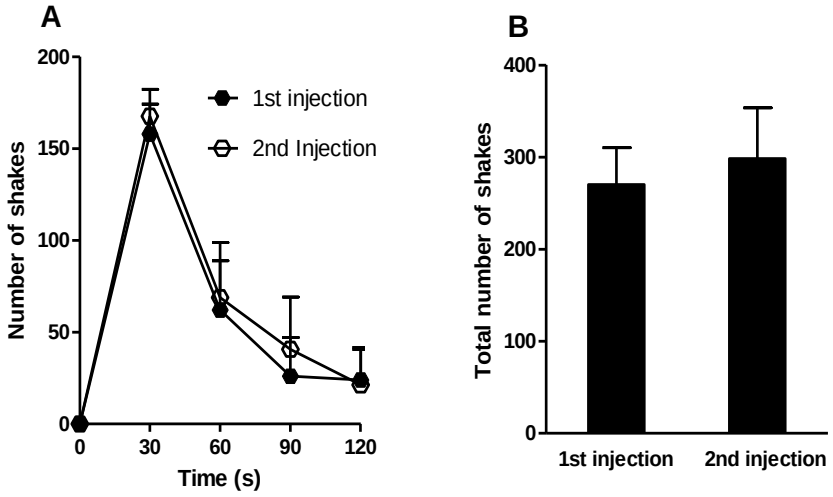


Figure 13. Time course of the hindpaw shaking behavior induced by two i.m. injections of 100 µl of HS in the same group of rats and reproducibility of the nociceptive response (A). HS was injected into the same masseter muscle twice at 30-min interval. The total number of hindpaw shakes was measured (B). Each point shows the mean ± SEM of paw shakes (n ≥ 6).

1.2 GASTROCNEMIUS AND TRICEPS MUSCLE PAIN

To carry out a spinally innervated muscle pain model in the most similar way as the masseter pain one, we tried injecting HS in the gastrocnemius and triceps of anesthetised rats; this did not induce any visible changes in the behavior, so these pain models had to be performed on conscious rats.

The first set of experiments tested whether an injection of 5% HS in the gastrocnemius or triceps muscle was able to cause any nociceptive behavior. Neither the pinch of these muscles (sham group) nor injection of 0.5 ml of saline solution induced any significant nociceptive response. Different volumes of HS (0.1, 0.25 and 0.5 ml) caused withdrawal or flexing of the hindpaw (gastrocnemius) or of the forepaw (triceps). The time (s) rats maintained these behaviors were dependent on the volume of HS injected (Fig. 14).

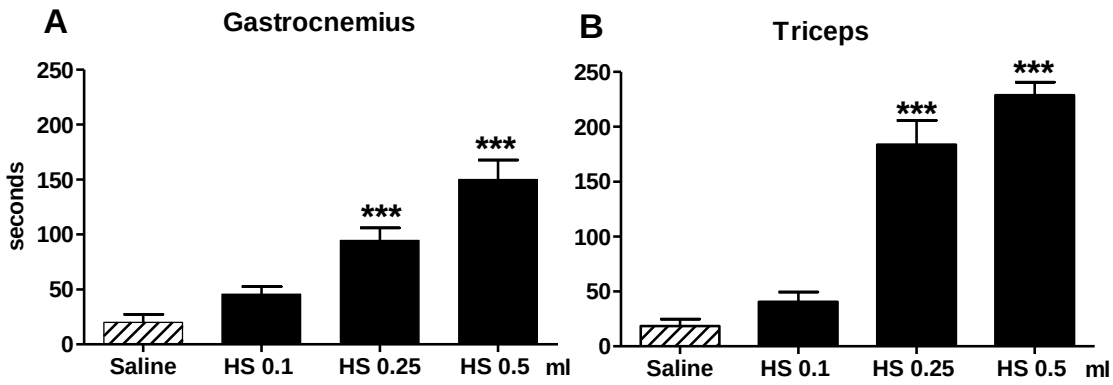


Figure 14. Nociceptive behavior induced by different volumes of hypertonic saline (HS) injected in the gastrocnemius (A) or triceps (B). Bars indicate the mean ± SEM of the time (s) that the rats spent with the injected paw withdrawn or flexed after administration of 0.5 ml of saline solution or different volumes of HS. *** $p < 0.001$ *vs* saline. (One-way ANOVA, n ≥ 6).

To demonstrate the reproducibility of this nociceptive behavior, the maximum volume of HS (0.5 ml) was injected three times, every 30 min, into the gastrocnemius or triceps (Table 10). No statistically significant differences were observed in the behavior obtained after the three injections.

	Gastrocnemius	Triceps
1st injection HS	149.8 ± 21.3	229 ± 9.9
2nd injection HS	179.2 ± 20.9	251 ± 3
3rd injection HS	192.5 ± 11.4	247.3 ± 7.8

Table 10. Time (s) that the animals remained with a lifted or retracted paw after the administration of 0.5 ml of HS three consecutive times at 30 min intervals. Data are presented as the means ± SEM, n ≥ 6.

All further experiments were carried out injecting 0.5 ml of HS in both muscles because this volume permitted us to observe correctly the antinociceptive effect of the drugs tested, was reproducible and did not induce sensitivity.

2. OPIOID ANTINOCICEPTIVE EFFECT

2.1 MASSETER

To evaluate the antinociceptive effect of opioid agonists in the masseter pain model two different types of opioids were used: morphine and loperamide.

The first thing was to assure that the vehicle of the drugs tested did not alter the nociceptive effect of HS. One group of rats was treated with cremophor (the vehicle of loperamide) and another with saline (the vehicle of morphine) 30 min before

HS infusion. I.p. administration of the vehicle did not significantly modify the shaking behavior induced by the i.m. injection of HS (Table 11).

Morphine and loperamide (0.6-2.5 mg/kg) were i.p. administered 30 min before the injection of HS in different groups of rats. Both drugs reduced the number of hindpaw shakes in a dose dependent manner when comparing them to the vehicle treated group (Fig. 15).

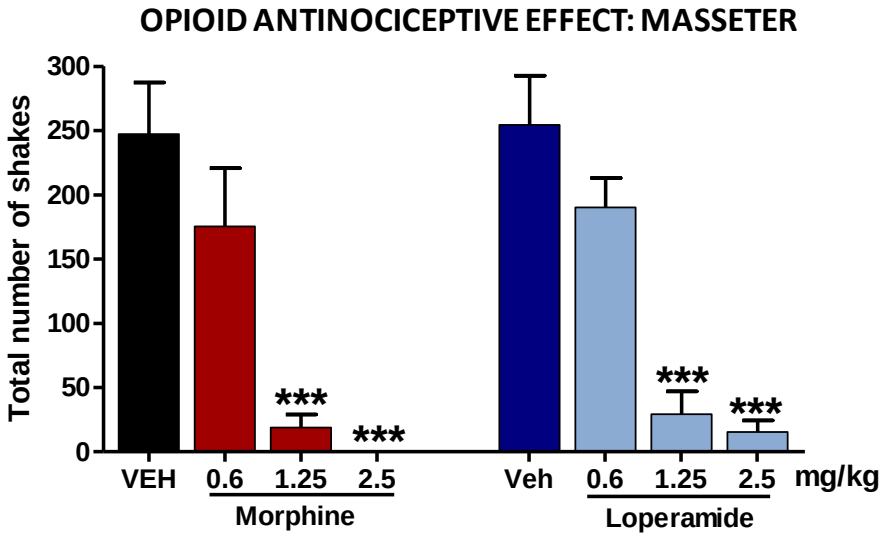


Figure 15. Antinociceptive effect of intraperitoneally administered morphine and loperamide on HS induced nociceptive behavior in the masseter muscle. Bars show the total number of shakes (mean ± SEM). ***p<0.001 *vs* vehicle (VEH). (One way ANOVA, n≥6)

To determine if central or only peripheral receptors were involved in the observed antinociception, naloxone and naloxone methiodide (0.5-2 mg/kg) were administered 20 min before 1.25 mg/kg morphine or loperamide. Naloxone 0.5 mg/kg was capable of antagonising, with similar efficacy, the morphine and loperamide induced antinociception. Naloxone methiodide 0.5 mg/kg reversed the antinociceptive effect of loperamide but not of morphine, when the administered dose of naloxone methiodide was increased to 1 mg/kg it prevented the effect of morphine (Fig. 16).

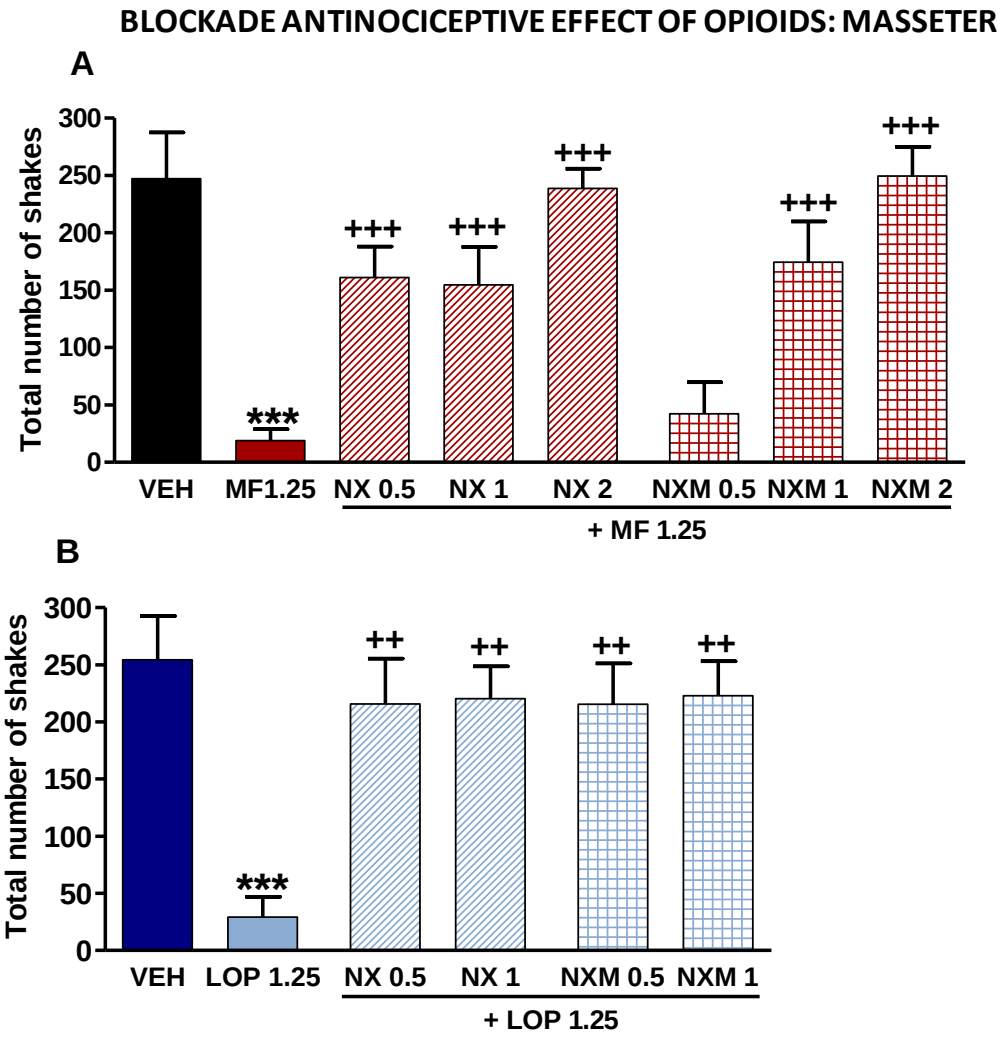


Figure 16. Blockade of the antinociceptive effect of 1.25 mg/kg morphine (MF) (A) and 1.25 mg/kg loperamide (LOP) (B) by naloxone (NX) and naloxone methiodide (NXM) (0.5-2 mg/kg). Bars represent the total number of shakes (mean ± SEM) induced by HS injection into the masseter. *** $p < 0.001$ *vs* vehicle (VEH), ++ $p < 0.01$, +++ $p < 0.001$ *vs* 1.25 MF or 1.25 LOP. (One-way ANOVA, n ≥ 6).

When antagonists were administered alone they did not modify the nociceptive behavior induced by HS (Table 11).

TREATMENT	MEAN NUMBER OF SHAKES
HS injection	219.5 ± 12.7
Saline i.p. + HS i.m.	247.0 ± 40.1
Cremophor i.p. + HS i.m.	253.8 ± 64.4
NX 1 mg/kg i.p. + HS injection i.m.	296.0 ± 63
NXM 1mg/kg i.p. + HS i.m.	328.0 ± 101

Table 11. Nociceptive effect of HS injection on the masseter pain model, after vehicles and antagonist administration.

2.2 GASTROCNEMIUS AND TRICEPS

Once proven that this new model was reliable to measure muscle nociception and could be reproduced, we determined if the administration of the vehicles could alter this nociceptive behavior; neither of them modified significantly the response to HS injection in the gastrocnemius or the triceps muscle (Table 12).

In both models we obtained similar results when morphine and loperamide were administered: morphine (2.5-10 mg/kg) was capable of reducing the time the rat spent with the paw flexed or withdrawn in a dose dependent manner after HS injection, whilst loperamide (5-10 mg/kg) showed no antinociceptive effect in either muscle (Fig. 17).

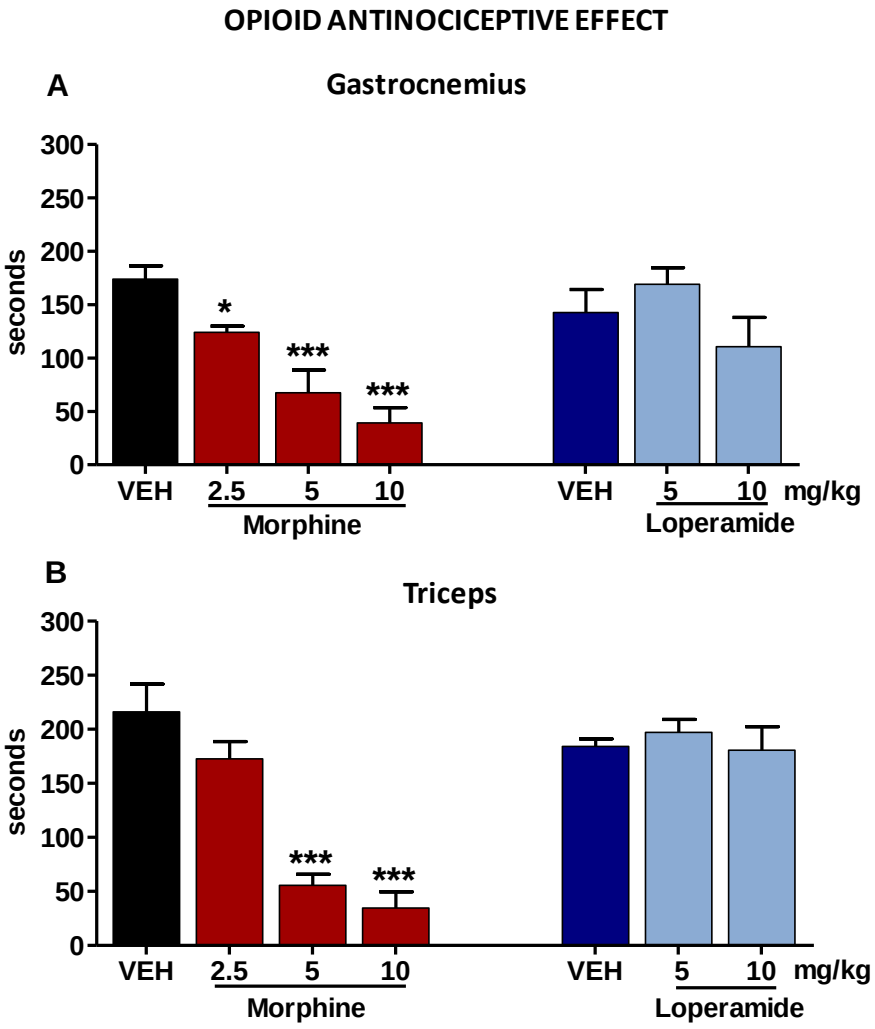


Figure 17. Antinociceptive effect of morphine and loperamide on gastrocnemius (A) and triceps (B) nociceptive behavior induced by 0.5 ml of HS. The effect of morphine (2.5, 5 and 10 mg/kg) and loperamide (5 and 10 mg/kg) is compared to their respective vehicle groups (VEH). Bars show the mean time (s) ± SEM spent with the injected paw withdrawn or flexed. * $p < 0.05$, *** $p < 0.001$ *vs* VEH. (One-way ANOVA, n ≥ 6).

To assess if morphine was acting through peripheral, central or a combination of both receptors its effect was antagonised with naloxone and naloxone methiodide. In both models naloxone but not naloxone methiodide (1 mg/kg) reversed the effect of morphine (10 mg/kg) (Fig 18).

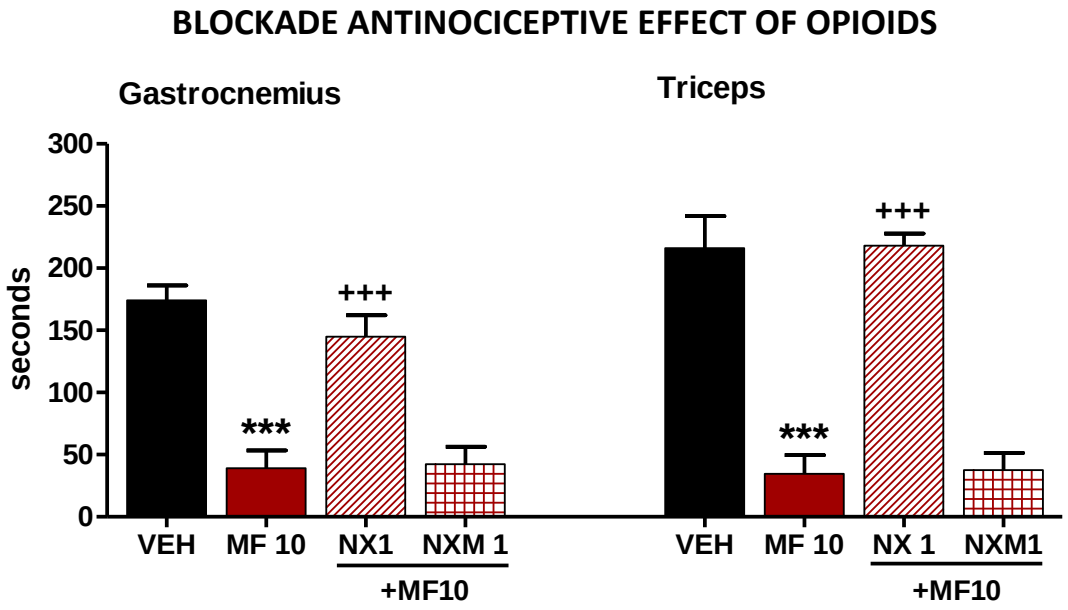


Figure 18. Blockade of the antinociceptive effect of 10 mg/kg morphine (MF) by 1 mg/kg naloxone (NX) or naloxone methiodide (NXM), in gastrocnemius or triceps pain induced by HS. Bars show the mean time (s) ± SEM that rats spent with the injected paw withdraw or flexed. *** $p < 0.001$ *vs* Vehicle (VEH), +++ $p < 0.001$ *vs* MF 10. (One-way ANOVA, n ≥ 6).

As expected, neither of the antagonists (1 mg/kg) modified the nociceptive response induced by HS either in the gastrocnemius or triceps muscle. (Table 12).

TREATMENT	SECONDS	
	Gastrocnemius	Triceps
HS i.m.	149.8 ± 21.3	229 ± 9.9
Saline i.p.+HS i.m.	174 ± 12.2	216 ± 26
Cremophor i.p.+HS i.m.	142.6 ± 21.5	184 ± 7
Naloxone 1mg/kg+HS i.m.	177.5 ± 14.37	241 ± 5.76
Naloxone methiodide + HS i.m.	171.5 ± 16.95	221.4 ± 17.31

Table 12. Time (s) that the animals remained with a lifted or retracted paw after the administration of 0.5 ml of HS in rats treated intraperitoneally with vehicles (saline or cremophor) or antagonists. Data are presented as the means ± SEM, n ≥ 6.

2.3 TAIL-FLICK TEST

The tail-flick test was performed, in both conscious and anesthetized rats, to rule out the possibility that the differences found in the antinociceptive effect of loperamide between the masseter and the gastrocnemius/triceps pain models were due to the anesthetized state of the rats

As had happened in the spinally innervated muscle pain models, morphine (2.5-10 mg/kg) showed an antinociceptive dose dependent effect but loperamide failed to exhibit this effect (Fig. 19).

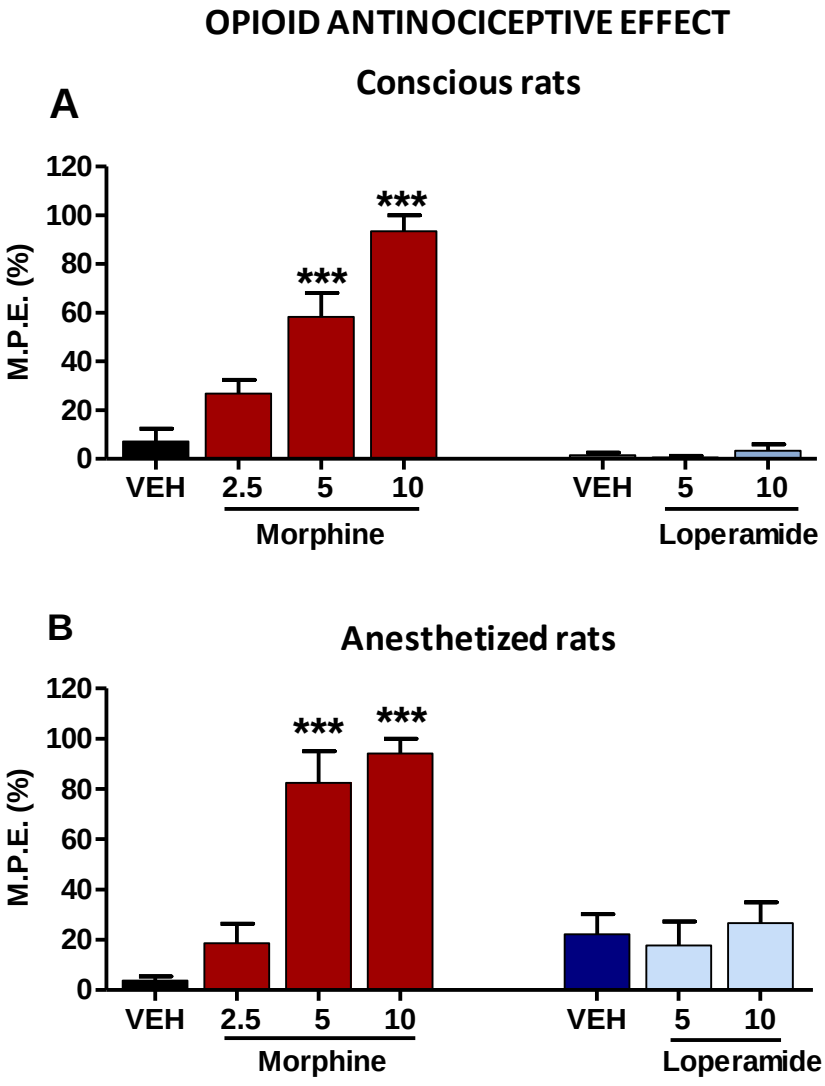


Figure 19. Antinociceptive effect of morphine (2.5, 5 and 10 mg/kg) and loperamide 5 and 10 mg/kg) in conscious (A) and lightly anaesthetized (B) rats in the tail-flick test. Bars show the mean ± SEM of the percentage of the maximum possible effect (M.P.E.). *** $p < 0.001$ *vs.* vehicle (VEH). (One-way ANOVA, $n \geq 6$).

To completely dismiss a possible implication of peripheral receptors in the antinociceptive effect of morphine in this test, naloxone and naloxone methiodide were used. Naloxone (0.5-1 mg/kg), but not naloxone methiodide (1-2 mg/kg), was able to prevent the antinociceptive effect of morphine (10 mg/kg) both in conscious and anesthetised rats (Fig. 20).

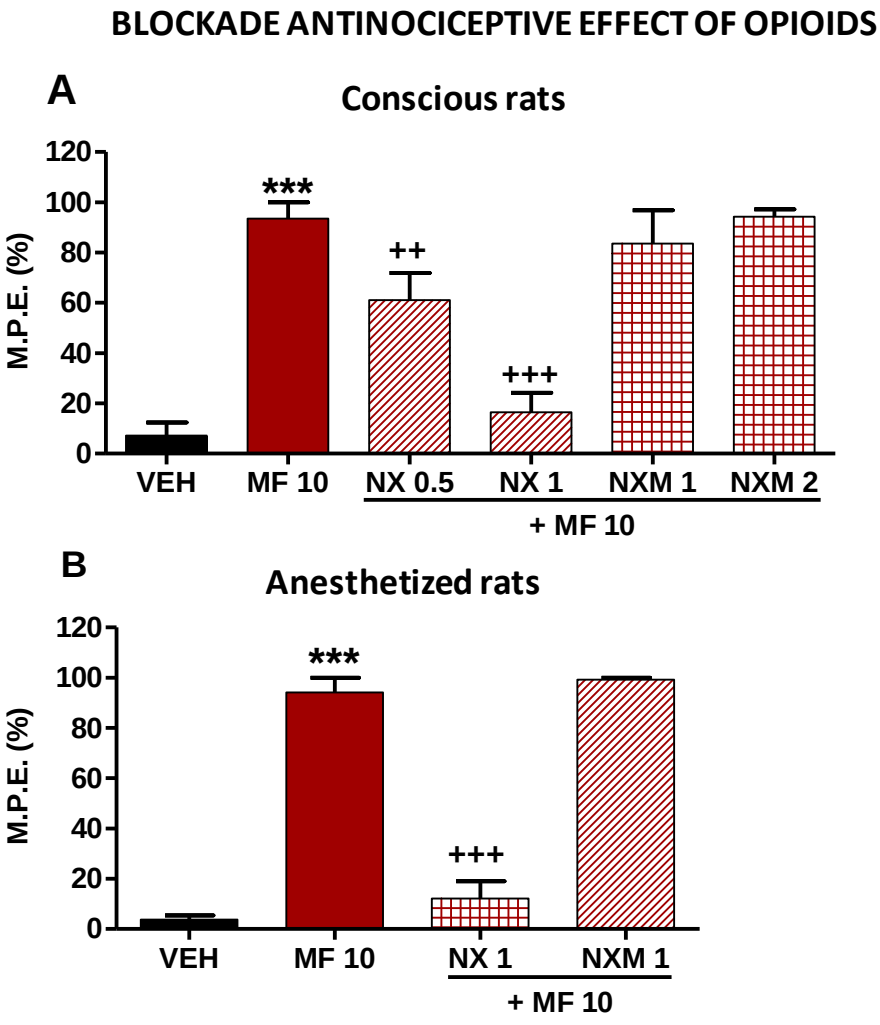


Figure 20. Blockade of the antinociceptive effect of morphine (10 mg/kg MF, i.p.) by different doses of naloxone (NX) (0.5-1 mg/kg) or naloxone methiodide (NXM) (1-2 mg/kg) in conscious (A) and lightly anaesthetised (B) rats evaluated by the tail-flick test. Bars represent the percentage of the maximum possible effect (M.P.E.) (mean ± SEM). *** $p < 0.001$ *vs* vehicle (VEH), ++ $p < 0.01$, +++ $p < 0.001$ *vs* MF 10. (One-way ANOVA, n ≥ 6).

3. CANNABINOID ANTINOCICEPTIVE EFFECT

3.1 MASSETER

These experiments were aimed to evaluate if cannabinoids have any antinociceptive effect in these muscle pain models. The first part consisted on evaluating the antinociceptive effect of systemically administered cannabinoids and in the second, they were administered locally.

3.1.1 Systemic antinociceptive effect of cannabinoids

The first cannabinoid used was WIN 55-212 (WIN) (0.25-3 mg/kg), which is a non-selective cannabinoid agonist. When administering it i.p. 30 min before HS injection, it reduced the number of hindpaw shakes in a statistically significant dose-dependent manner (Fig 21).

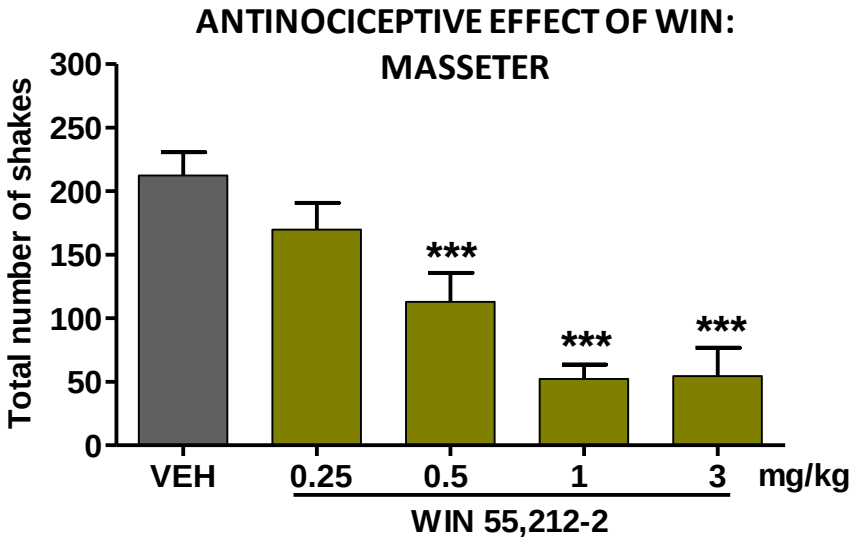


Figure 21. Antinociceptive effect of i.p. administered WIN on HS induced nociceptive behavior in the masseter pain model. Bars show the total number of shakes induced by HS injection in the masseter muscle (mean ±SEM). ***p<0.001 *vs* vehicle (VEH). (One way ANOVA, n≥7)

Since WIN is a non-selective cannabinoid agonist, it displays affinity for both CB_1 and CB_2 receptors (Pertwee, 1999), selective antagonists for each subtype of cannabinoid receptor were administered i.p. 20 min before WIN (1 mg/kg i.p.). Both CB_1 and CB_2 receptor antagonist, AM 251 and AM 630 (0.5 mg/kg i.p.) respectively, were able to prevent the antinociceptive effect of WIN (Fig. 22).

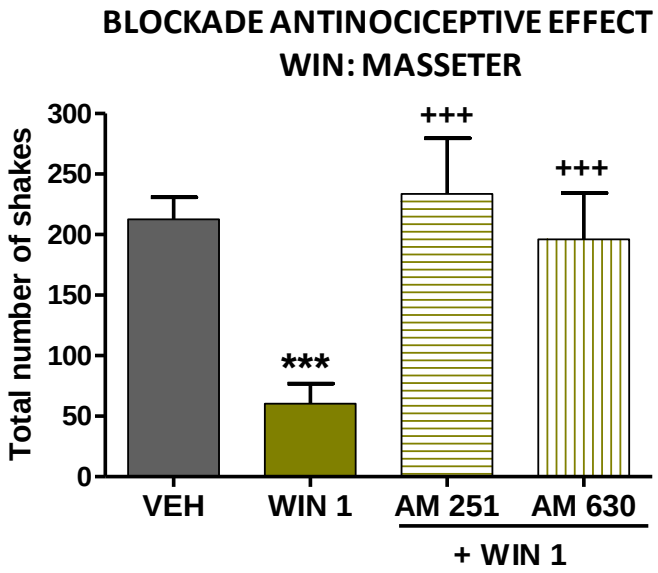


Figure 22. Blockade of the antinociceptive effect of 1 mg/kg WIN by AM251 and AM630 (0.5 mg/kg) on HS induced nociceptive behavior in the masseter pain model. Bars show the total number of shakes induced by HS injection in the masseter muscle (mean±SEM). ***p<0.001 *vs* vehicle (VEH); +++ p<0.001 vs WIN. (One way ANOVA, n≥6)

Neither cannabinoid vehicles nor antagonists (AM 251, AM 630) elicited any significant effect on HS induced nociceptive behavior when they were administered alone (Table 13).

TREATMENT	MEAN NUMBER OF SHAKES
HS i.m.	211±37.5
Saline + HS i.m.	195±21.6
Tween ethanol + HS i.m.	212±18.3
Tocrisolve + HS i.m.	208±34.4
AM251 0.5mg/kg + HS i.m.	202±31.1
AM630 0.5mg/kg + HS i.m.	179±22.7

Table 13. Effect of i.p. administered vehicles and antagonists on HS induced nociceptive behavior in the masseter pain model. Data are presented as the means ± SEM, n ≥ 6.

To further study the implication of CB_1 and CB_2 receptors in this masseter pain model and as a cross-check to the results obtained, CB_1 and CB_2 receptor agonists, ACEA (0.125-3 mg/kg) and JWH015 (1-5 mg/kg) were used. Both agonists displayed a, dose dependent, antinociceptive effect: ACEA was able to reduce the number of hindpaw shakes in a statistically significant way at a dose of 0.5 mg/kg and JWH015 at 5 mg/kg when compared to their respective vehicle groups (Fig. 23).

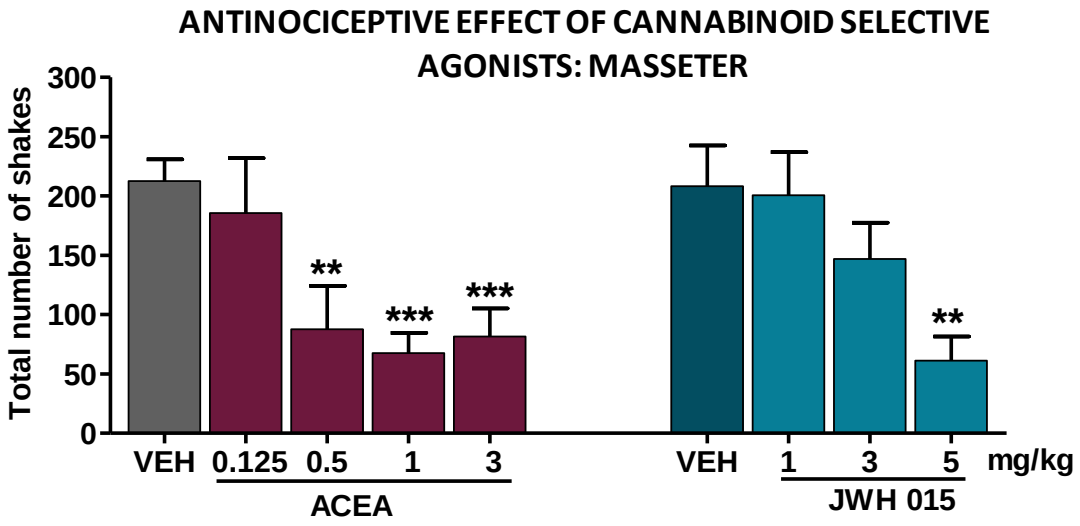


Figure 23. Antinociceptive effect of i.p. administered ACEA and JWH015 on HS induced nociceptive behavior in the masseter pain model. Bars show the total number of shakes induced by HS injection in the masseter muscle (mean ±SEM). ***p*<0.01, ****p*<0.001 *vs* vehicle (VEH). (One way ANOVA, n≥7)

When selective CB_1 and CB_2 antagonists (0.5mg/kg) were administered i.p. 20 min before their respective agonists, they antagonised the antinociceptive effect of 1 mg/kg ACEA and 5 mg/kg JWH 015 (Fig 24).

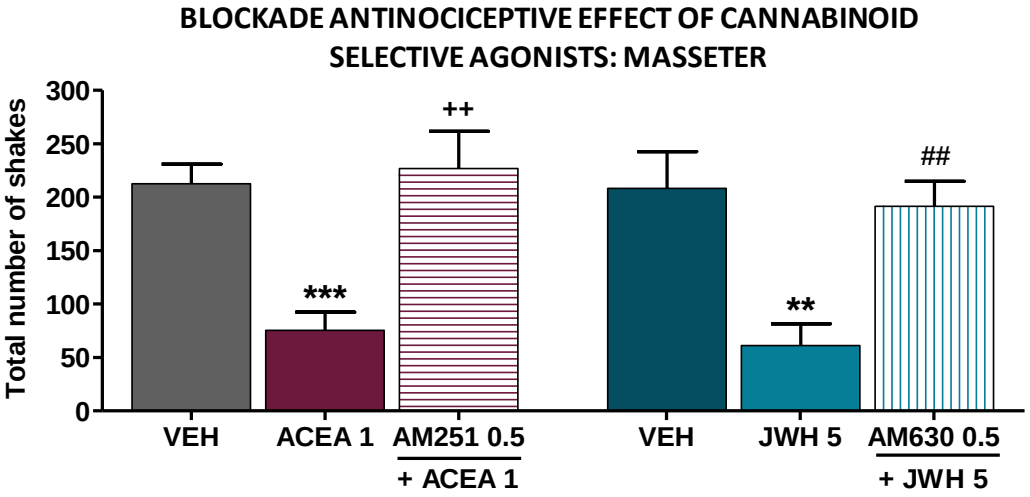


Figure 24. Blockade of the antinociceptive effect of 1 mg/kg ACEA and 5 mg/kg of JWH015 (JWH) on HS induced nociceptive behavior in the masseter muscle. Bars show the total number of shakes induced by HS injection in the masseter muscle (mean ±SEM). ***p*<0.01, ****p*<0.001 *vs* vehicle (VEH); ++ *p*<0.01 *vs* ACEA 1; ##*p*<0.01 *vs* JWH015 5. (One way ANOVA, n≥6)

3.1.2 Peripheral antinociceptive effect of cannabinoids

The next set of experiments was conducted to evaluate if i.m. administered cannabinoids also showed an antinociceptive effect on the HS induced nociceptive behavior.

First of all control experiments were carried out to evaluate if saline and tween ethanol altered the nociceptive effect induced by HS (Table X). In about 36% of the rats there was some degree of hindpaw shaking behavior, which is consistent with the data published by Ro et al., 2003. Of these, 15% of the rats responded with more than 50 shakes, which we considered could conceal a possible antinociceptive effect of the cannabinoid drug, and therefore we took out of the statistical analysis all rats which responded to the vehicle or cannabinoid administration with more than 50 hindpaw shakes.

When WIN (0.05-0.2mg/kg) was administered 30 min before HS infusion, it reduced the number of hindpaw shakes. Then, WIN was also infused 5 min before HS in different groups of rats and also showed an antinociceptive effect (Fig. 25).

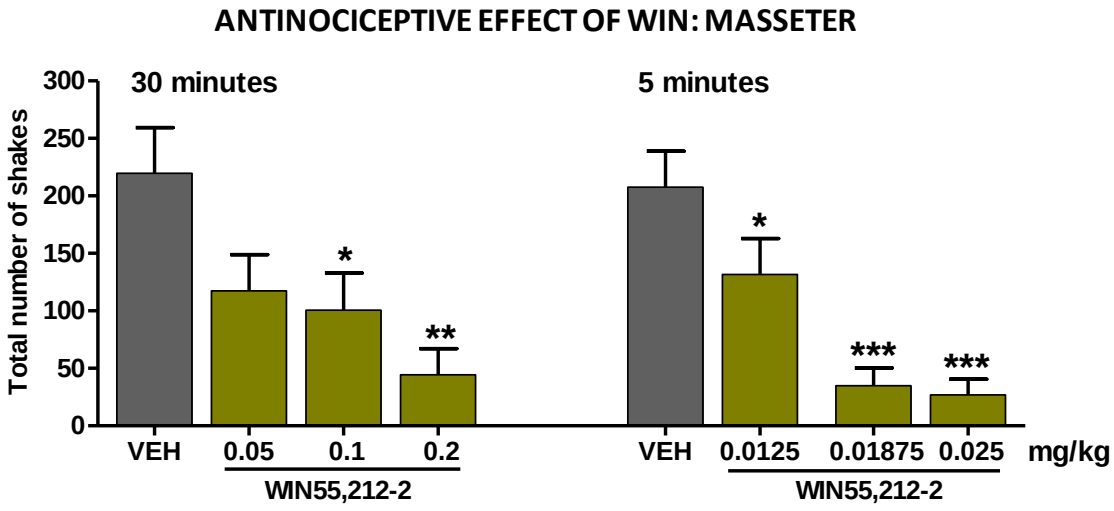


Figure 25. Antinociceptive effect of i.m. administered WIN 5 and 30 min before HS injection. Bars show the total number of shakes induced by HS injection in the masseter muscle (mean±SEM). **p*<0.05, ***p*<0.01, ****p*<0.001 *vs* vehicle (VEH). (One way ANOVA, n≥7)

Taking into account these results, all future experiments were carried out evaluating the antinociceptive effect of cannabinoid agonists 5 min after their administration.

To elucidate if the effect of WIN was mediated via CB_1 or CB_2 receptor, rats were pretreated with AM251 and AM630 (0.01-0.02 mg/kg) i.m. before WIN (0.01875 mg/kg). Only the dose of 0.2 mg/kg antagonized the effect of WIN (Fig. 26).

Antagonists when administered alone did not modify the nociceptive response to HS (Table 14).

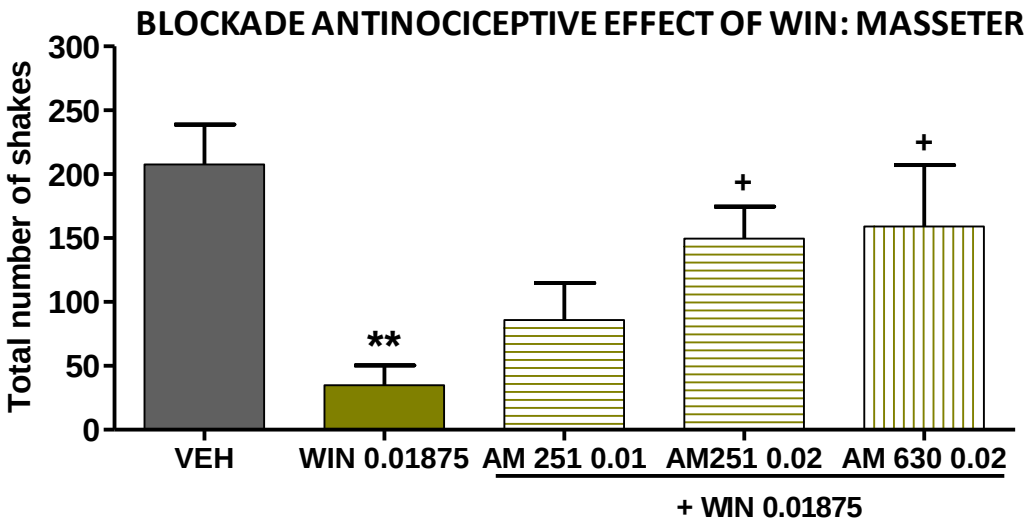


Figure 26. Blockade of the antinociceptive effect of WIN 0.01875 mg/kg i.m. on HS induced nociceptive behavior in the masseter pain model. Bars show the total number of shakes induced by HS injection in the masseter muscle (mean ±SEM). **p<0.01 *vs* vehicle (VEH); + p<0.05 *vs* WIN (One way ANOVA, n≥6).

TREATMENT	MEAN NUMBER OF SHAKES
HS i.m.	211.0 ± 37.5
IS i.m. + HS i.m.	189.9 ± 44.8
TE i.m. + HS i.m.	207.5 ± 31.4
AM251 0.02mg/kg. i.m. + HS i.m.	173.7 ± 47.3
AM251 0.04mg/kg. i.m. + HS i.m.	188.4 ± 40.6
AM251 0.5mg/kg i.p. + HS i.m.	202 ± 31.1
AM630 0.02mg/kg i.m. + HS i.m.	171.2 ± 53.8

Table 14. Effect of i.m. administered vehicles and i.m. and i.p. administered antagonists on HS induced nociceptive behavior in the masseter pain model. Data are presented as the means ± SEM, n ≥ 6.

When studying the effect of the selective cannabinoids agonists, both ACEA (0.00625-0.025 mg/kg) and JWH015 (0.025-0.05 mg/kg) reduced the number of hindpaw shakes being the effect statistically significant at doses 0.0125 mg/kg for ACEA and 0.025 mg/kg for JWH015 (Fig. 27).

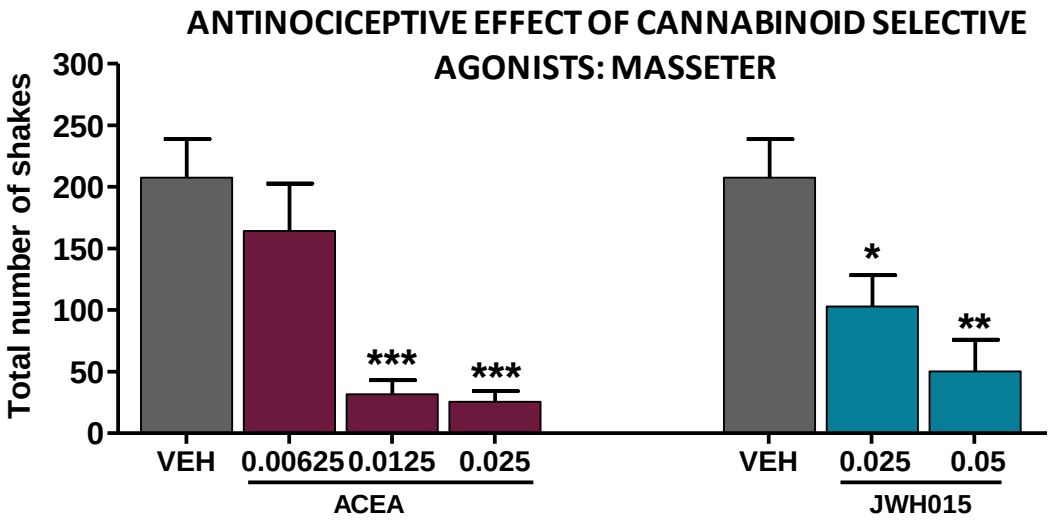


Figure 27. Antinociceptive effect of i.m. administered WIN on HS injection. Bars show the total number of shakes induced by HS injection in the masseter pain model (mean ±SEM). *p<0.05, **p<0.01, ***p<0.001 *vs* Vehicle (VEH). (One way ANOVA, n≥7)

Selective antagonist AM 630 (0.02mg/kg) was capable of preventing the antinociceptive effect of JWH015, but AM251 0.01 mg/kg did not reverse the effect of ACEA. When we increased the dose of AM251 to 0.02 and 0.04 mg/kg, 36 and 43% of the rats, respectively, responded with more than 50 shakes to the injection of the antagonist or agonists. With these results we consider that the antagonism of ACEA cannot be studied locally so, finally, ACEA was reversed by AM251 (0.5 mg/kg) injected i.p. 30 min before the administration ACEA (Fig. 28).

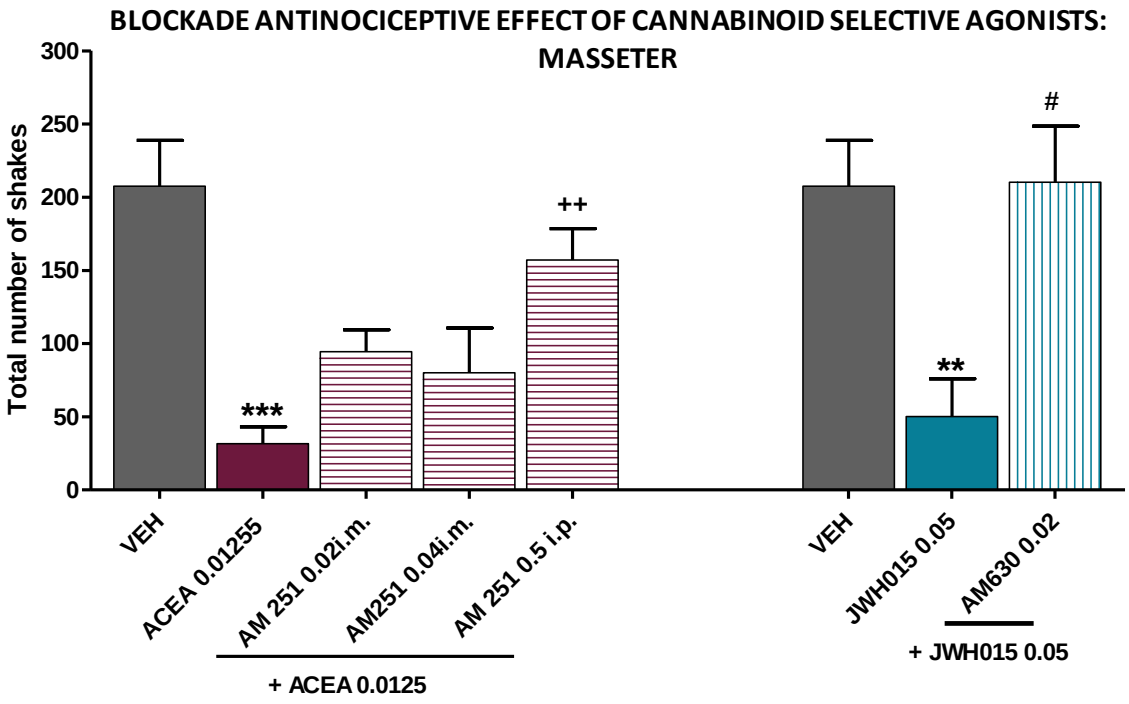


Figure 28. Blockade of the antinociceptive effect of 0.0125 mg/kg ACEA and 0.05 mg/kg of JWH015 on HS induced nociceptive behavior in the masseter pain model. Bars show the total number of shakes induced by HS injection in the masseter muscle (mean ±SEM). **p<0.01, ***p<0.001 *vs* vehicle (VEH); ++ p<0.01 *vs* ACEA; #p<0.05 *vs* JWH015. (One way ANOVA, n≥6).

3.2 GASTROCNEMIUS

3.2.1 Systemic antinociceptive effect of cannabinoids

First of all, saline and the vehicle of cannabinoids were tested to be sure they did not alter the nociceptive effect induced by HS (Table 15).

Then, following the same protocol as in the masseter pain model, WIN (0.5-3mg/kg) was administered 30 min before HS injection in the gastrocnemius muscle. All tested doses significantly decreased the time the animals maintained their paw withdrawn (Fig. 29), and this effect was antagonised by AM251 (0.05 mg/kg), but not by AM630 (Fig. 30).

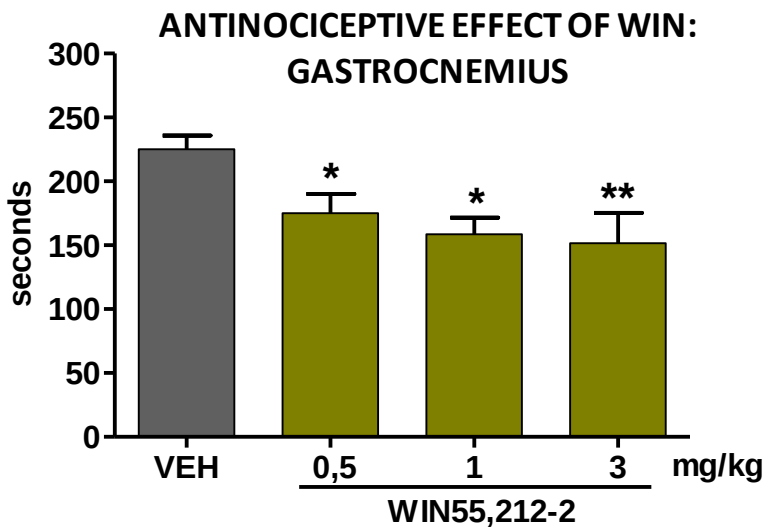


Figure 29. Antinociceptive effect of i.p. administered WIN on HS injection in the gastrocnemius pain model. Bars show the total number of seconds the rat maintained the paw withdrawn after HS injection (mean ±SEM). **p*<0.05, ***p*<0.01 *vs* vehicle (VEH). (One way ANOVA, n≥6).

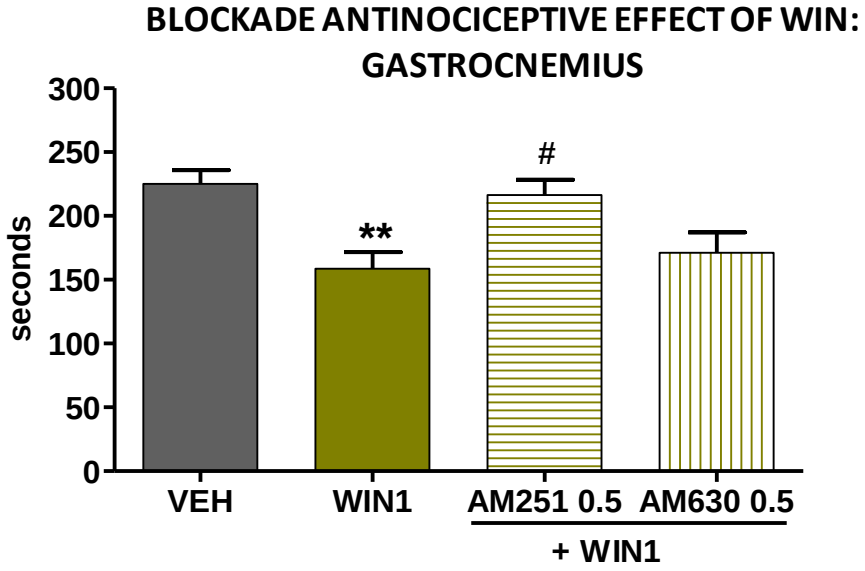


Figure 30. Blockade of the antinociceptive effect of WIN 1 mg/kg on HS induced nociceptive behavior in the gastrocnemius pain model. Bars show the total number of seconds the rat maintained the paw withdrawn after HS injection (mean ±SEM). **p<0.01 *vs* vehicle (VEH); + p<0.05 *vs* ACEA 0.5. (One way ANOVA, n≥6).

When testing cannabinoid selective agonists ACEA (0.5-1 mg/kg) and JWH015 (3-5 mg/kg), only CB_1 receptor agonist, ACEA, reduced the nociceptive behavior induced by HS injection (Fig. 31). As could be expected this effect was reversed with its selective antagonist AM251 (0.5mg/kg) (Fig. 32).

AM251 administered alone had no effect over the HS induced nociceptive behavior (Table 14).

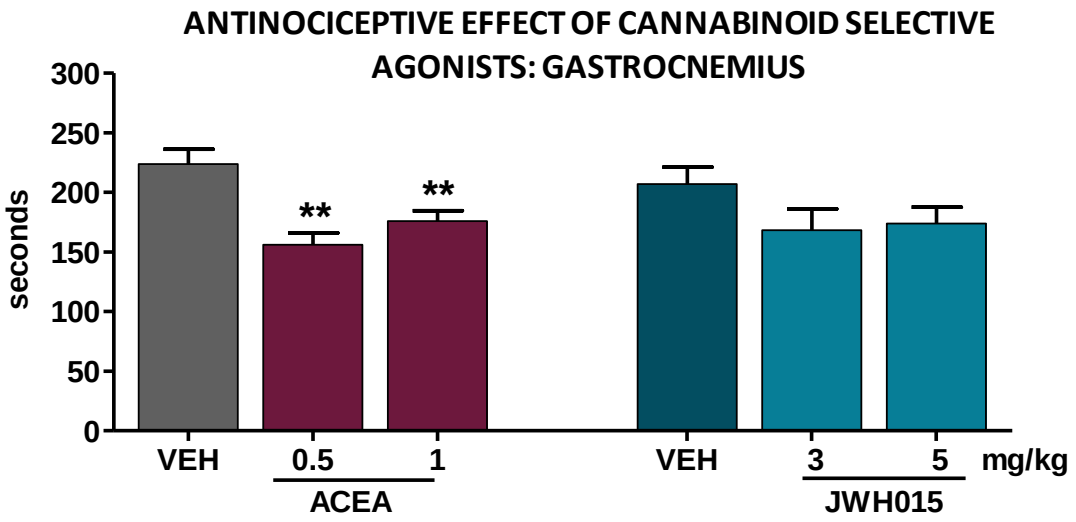


Figure 31. Antinociceptive effect of i.p. administered ACEA and JWH015 on HS injection. Bars show the total number of seconds the rat maintained the paw withdrawn after HS injection (mean ±SEM). ***p*<0.01 *vs* vehicle (VEH). (One way ANOVA, n≥6).

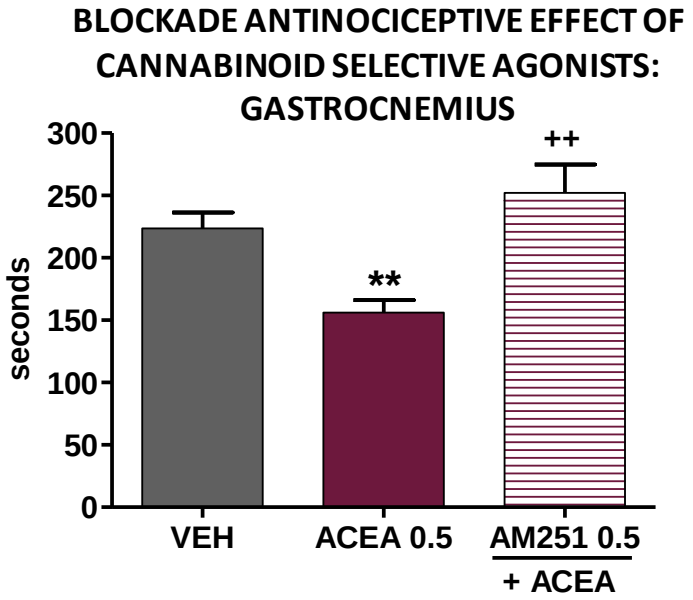


Figure 32. Blockade of the antinociceptive effect of ACEA 0.5 mg/kg on HS induced nociceptive behavior in the gastrocnemius pain model. Bars show the total number of seconds the rat maintained the paw withdrawn after HS injection (mean ±SEM). **p<0.01 *vs* vehicle (VEH); ++ p<0.01 *vs* ACEA 0.5. (One way ANOVA, n≥6).

TREATMENT	MEAN NUMBER OF SECONDS
HS i.m.	221.3±21.8
Saline + HS i.m.	211.1±11.5
Tween ethanol + HS i.m.	223.7±12.6
Tocrisolve + HS i.m.	206.9±14.3
AM251 0.5mg/kg + HS i.m.	220.2±19.1

Table 15. Effect of i.p. administered vehicles and antagonists on HS induced nociceptive behavior in the gastrocnemius pain model. Data are presented as the mean time that the animals remained with the paw flexed or withdrawn measured for up to 5 min (mean ± SEM, n ≥ 6).

3.2.2 Peripheral antinociceptive effect of cannabinoids

The last experiments were conducted to evaluate whether cannabinoids presented an antinociceptive effect when administered locally in the gastrocnemius pain model.

The administration of cannabinoid vehicle i.m. 5 min before HS did not modify the nociceptive behavior compared to the saline treated group (Table 16).

The i.m. injection of WIN (0.0125-0.1mg/kg) reduced the number of seconds the rat maintained the paw withdrawn or flexed due to HS injection. The maximum antinociceptive effect was reached with WIN 0.05 mg/kg (Fig. 33).

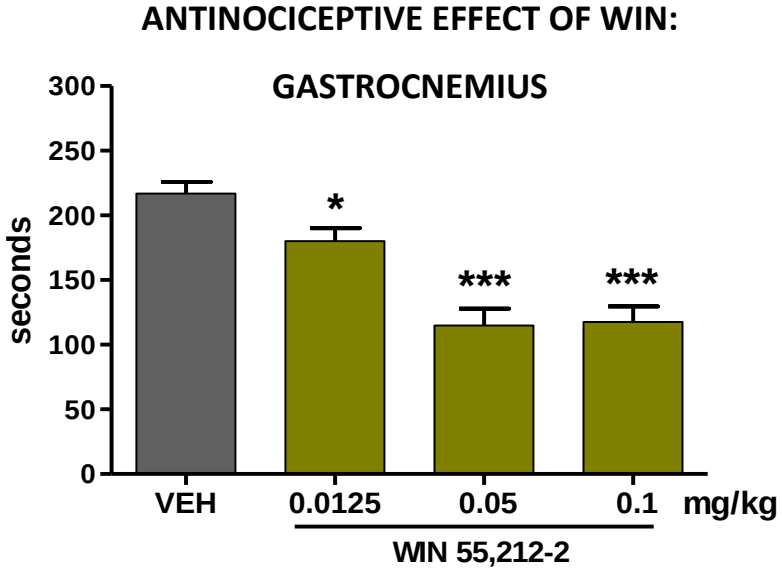


Figure 33. Antinociceptive effect of i.m. administered WIN 5 min before HS injection in the gastrocnemius pain model. Bars show the total number of seconds the rat maintained the paw withdrawn after HS injection (mean ±SEM). *p<0.05, ***p<0.001 vs vehicle (VEH). (One way ANOVA, n≥6).

At the dose of 0.02 mg/kg only AM251 reversed the effect of WIN, the dose of AM630 had to be increased to 0.04 mg/kg to antagonize this effect (Fig. 34).

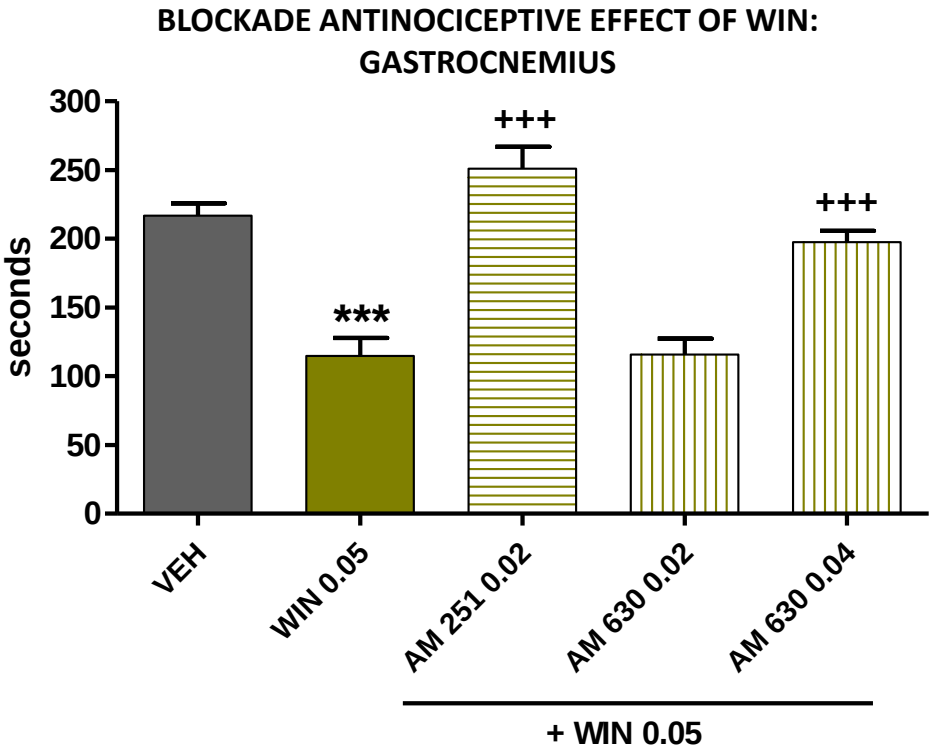


Figure 34. Blockade of the antinociceptive effect of 0.05 mg/kg WIN on HS induced nociceptive behavior in the gastrocnemius pain model. Bars show the total number of seconds the rat maintained the paw withdrawn after HS injection (mean ±SEM). ***p<0.01 *vs* vehicle (VEH); +++ p<0.01 *vs* WIN. (One way ANOVA, n≥6).

TREATMENT	MEAN NUMBER OF SECONDS
HS i.m.	221.3±21.81
saline i.p.+ HS i.m.	210.5±12.2
Tween ethanol i.m.+HS i.m.	216.8±9.0
AM251 i.m. + HS i.m.	210.3±13.6
AM630 i.m. + HS i.m.	217.8±26.6

Table 16. Effect of i.m. administration of vehicle and antagonists on HS induced nociceptive behavior in the gastrocnemius pain model. Data are presented as the means ± SEM, n ≥ 6.

Both selective agonists, ACEA and JWH015 (0.0125-0.05 mg/kg) presented an antinociceptive effect in this pain model (Fig. 35), that was antagonized with their corresponding selective antagonist (AM251 0.02 mg/kg and AM630 0.02-0.04 mg/kg) (Fig. 36).

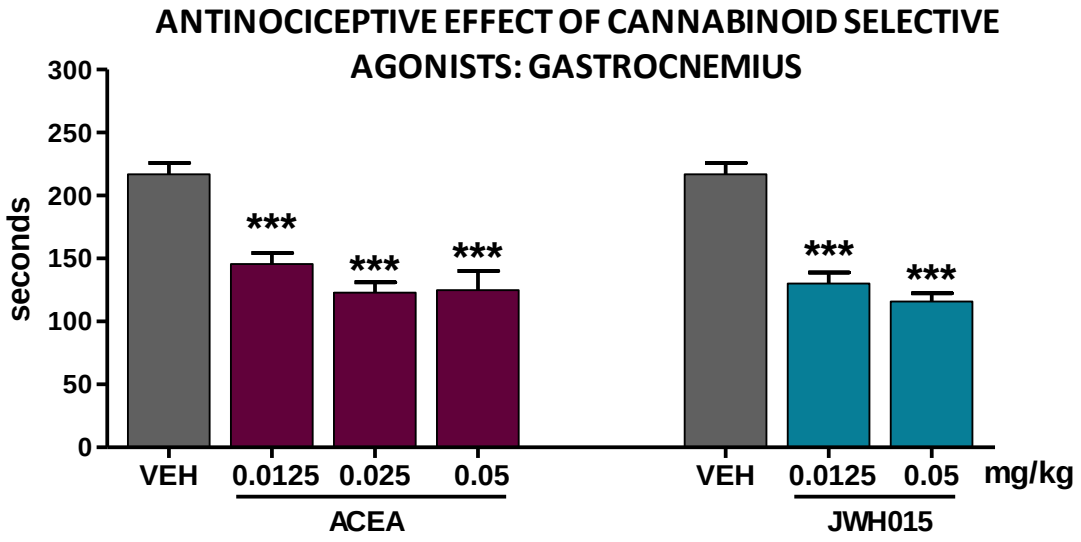


Figure 35. Antinociceptive effect of i.m. administered ACEA and JWH015 (0.0125-0.05 mg/kg) 5 min before HS injection in the gastrocnemius pain model. Bars show the total number of seconds the rat maintained the paw withdrawn after HS injection (mean ±SEM). ***p<0.001 *vs* vehicle (VEH). (One way ANOVA, n≥6).

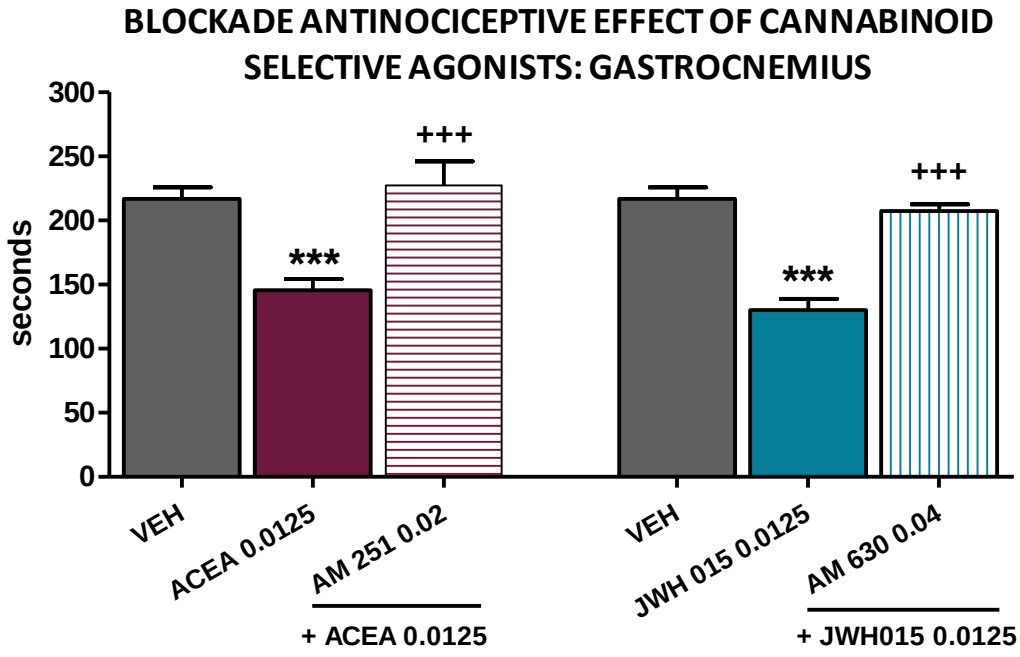


Figure 36. Blockade of the antinociceptive effect of 0.0125 mg/kg ACEA and 0.0125 mg/kg of JWH015 on HS induced nociceptive behavior in the gastrocnemius pain model. Bars show the total number of seconds the rat maintained the paw withdrawn after HS injection (mean ±SEM). ***p<0.001 *vs* vehicle (VEH); +++ p<0.01 *vs* ACEA 0.0125; ###p<0.01 *vs* JWH015 0.0125. (One way ANOVA, n≥6).

3.3 CANNABINOID TETRAD

The cannabinoid tetrad was carried out on rats treated with saline and TE, in both groups we obtained similar results for the analgesic, temperature, catalepsy and motor impairment tests.

WIN (0.5-1 mg/kg) did not modify the temperature nor induce catalepsy whilst it did show an antinociceptive effect on the Randall-Selitto test in a dose dependent manner. WIN 0.5mg/kg did not modify the locomotor activity but when 1 mg/kg was administered rats crossed less times the photocell beams than those treated only with TE reaching a small, but significant statistical differences (Fig. 37).

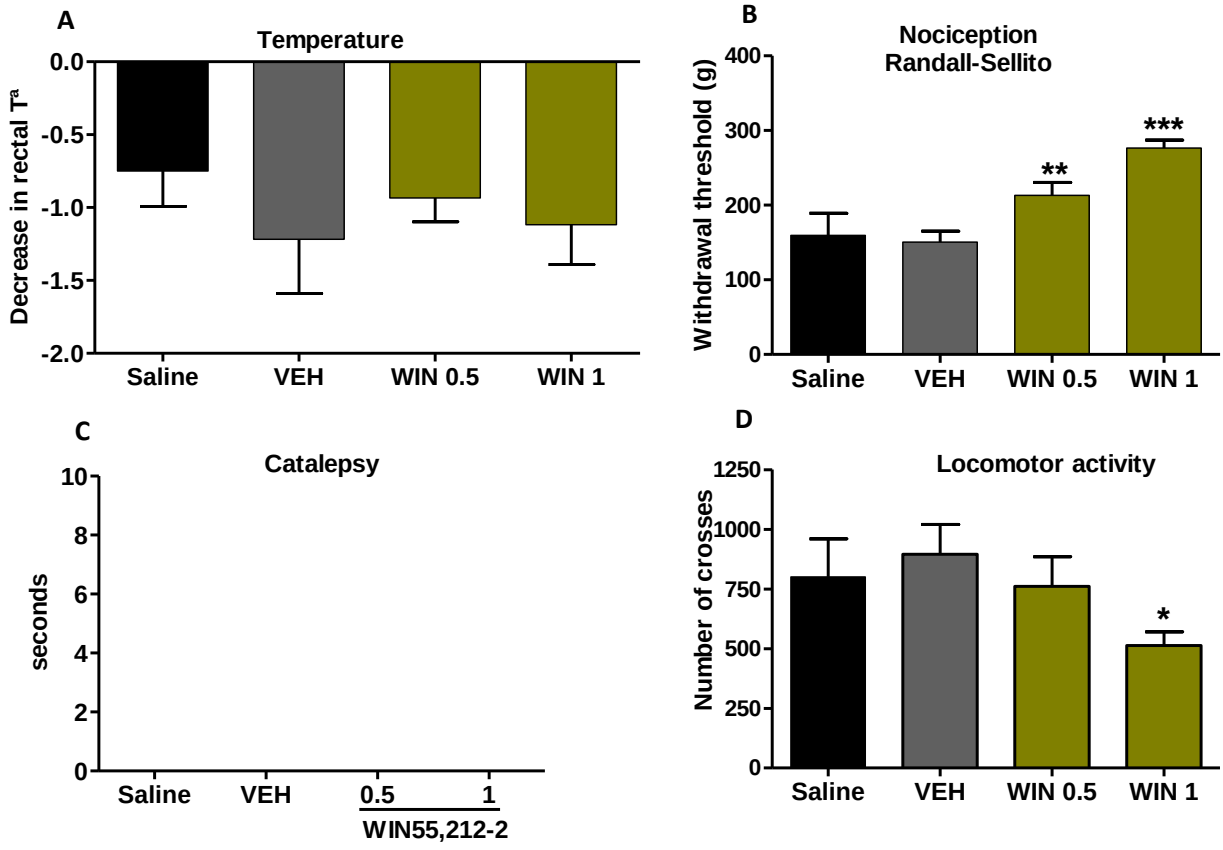


Figure 37. Effects of WIN 0.5 and 1 mg/kg administration on temperature (A), nociception (B), catalepsy (C) and locomotor activity (D). Bars show the temperature difference with respect to baseline (ºC) (A), grams exerted on the hindpaw of the rat before it withdrew it (B), seconds that rat remained immobilized in the ring test (C) and the number of interruptions of photocell beams recorded over a 30-min period (D) (mean ±SEM). *p<0.05, **p<0.01 ***p<0.001 *vs* vehicle (VEH) (One way ANOVA, n≥6).

DISCUSSION

The craniofacial sensory system has many differences from the spinal sensory system, as stated in the introduction of this thesis, such as a higher innervation density in many craniofacial tissues than in most spinally innervated ones or shorter conduction distances of peripheral nerve pathways, so findings in one system cannot be extrapolated directly to the other (Sessle, 2008). Taking this into account, pharmacological effects of different drugs on one system should also not be assumed to be the same in the other.

Recently a new behavioral assessment of craniofacial muscle pain has been described (Ro et al., 2003). The injection of different algesic substances (i.e. HS, glutamate, or formalin) evokes a nociceptive response which consists on vigorous hindpaw shakes, as an attempt to rub or scratch the affected region, which is stereotypical nocifensive behavior in intact animals. Experimental manipulation of the TMJ or masticatory muscles is particularly difficult in awake animals, and also difficult to provide prolonged or multiple deliveries of drugs without anesthetizing the animals, this model allows an easier and reliable access to deep craniofacial tissues for peripheral application of several pharmacological agents (Ro et al., 2003).

Among the algesic substances used, HS has been extensively used in human studies since it was first introduced by Kellgren (Kellgren, 1938) as the quality of the induced pain is comparable to acute clinical muscle pain, shows localized and referred pain characteristics and is used in humans without side effects (Stohler and Lund, 1994) and its effect on the rat masseter muscle is easily reproducible (Ro et al., 2003).

To compare results obtained in the orofacial region with results in spinally innervated muscle, the noxious stimulation must be the same. It was unexpected to find that none of the muscle pain models have been used to investigate simultaneously nociception in orofacial and spinally innervated muscles. So our first aim was to establish a simple model which allowed a reliable nociceptive response after painful stimulation of several spinally innervated muscles.

To reproduce the orofacial model described by Ro et al. in the most similar way as in spinally innervated muscles, HS was injected into the gastrocnemius and triceps muscles of anesthetised rats; this did not induce any visible nociceptive response. Conversely, a reliable volume and time-dependent nociceptive response was recorded in conscious animals, which consisted on paw withdrawal. Moreover, considering that

the stimulus is the same in the limb and orofacial muscles, it could be useful in comparing the characteristics of pain at these locations.

So, with these results our first aim was reached: we described two reliable models of pain in spinally innervated muscles using the same noxious stimulation that was used in the orofacial muscle pain model, previously published.

These models of acute pain have been used as a first experimental approach to compare orofacial muscle pain with that of spinally innervated muscles. Nevertheless the use of models of chronic muscle pain, in which inflammation and/or hyperalgesia appear, could be more interesting because of their clinical repercussions.

1. EFFECT OF CENTRAL AND PERIPHERAL OPIOIDS ON ACUTE MUSCLE NOCICEPTION

The first experiments of this thesis aimed to compare the participation of both, peripheral and central opioid receptors in an orofacial (masseter) and spinally innervated (gastrocnemius and triceps) muscles.

As expected, i.p. administration of morphine decreased the nociceptive behavior in the masseter as well as in limb muscles, and i.p. naloxone antagonised the effect of morphine at the three sites tested. Additionally, naloxone methiodide, an antagonist that does not cross the blood-brain barrier (Russell et al., 1982; Lewanowitsch and Irvine, 2002), antagonised the effect of morphine in the masseter muscle but not in the gastrocnemius or triceps muscles. These results suggest the participation of peripheral opioid receptors in acute nociception in the orofacial region, but not in limb muscles. To assess this possibility the effect of a peripheral agonist, loperamide, was tested and, in agreement with the obtained results it induced antinociception in the masseter, but not in the gastrocnemius or triceps. The effect of loperamide was antagonised by naloxone and by naloxone methiodide, confirming the participation of peripheral opioid receptors.

Although 10 mg/kg of morphine may be considered a high dose, its antinociceptive effect cannot be attributed to motor impairment because significant motor behavior impairments have been previously discarded in our laboratory (Burgos et al., 2010).

Finally, the effect of the drugs was tested using the classic tail flick test (D'amour and Smith, 1941), where opioids are effective, mainly, through the stimulation of spinally located opioid receptors (Le Bars et al., 2001). The obtained data was in accordance with that obtained in spinally innervated muscles; i.p. administered morphine induced dose-dependent antinociception that was antagonised only by naloxone and neither loperamide or naloxone methiodide were effective. Moreover, these results were similar in conscious and anesthetised rats, which eliminate the possibility that the anaesthesia was the cause of the different responses to opioids in the different muscles assayed.

The results obtained in the masseter are in line with the results of previous studies which demonstrate that i.m. administered morphine reduces the shaking behavior induced by mustard oil (MO) injection into the masseter (Ro et al., 2003; Han et al., 2008). Local administration of morphine into the masseter decreases the nociceptive shaking behavior after injection of MO in lightly anaesthetised rats, suggesting that this effect is mediated by a peripheral opioid receptor (Han et al., 2008). Moreover, it has been demonstrated that the intramuscular administration of a selective mu opioid agonist, DAMGO, is able to reduce the nociceptive response induced by HS in the masseter, and the effect of this drug is significantly enhanced under inflammatory conditions (Nunez et al., 2007), during which the expression of mRNA and protein for the mu-opioid receptor are increased in the trigeminal ganglion. In addition, the role of peripheral opioid receptors has also been previously suggested by more authors using other orofacial animal models, such as the face grooming provoked by subcutaneous capsaicin (Pelissier et al., 2002).

Under our experimental conditions opioids were not topically, but systemically administered. The importance of the peripheral component was pointed out because the effect of morphine was completely antagonised by the administration of naloxone methiodide, which does not reach the central nervous system, and because of the effectiveness of loperamide.

The effectiveness of the peripheral opioid analgesia has also been demonstrated in visceral tissues using the acetic acid test in mice (Labuz et al., 2007) and also in musculoskeletal structures using inflammatory joint pain models (Mecs et al., 2009; Pereira Santos et al., 2009).

Regarding opioid effects on limb muscles, which are spinally innervated, the involvement of peripheral opioid analgesia does not seem to play an important role, under our experimental conditions. Previous studies in the spinally innervated system have observed that antinociception is not commonly observed when opioids are administered locally into uninflamed tissues (Stein et al., 1989). Locally administered loperamide has shown an antinociceptive effect on the tonic phase of formalin induced nociception in the paw but not in the early phase (Dehaven-Hudkins et al., 1999) which supports the hypothesis that the inflammatory component is necessary for the full expression of peripheral antinociception in spinally innervated muscles. It is well known that peripheral opioid receptors participate in different degrees in the control of the nociceptive information and in the development of secondary allodynia and hyperalgesia in various experimental paradigms, such as the inflammation in the rat´s paw induced by formalin (Sevostianova et al., 2005; Ambriz-Tututi et al., 2009). In this inflammatory model, peripheral analgesia is associated with enhanced axonal transport of opioid receptors toward the periphery, increased mRNA transcription and a higher opioid receptor density in DRG, as well as increased opioid receptor binding and G protein coupling (Obara et al., 2009).

It has been demonstrated that spinal mu and delta opioids but not kappa, are involved in opioid analgesia in a model of chronic pain induced by the repeated administration of acidic saline (Sluka et al., 2002). It has been proposed that central, but not peripheral, mechanisms are involved in the inhibition of the nociceptive reflex, after morphine administration, in the gastrocnemius muscle injected with complete Freund`s adjuvant (Li and Zhao, 2003).

To summarize: our experimental model is a model of acute nociceptive pain, so there are not adaptive changes, such as de expression of new opioid receptors that enhance the peripheral opioid effect, on the other hand the present data are consistent with previous results which also suggest that in cases of acute nociceptive pain, central mechanisms are involved in opioid analgesia.

It is important to point out that in the masseter pain model where peripheral opioid receptors are mainly implicated, the doses of morphine necessary to induce antinociception are lower than those at which central opioid receptors become involved (gastrocnemius, triceps and tail-flick test). This is interesting because low

doses of opioids imply fewer adverse effects, such as respiratory depression, dependence and tolerance, which represent an important advantage.

The difference between the antinociceptive effect recorded in orofacial muscle and in limb muscles could be attributed to the experimental model. In the first case animals were lightly anaesthetised whereas in the second they were awake, it could be possible to suggest that central components are reduced in the masseter pain model and this could make more evident the peripheral analgesia. Nevertheless the absence of differences in the results obtained in the tail flick tests in anaesthetised and awake rats suggest that the presence of peripheral opioid analgesia does not depend on the administration of the anaesthesia and, in any case, our results demonstrate that peripheral opioid receptors play a role on acute muscle pain in the masseter.

In summary, our data provide evidence that morphine and loperamide differentially modulate hypertonic saline-induced nociceptive behavior after its injection into the masseter, gastrocnemius or triceps muscle. This suggests that differences in underlying mechanisms may exist among these two types of muscle pain.

2. EFFECT OF CENTRAL AND PERIPHERAL CANNABINOIDS ON ACUTE MUSCULAR NOCICEPTION

Along the same line with the first set of experiments of this thesis, the second set aimed to compare the participation of both, peripheral and central CB_1 and CB_2 cannabinoid receptors in the masseter and gastrocnemius muscles, using also the same models

It is well known that cannabinoid agonists are antinociceptive and antihyperalgesic in a variety of animal models of acute and chronic pain and human studies (Martin Fontelles and Goicoechea Garcia, 2008; Elikkottil et al., 2009; Karst et al., 2010; Thaler et al., 2011).

These experiments demonstrate that cannabinoids have the capacity to attenuate nociception induced by HS in masseter and gastrocnemius. This effect was studied both by systemic (i.p.) and local (i.m.) administration of non-selective and selective agonists of CB_1 and CB_2 receptors.

The obtained results demonstrate that the non-selective cannabinoid agonist, WIN 55,212-2, and the selective CB_1 and CB_2 agonists, ACEA and JWH 015, are capable of reducing the nociceptive shaking behavior induced by HS in the masseter muscle, when administered systemically and locally.

The i.m. WIN 55,212-2 effect was tested 5 and 30 min after its administration because a local effect appears faster than a systemic one. As expected, when the effect of WIN 55,212-2 was tested at the shorter interval of time smaller doses were required. So, all i.m experiments were carried out 5 min after cannabinoid agonists' administration.

The selective antagonists, AM 251 and AM 630, blocked the antinociceptive effect of WIN 55,212-2 and of the selective agonists, suggesting the involvement of CB_1 and CB_2 receptors in this orofacial muscle pain model.

There are studies that demonstrate that cannabinoid receptor agonists may be effective agents for craniofacial pain (Papanastassiou et al., 2004). Some previous data, obtained in this area, suggest that the antinociception is mainly mediated though CB_1 receptors whereas our results indicate that both CB_1 and CB_2 receptors are involved in the antinociceptive effect. This difference could be attributed to the different tests and tissues used: temporomandibular joint and formalin test (Burgos et al., 2010), infraorbital nerve constriction (Liang et al., 2007), although the innervation is trigeminal in all these cases.

Moreover, results concerning the involvement of different subtypes of CB receptors are in good agreement with the data obtained in gastrocnemius, which suggest that both CB_1 and CB_2 receptors are localised in muscle, and with the data of Cavuoto et al. (Cavuoto et al., 2007) that demonstrated the expression of CB_1 and CB_2 receptors in human and rodent skeletal muscle.

When the antinociceptive effect of the cannabinoids was studied, using the gastrocnemius pain model, we observed that i.p. WIN 55,212-2 induced an antinociceptive effect that was only blocked by the CB_1 antagonist.

To test if i.p. administration of cannabinoids produces psychoactive effects that can limit the usefulness of the cannabinoid analgesia the cannabinoid tetrad was performed. This evaluates antinociception, temperature, motility and catalepsy, after WIN 55,212-2 i.p. administration (Pertwee, 1972). Moreover information about motor

impairments could be obtained, which could limit the validity of studies involving motor responses (Sanudo-Pena et al., 2000), and also assess nociception using another test.

The classical evaluation of nociception with the hot-plate test was changed by the Randall-Sellito test that can be applied on the gastrocnemius muscle. At the tested doses, WIN 55,212-2 did not induce catalepsy or hypothermia but it produced analgesia and at dose of 1 mg/kg it reduced the locomotor activity; this slight hypomotility could explain the reduced antinociception observed after the systemic administration of WIN 55,212-2 in the gastrocnemius pain model.

Moreover, when the effect of i.p. selective agonists was studied, only the CB_1 selective agonist, ACEA, reduced the HS-induced nocifensive behavior in the gastrocnemius, whilst JWH 015 reduced the painful response without reaching statistical significance; this effect could be unspecific because of the doses required are higher. The ACEA effect was fully blocked by AM 251, confirming the participation of CB_1 receptors.

When animals were treated with the three cannabinoid agonists via i.m. administration in the gastrocnemius, all of them showed analgesic efficacy which was reversed by both CB_1 and CB_2 antagonists, suggesting the implication of the two types of CB peripheral receptors in this pain model.

Therefore, the antinociceptive effect of systemic cannabinoids in the gastrocnemius pain model appears to involve mainly CB_1 receptors, although the participation of CB_2 cannot be completely dismissed considering the evidence of the participation of both CB_1 and CB_2 receptors when the local administration is used. In the same line, dealing with the participation of both kinds of receptors, a previous study of inflammatory muscle pain in triceps demonstrated that the antihyperalgesic effect, induced by i.p. WIN 55,212-2 was mediated by both CB_1 and CB_2 receptors (Kehl et al., 2003).

Regarding the comparison of the muscle pain models, cannabinoids are more effective on masseter than on gastrocnemius, and the doses needed to induce antinociception are also smaller in masseter; these data are in agreement with those previously reported (Burgos et al., 2010) that demonstrated that the potency and effectiveness of WIN 55,212-2 in models of inflammatory pain was greater in the

trigeminal territory than in spinal nerve-innervated areas and confirm the differences between nociceptive transmission in trigeminal and spinal territories (Sessle, 2005; Takemura et al., 2006).

There is increasing evidence that action of CB_1 agonists or CB_1/CB_2 dual agonists at peripheral sites produce analgesic effects without CNS side effects. Cannabinoid agonists (natural or synthetic) have demonstrated to be able to modulate peripheral nociception in a wide number of models of animal nociception in inflammatory, thermal and neuropathic pain (Guindon et al., 2007; Kress and Kuner, 2009). These works include reduction of hyperalgesia and/ allodynia recorded in very different models such as: carrageenan-induced nociception, formalin-induced nociception, heat injury, injection of capsaicin, partial sciatic nerve ligation, CFA-induced thermal hyperalgesia (for a review see (Cheng and Hitchcock, 2007).

Also there is clinical data, in human trials, to support peripheral action of cannabinoid agonists. Topical application of the synthetic CB_1 and CB_2 agonist, HU-210, significantly suppress capsaicin-induced burning pain and no psychoactive effects are observed (Dvorak et al., 2003; Rukwied et al., 2003) and recently, it has been demonstrated that topical cannabinoid receptor agonists are an effective and well-tolerated adjuvant therapy option in postherpetic neuralgia in patients (Phan et al., 2010).

However, to our knowledge, there are not data about the local cannabinoid effectiveness in muscle pain. Our results obtained after the i.m. administration of WIN 55,212-2 (CB_1-CB_2 agonist) and ACEA (CB_1 selective agonist) are in line with those above described and, moreover, we demonstrated that i.m. JWH 015 is also effective to alleviate the HS-induced muscle pain which may be interesting from a therapeutic point of view. It is well known that activation of CB_2 receptors by a selective agonist results in relief of acute and chronic pain in preclinical models of inflammatory, neuropathic and visceral pain (Valenzano et al., 2005; Cheng and Hitchcock, 2007; Anand et al., 2010), and our results confirm CB_2-mediated peripheral antinociception in the two models of muscle pain studied. All these data are important, since CB_2 receptor-selective agonists lack centrally mediated adverse effects, which may increase their therapeutic potential (Guindon and Hohmann, 2008).

On the other hand, although some authors have demonstrated that there is an endocannabinoid tone in a model of osteoarthritis (Schuelert and McDougall, 2008)in our study we have not observed behavioral alterations when the antagonists were administered alone; this could be due to the different pain models used.

The most interesting conclusion from our work is the demonstration of the peripheral component in muscle of the antinociceptive effect of all the cannabinoids tested. Moreover, taking into account the effect of selective agonists and antagonists, it could be suggested that in this effect participate CB_1 and CB_2 receptors.

It is worth noting orofacial pain requires lower doses of both types of opioids and cannabinoids to reduce the nociceptive behavior, this is interesting because clinically low doses of morphine and CB_1 agonists (WIN and ACEA) imply fewer adverse effects.

Also, although our results are not final because it would be interesting to know if the differences found in the opioid experiments also exist in humans, it can be suggested that peripheral opioid receptors are potential targets for the treatment of muscular orofacial pain, and peripheral CB_1 and CB_2 cannabinoid receptors for both orofacial and spinally innervated muscles avoiding undesirable central effects of these drugs.

CONDITIONED PAIN MODULATION IN MECHANICALLY AND CHEMICALLY EVOKED MUSCLE PAIN

METHODS

1. PARTICIPANTS

Twelve males and twelve females, with no difference in age between groups (mean age±SEM 24.75±0.89) participated in the study. Volunteers were recruited via e-mail sent to all students studying science at the time of the experiment at Aalborg University, the first 12 males and 12 females which responded, and did not present any of the exclusion criteria were included in the study. Exclusion criteria were: pain medication or any alteration, disease or previous injury that could interfere in normal somatosensory functioning. All experiments were performed in the orofacial pain lab at the Center for Sensory-Motor Interaction. The study was approved by the Local Ethics Committee (VN: 20080057). Informed consent was obtained from each subject before study inclusion.

2. NOCICEPTIVE TEST STIMULATION

2.1 THERMAL TESTING

To determine thermal pain thresholds (TPT) the thermal sensory device TSA 2001 II (CHEPS-MEDOC) was used. The contact area of the thermode had a surface of 9 cm^2. Measurements were made through the method of limits (Matos et al., 2011). The baseline temperature for the thermode was set at +32 °C (centre of neutral range) and thermal stimulation limits were preset to +0 °C as the coolest level, and +50 °C as the warmest. The rate of temperature increase or decrease was 1 °C/s and when returning to baseline 3 °C/s. Subjects were instructed to push the button with their right hand when the heat or cold stimulus felt barely painful.

2.2 PRESSURE PAIN TESTING

Pressure pain thresholds (PPT) and pressure pain tolerance (PPTol) were obtained with a handheld electronic algometer (SomedicAB, Stockholm, Sweden). The tip which contacts the skin is mounted with a 1 cm diameter circular rubber probe to avoid metal edges being painful. Pressure was applied with the probe perpendicular to the test site and with a constant rate of 30 kPa/s, with a safety limit of 2200 kPa.

Participants were instructed to press the stop button as soon as the pressure felt painful (PPT) or when they felt the maximum pain they could tolerate (PPTol).

TPT, PPTs and PPTol were always measured at the right masseter and left forearm.

3. CONDITIONING STIMULI

Two types of conditioning stimuli were used:

1. Mechanical pain (MP) inducing device: pain was induced by a specially designed compressive device connected to a force transducer as previously described (Sowman et al., 2011). The head compression device was positioned so that the compressive points contacted the head 4 cm above the upper point of the ear lobes bilaterally and 4 cm above both the occiput and the nasion posteriorly and anteriorly respectively (Fig. 38). Once the compression device was correctly positioned over the head, similar pressure was made through the lateral probes over both temporalis muscles. The force applied on the probes could be seen through the transducer and pressure was made over the temporalis muscles until the subject felt pain graded as a 6 on the visual analogue scale (VAS) or reached the safety limit (60N).

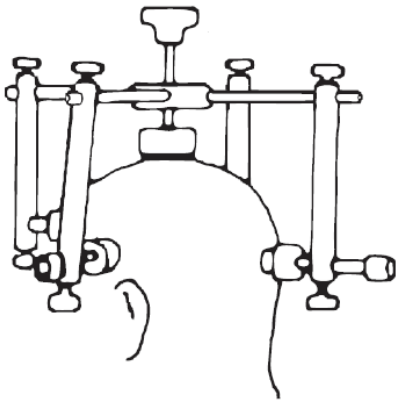


Figure 38. Drawing of the mechanical compressing device used in the experiments (Sowman et al., 2011).

2. The second conditioning stimulus consisted on an injection of 1 ml of hypertonic saline 0.5% (HS) into the right temporalis muscle.

The control sessions of the painful ones consisted on an injection of 1 ml of isotonic saline (IS) and the adjustment of same mechanical compressive device but without exerting any pressure (mechanical control or MC).

Right after the painful stimulus was halted, volunteers were asked to draw the distribution of the painful sensation on a pain map and rate on a VAS scale the displeasure felt by the pain induced by the conditioning stimulus.

4. PAIN INTENSITY RATING

For the participants to provide ongoing feedback regarding pain experience due to the conditioning stimulus, an electronic visual analogue scale (VAS) was continuously controlled by the participants and its output was recorded by a computer

(sample rate 0.2 Hz). Volunteers were asked to rate the pain intensity as soon as conditioning stimulus or control was applied until no pain was felt or sensory testing was finished. The area under the VAS curve (AUC) was analyzed to determine pain intensity during application of conditioning stimuli or control.

5. PROTOCOL

Subjects were asked to attend four sessions: two painful in which conditioning stimuli were applied and two controls. The nature of the first session was randomly allocated. During the whole experiment volunteers sat, comfortably, on a chair with arm rest and feet on the floor.

TPT, followed by PPTs and PPTol were taken at baseline, TPT and PPTs were obtained as the average of three consecutive stimuli. PPTol were only taken once. Then pain or control was applied depending on the nature of the session. The same pain thresholds were taken again, while the subject recorded the pain induced by the conditioning stimulus on the VAS. Once pain disappeared PPTs were taken every two minutes for 20 minutes, to avoid temporal summation or sensitization of the areas tested, only one evaluation of PPT was performed each time. After these twenty minutes again TPTs, PPTs and PPTol were taken (Table 17). Measurements were always taken over the right masseter and left forearm, though the sequence of the measurements were randomly allocated as were the sequence of the sessions.

Baseline	During pain	After pain											
		0 min Post1	2 min Post2	4min Post3	6min Post4	8 min Post5	10min Post6	12min Post7	14min Post8	16min Post9	18min Post10	20min Post11	22min Post12
TPT PPT PPTol	TPT PPT PPTol	PPT											TPT PPT PPTol

Table 17. Experimental timeline. At baseline thermal pain thresholds (TPT) were measured followed by pressure pain thresholds and pressure pain tolerance (PPT and PPTol). Then pain was induced or control applied and again TPT, PPTs and PPTol were assessed in the same order. Once the compressive device was removed and the volunteer felt no pain (due to HS injection or mechanical compression) PPTs were evaluated every two minutes, after these twenty minutes again thermal PPTs and PPTol were assessed. Measurements were always performed over the masseter and forearm

6. STATISTICAL ANALYSIS

First of all, to examine the effect of the conditioning stimuli on pain perception, HPT, PPTs and PPTol values were normalised prior to the analysis by expressing their magnitudes as a multiple of the baseline measurement.

To determine the effect of the conditioning stimuli over to pain thresholds two different analysis were performed. First, to study if the conditioning stimulus altered the pain thresholds while it was exerting pain a t-test was performed, then a two way repeated measures ANOVA was used to analyse if the pain thresholds differed between the control and conditioning painful session after the conditioning stimulus was removed, the two variables analysed were condition (painful or control) and time.

When different results for males and females were obtained gender differences were analyzed, this was done through the maximum possible effect (M.P.E.). Again the same analyses were performed but in the t-test the variable analyzed was gender and in the repeated measures two way ANOVA they were time and gender. Statistical significance was set at 5% and posthoc mean comparisons were corrected by the Bonferroni method. Data are presented as mean ± SEM.

RESULTS

The first analyses performed were aimed to evaluate differences between the painful conditioning sessions and their respective controls, but no differences were found. When observing the raw data certain differences between males and females were observed, so the analysis was performed separately between genders, these results are shown and discussed below.

1. BASELINE THERMAL AND PRESSURE PAIN THRESHOLDS

The results obtained from the four sessions were analysed to determine whether there are gender differences in:

- Baseline pain threshold to different stimuli (thermal and mechanical)
- Pain intensity induced by the two different conditioning stimuli.

Two way ANOVA showed that females had lower baseline pain thresholds than males at the two sites tested, masseter and forearm, to pressure stimuli (masseter ANOVA: $F=7.039$, $p<0.05$, forearm ANOVA: $F=14.78$ $p<0.001$), pressure tolerance (masseter; ANOVA: $F=21.75$ $p<0.001$, forearm; ANOVA: $F=45.21$ $p<0.001$) and heat at the masseter (ANOVA: $F=5.679$ $p<0.05$). No statistically significant differences to forearm heat pain thresholds were found between males and females (ANOVA: $F=2.048$ $p>0.05$). Post hoc analyses are shown on graphs (Fig. 39).

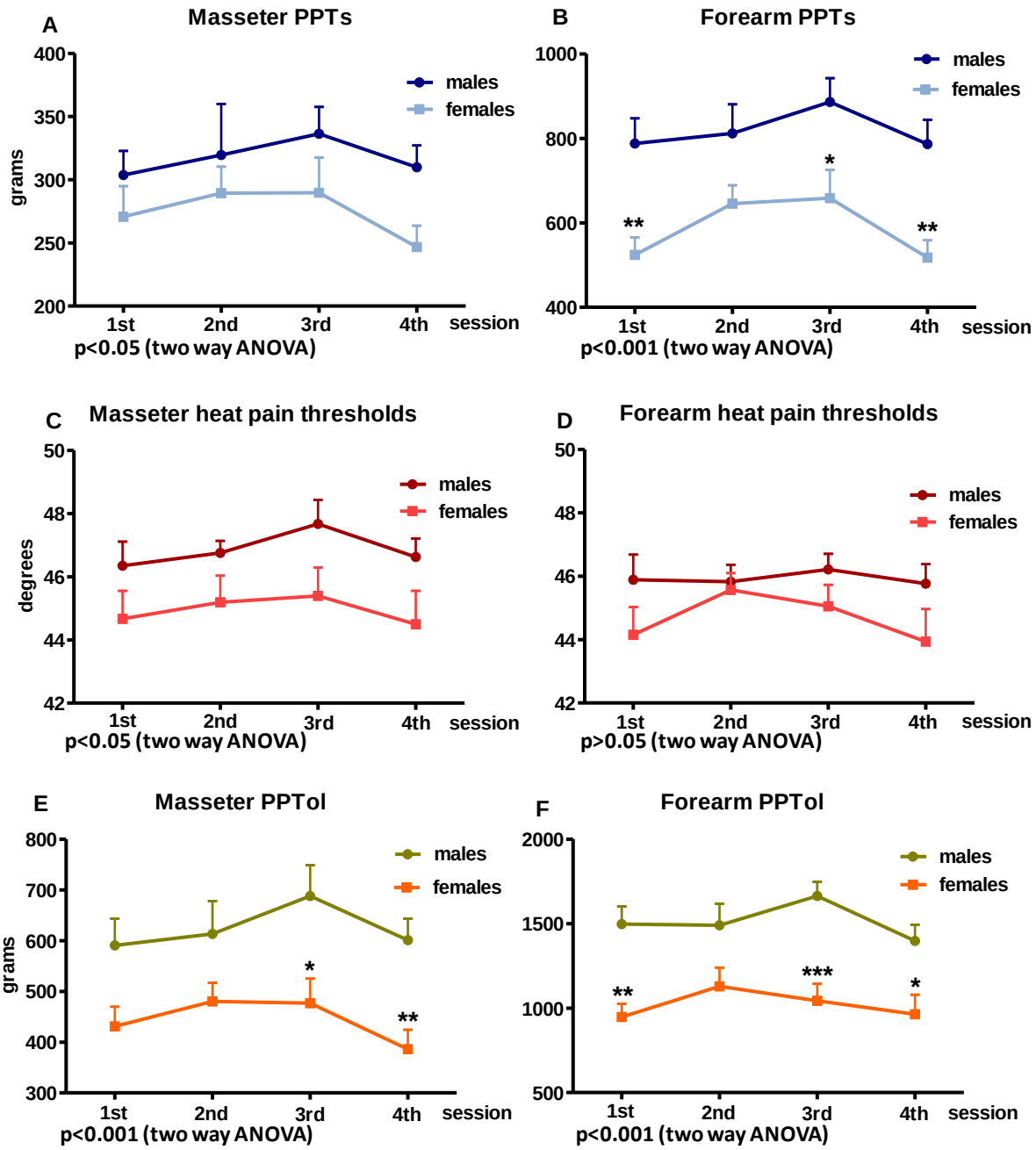


Figure 39. Gender differences in baseline PPT, PPTol and HPT. Points indicate the mean±SEM of masseter PPT (A), forearm PPT (B), masseter HPT (C), forearm HPT (D), masseter PPTol (E) and forearm PPTol (F). (Two-way ANOVA, n= 12, Bonferroni posthoc analisys) *p<0.05, p<0.01, p<0.001 *vs* males.

2. VISUAL ANALOGUE SCALE SCORE

To determine the intensity of the pain induced by each of the conditioning stimuli the mean AUC was analyzed for each gender, with the mean AUC two different comparisons were performed:

- Pain intensity induced by the same stimulus between genders
- Pain intensity between conditioning stimuli in the same gender

The pain intensity induced by the mechanical compressing device generated a similar AUC in females and males (males: 2215±234 females: 2586±230 unpaired t-test p=0.34). The AUC, when pain was induced by HS, was found to be increased in females when compared to males in a statistically significant manner (males: 1230±167 females: 1926±293 p=0<0.05) (Fig. 40).

Under our experimental conditions, the AUC was higher, both in males and females, when assessing pain evoked by mechanical compressing device than by HS (males: p<0.01, females: p<0.05) (Fig 41).

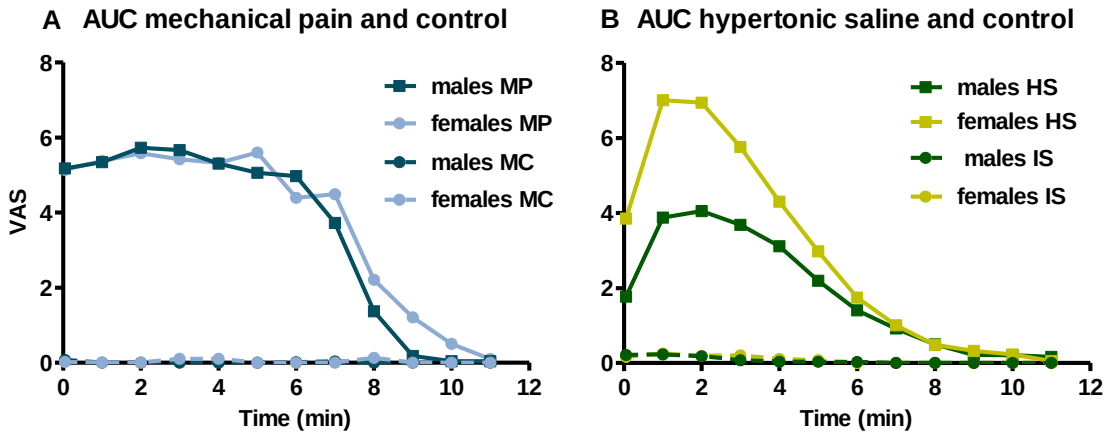


Figure 40. Continuous visual analogue scale (VAS) rating of pain intensity during head compression (MP) and its control (MC) (A) or hypertonic saline (HS) and its respective control (IS) injection (B). Data are shown as the average VAS score of all subjects at one minute intervals.

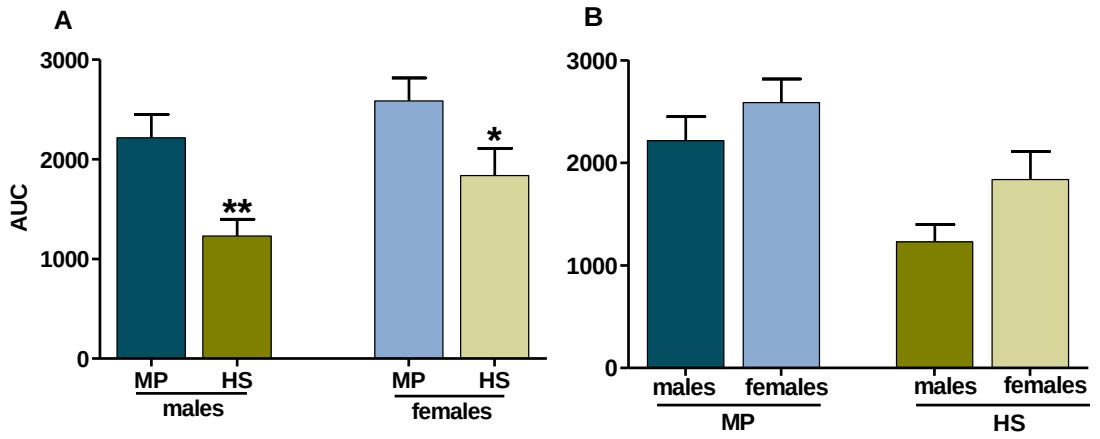


Figure 41. Differences in the area under the VAS curve (AUC) reported for mechanical and hypertonic pain in male and female volunteers (A) and differences between genders (B). Data are shown as the average VAS score for each gender±SEM. *p<0.05, **p<0.01 *vs* MP (unpaired t test n=12).

3. PAIN MAPS

Pain maps show that the area of distribution of pain induced by the conditioning stimuli is varied amongst the participants (Fig 42). With the mechanical compressing device: pain was localized to the areas where the contact probes of the device were placed or referred to adjacent areas but always in the lateral side of the head. The same thing happened to pain induced by HS: pain was, or localized to the site of injection or referred to adjacent areas but always in the lateral side of the head where the injection was performed.

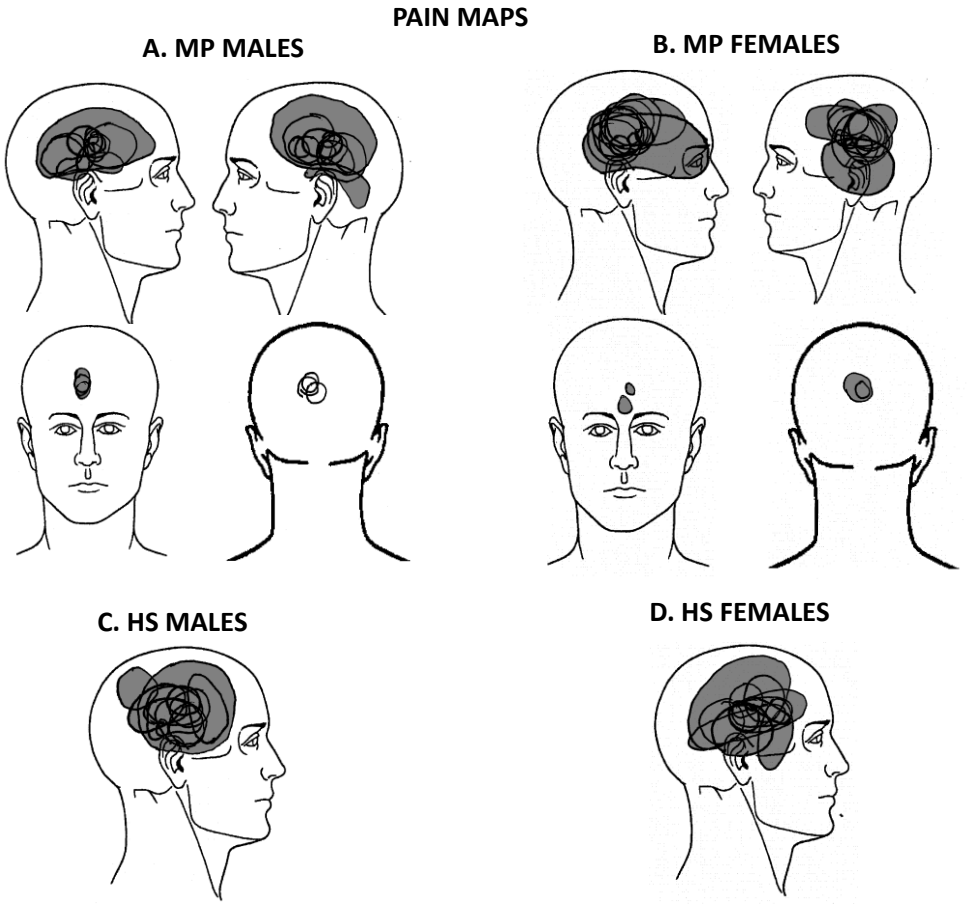


Figure 42. Pain maps drawn by male (A) and female (B) volunteers during the mechanical pain session and male (C) and female (D) subjects during the hypertonic saline (HS) session.

4. EFFECTS OF THE CONDITIONING STIMULI ON PAIN THRESHOLDS

The effect of each conditioning stimulus on HPT, PPT and PPTol thresholds was studied at two different time points: while the MCD and HS were inducing pain and after the pain had subsided.

Variation of cold pain thresholds were not studied as a high percentage of male volunteers did not feel pain at the safety limit, so variation in pain thresholds could not be studied.

4.1 EFFECTS OF CONDITIONING STIMULI DURING PAIN ON:

4.1.1 Heat pain thresholds

No significant variation of HPT, during the MP and HS sessions, was observed when compared to their respective control ones at any of the two sites tested,

(masseter and forearm), in either gender. The variation of HPT during the four different sessions is shown on Table 18.

		IS	HS	MC	MP
Males	Masseter	1.0±0.01	1.0±0.02	1.0±0.01	1.0±0.02
	Forearm	1.0±0.01	1.0±0.01	1.0±0.01	1.0±0.01
Females	Masseter	1.0±0.03	1.0±0.02	1.0±0.01	1.0±0.02
	Forearm	1.0±0.02	1.0±0.01	1.0±0.02	1.0±0.02

Table 18. Variation of heat pain threshold values during mechanical pain (MP), hypertonic saline (HS) or their respective controls: mechanical control (MC) and isotonic saline (IS) (n=12). Data are shown as mean ± SEM (n=12).

4.1.2 Pressure pain thresholds

When applying the mechanical compressing device, in male volunteers, masseter PPT decreased during MP session compared to the control one ($p<0.001$) and tended also to decrease in the forearm, though not in a statistically significant manner ($p=0.05$). In female volunteers masseter PPT did not vary ($p>0.05$), but in the forearm there was a significant increase compared to the control session ($p<0.05$).

HS injected in the temporalis muscle of male volunteers did not vary the PPT at the masseter or forearm ($p>0.05$) when compared to the IS session. In females PPT were decreased in the HS session when compared to the IS one, these decreases were at both sites tested, masseter ($p<0.01$) and forearm ($p<0.05$) (Fig. 43).

A summary on the main findings can be seen on Tables 24 and 25.

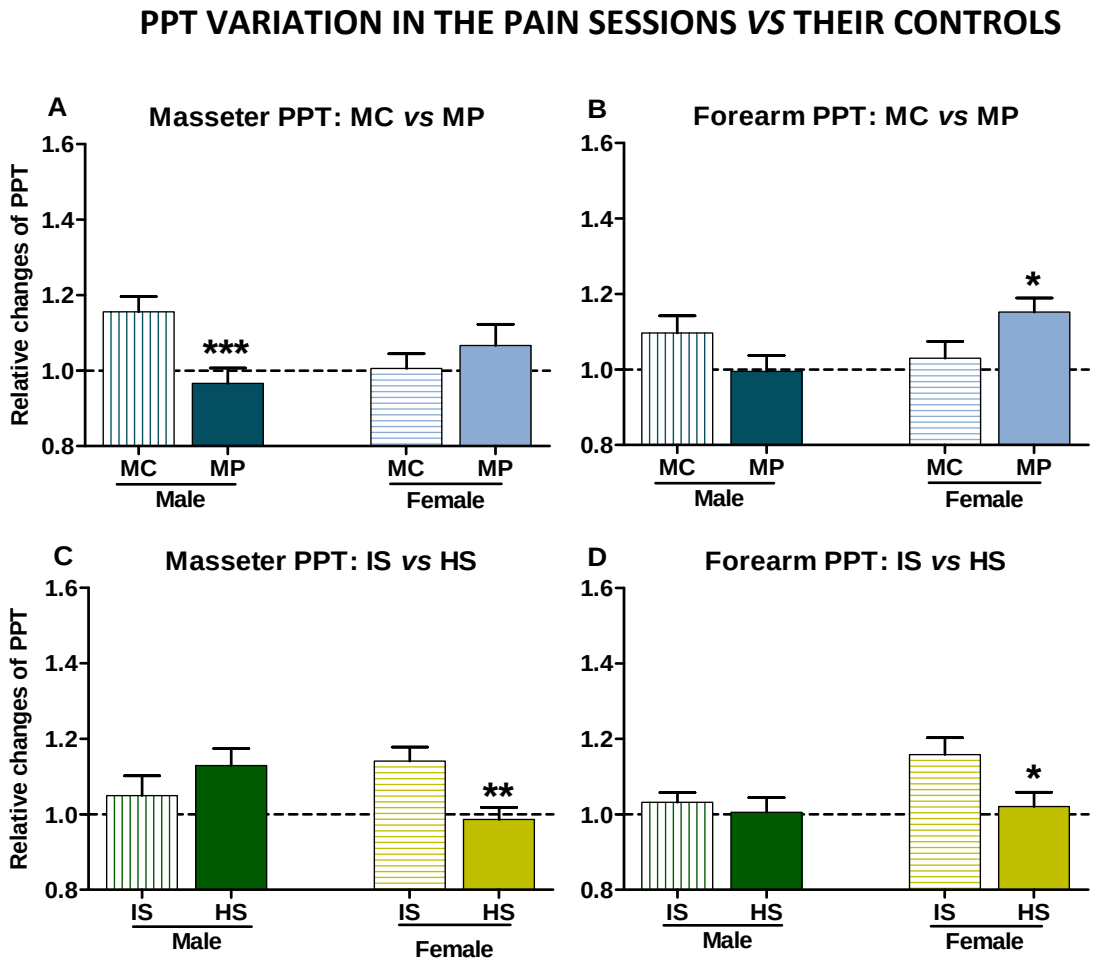


Figure 43. Variation in pressure pain thresholds (PPT) male and female masseter (A) and forearm (B) during mechanical pain (MP) session compared to the mechanical control one (MC) (n=11). And variation in male and female masseter (C) and forearm (D) pressure pain thresholds (PPT) during hypertonic saline (HS) session compared to the isotonic saline one (IS) (males: n=12, females n=11). Bars represent relative changes of PPT (normalized values to baseline). ***p<0.001, **p<0.01,* p<0.05 *vs* its respective control.

The number of subjects that responded in each session varied depending on the stimulus and gender (Table 19). A subject was considered as a responder when he/she presented an increase in PPT of more than 10% when compared to baseline values (Oono et al., 2011).

		IS	HS	MC	MP
Males	Masseter	58	25	42	58
	Forearm	42	25	33	25
Females	Masseter	42	42	75	25
	Forearm	33	50	58	42

Table 19. Percentage of subjects who were considered as responders during the isotonic saline (IS), hypertonic saline (HS), mechanical control (MC) and mechanical pain (MP) sessions. A subject was considered as responder when presenting an increase in PPT of more than 10% when compared to baseline.

4.1.2.1 Gender differences

To determine gender differences the percentage of the maximum possible effect (% M.P.E.) was calculated: % M.P.E.= (test-baseline)/(cutoff-baseline) x 100, where test is the response to the test stimulus, baseline is the response to the test stimulus before pain or control was applied, and cutoff is the preset grams at which the test ends in the absence of a response.

When comparing the response of males and females to the conditioning stimulus or control no statistically significant differences were found, although males tended to have increased PPT variations in the masseter during HS (p= 0.07), and during the MC session (p=0.07) when compared to females (Fig. 44).

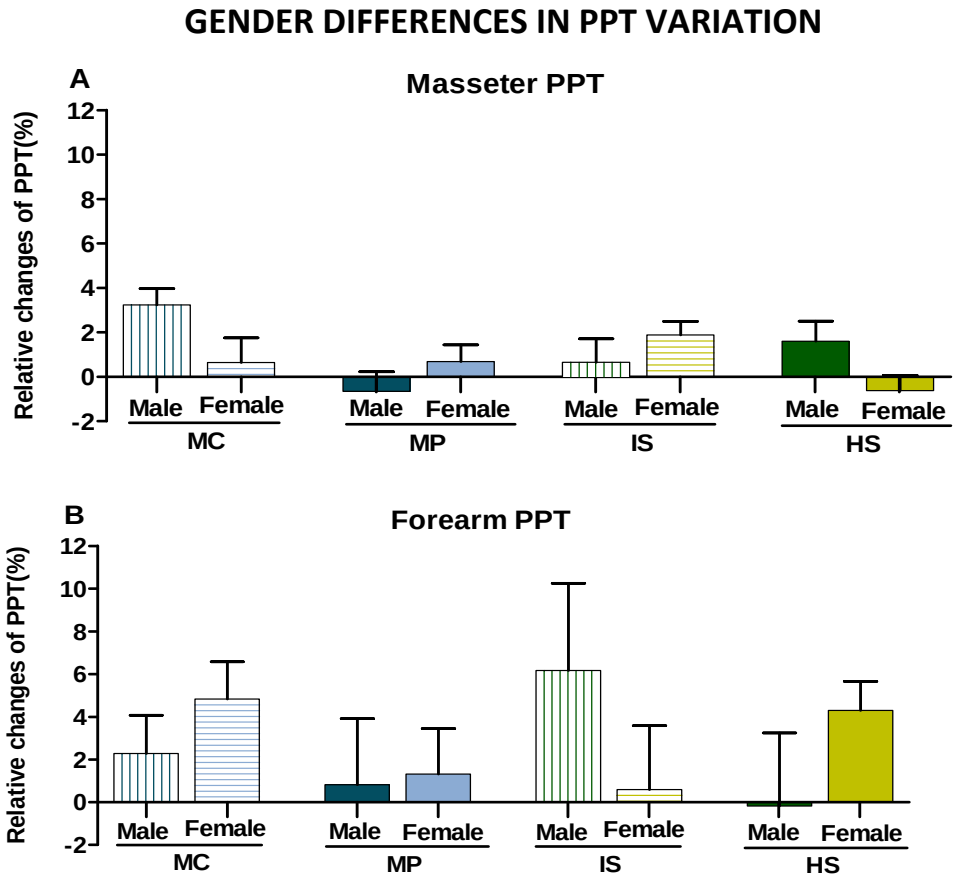


Figure 44. Gender differences in the variation of pressure pain thresholds (PPT) in masseter (A) and forearm (B) pressure pain thresholds during mechanical control (MC) and mechanical pain (MP) session (n=11). Gender differences in the variation in masseter (C) and forearm (D) pressure pain thresholds during isotonic saline session (IS) and hypertonic saline one (HS) (males n=12, females n=11). Bars represent the maximum possible effect±SEM.

4.1.3 Pressure pain tolerance

Male and female volunteers experienced no significant variation of PPTol during the MP and HS sessions when compared to their respective control sessions at any of the two sites tested, masseter and forearm. The variations of PPTol during the four different sessions are shown on Table 20.

		IS	HS	MC	MP
Males	Masseter	1.0±0.2	1.1±0.2	1.0±0.2	0.9±0.3
	Forearm	0.9±0.02	0.9±0.03	0.9±0.04	1.0±0.1
Females	Masseter	1.2±0.1	1.1±0.1	1.2±0.1	1.2±0.1
	Forearm	0.9±0.04	1.0±0.07	1.1±0.1	1.1±0.1

Table 20. Variation of PPTol at masseter and forearm while applying hypertonic saline (HS), mechanical pain (MP) or their respective controls: isotonic saline (IS) and mechanical control (MC) in each gender (n=12). Data are shown as mean relative changes of PPTol (normalized values to baseline)± SEM.

4.2 PPT VARIATION AFTER PAIN OR CONTROL:

After the assessment of the thermal and pressure pain thresholds, during pain induced by the conditioning stimuli or the two controls, PPT were assessed every two minutes for twenty minutes. PPT at the forearm or masseter did not vary, when compared to their respective control, in either gender. The variation of PPT for each session is shown on Table 21.

A	**MALES**							
	Masseter				**Forearm**			
	IS	**HS**	**MC**	**MP**	**IS**	**HS**	**MC**	**MP**
Post1	0,9±0,06	1.2±0,08	1.0±0.06	1.0±0.07	0,9±0,06	1.0±0.06	1.0±0.05	9.0±0.08
Post2	0,9±0,05	1.1±0,10	1.0±0.04	1.1±0.07	1.0±0.05	1.0±0.07	1.0±0.08	1.0±0.11
Post3	1.1±0.09	1.2±0.12	1.0±0.04	1.1±0.07	1.0±0.08	1.1±0.06	1.0±0.09	1.0±0.9
Post4	0,9±0,06	1.1±0.11	1.2±0.06	1.1±0.07	1.0±0.08	1.0±0.06	1.0±0.06	1.0±0.10
Post5	1.0±0.04	1.2±0.07	1.2±0.06	1.1±0.09	1.0±0.06	0,9±0,05	1.2±0.08	1.1±0.07
Post6	1.0±0.05	1.1±0.09	1.0±0.07	1.0±0.06	1.0±0.06	1.0±0.04	1.0±0.08	1.1±0.10
Post7	1.0±0.05	1.2±0.11	1.0±0.08	1.1±0.11	1.0±0.06	1.0±0.06	1.1±0.08	1.0±0.09
Post8	0.9±0.05	1.1±0.09	1.1±0.08	1.0±0.08	1.0±0.06	1.0±0.07	1.0±0.05	1.0±0.10
Post9	1.0±0.07	1.0±0.08	1.1±0.08	1.0±0.01	1.0±0.05	1.0±0.09	1.1±0.09	1.0±0.12
Post10	1.1±0.08	1.1±0.08	1.1±0.08	1.0±0.10	1.0±0.09	1.0±0.08	1.1±0.06	1.1±0.12
Post11	1.1±0.06	1.1±0.07	1.1±0.09	1.0±0.07	1.0±0.08	1.0±0.09	1.1±0.08	1.2±0.14
Post12	1.0±0.04	1.1±0.06	1.1±0.06	1.1±0.09	1.0±0.05	1.0±0.07	1.0±0.06	1.0±0.10

B	FEMALES							
	Masseter				Forearm			
	IS	HS	MC	MP	IS	HS	MC	MP
Post1	1.2±0.07	1.2±0.08	1.1±0.10	1.2±0.08	1.1±0.09	1.1±0.09	1.1±0.09	1.1±0.07
Post2	1.2±0.11	1.1±0.08	1.1±0.08	1.2±0.08	1.1±0.08	0.9±0.07	1.2±0.09	1.1±0.05
Post3	1.2±0.10	1.1±0.04	1.2±0.06	1.1±0.07	1.2±0.09	1.1±0.09	1.1±0.08	1.1±0.09
Post4	1.2±0.06	1.2±0.08	1.0±0.04	1.2±0.13	1.1±0.07	1.1±0.10	1.1±0.07	1.2±0.11
Post5	1.1±0.07	1.2±0.07	1.1±0.08	1.2±0.11	1.1±0.07	1.1±0.06	1.0±0.09	1.1±0.10
Post6	1.2±0.07	1.2±0.09	1.2±0.06	1.1±0.10	1.1±0.09	1.0±0.08	1.1±0.06	1.2±0.13
Post7	1.2±0.10	1.1±0.10	1.1±0.06	1.3±0.12	1.1±0.09	1.1±0.08	1.0±0.08	1.0±0.11
Post8	1.2±0.07	1.1±0.10	1.1±0.06	1.3±0.09	1.4±0.12	1.0±0.12	1.1±0.06	1.1±0.09
Post9	1.1±0.06	1.1±0.07	1.1±0.07	1.3±0.13	1.2±0.11	1.0±0.10	1.0±0.06	1.1±0.07
Post10	1.3±0.10	1.1±0.08	1.2±0.07	1.2±0.08	1.1±0.05	1.0±0.10	1.1±0.07	1.2±0.10
Post11	1.1±0.06	1.1±0.09	1.1±0.07	1.1±0.05	1.2±0.07	1.0±0.11	1.1±0.07	1.1±0.08
Post12	1.2±0.10	1.2±0.10	1.2±0.04	1.2±0.07	1.1±0.07	0.9±0.08	1.0±0.05	1.1±0.08

Table 21. Variation in male (A) and female (B pressure pain thresholds (PPT) after pain induced by mechanical compression during the pain session (MP) compared to the control session (MC) (n=12). Data represent relative changes of PPTs±SEM (normalized values to baseline). Measurements were performed eleven times at 2 min intervals after compressive device was removed and volunteers did not feel any remaining pain (Post 1–11) (two-way ANOVA).

4.3 FINAL ASSESSMENTS OF TPT, PPT AND PPTOL

4.3.1 Heat pain thresholds

As during pain assessments, heat pain thresholds did not vary in the HS or MP sessions when compared to their respective controls. This lack of statistically significant variation occurred both in males and females at both sites tested (Table 22).

		IS	HS	MC	MP
Males	Masseter	1.0±0.01	1.0±0.01	1.0±0.01	1.0±0.01
	Forearm	1.0±0.01	1.0±0.01	1.0±0.01	1.0±0.01
Females	Masseter	1.0±0.03	1.0±0.04	1.0±0.04	1.0±0.02
	Forearm	1.0±0.02	1.0±0.03	1.0±0.01	1.0±0.01

Table 22. Variation of heat pain thresholds at the masseter and forearm after hypertonic saline (HS), mechanical pain (MP) or their respective controls: isotonic saline (IS) and mechanical control (MC) in each gender (n=12). Data are shown as mean relative changes of PPTol (normalized values to baseline) ± SEM.

4.3.2 Pressure pain thresholds

In the final PPT assessments no differences were found when comparing the variation of PPT during the pain session with their respective controls. This lack of differences happened at the masseter and forearm and in both genders (Fig. 45).

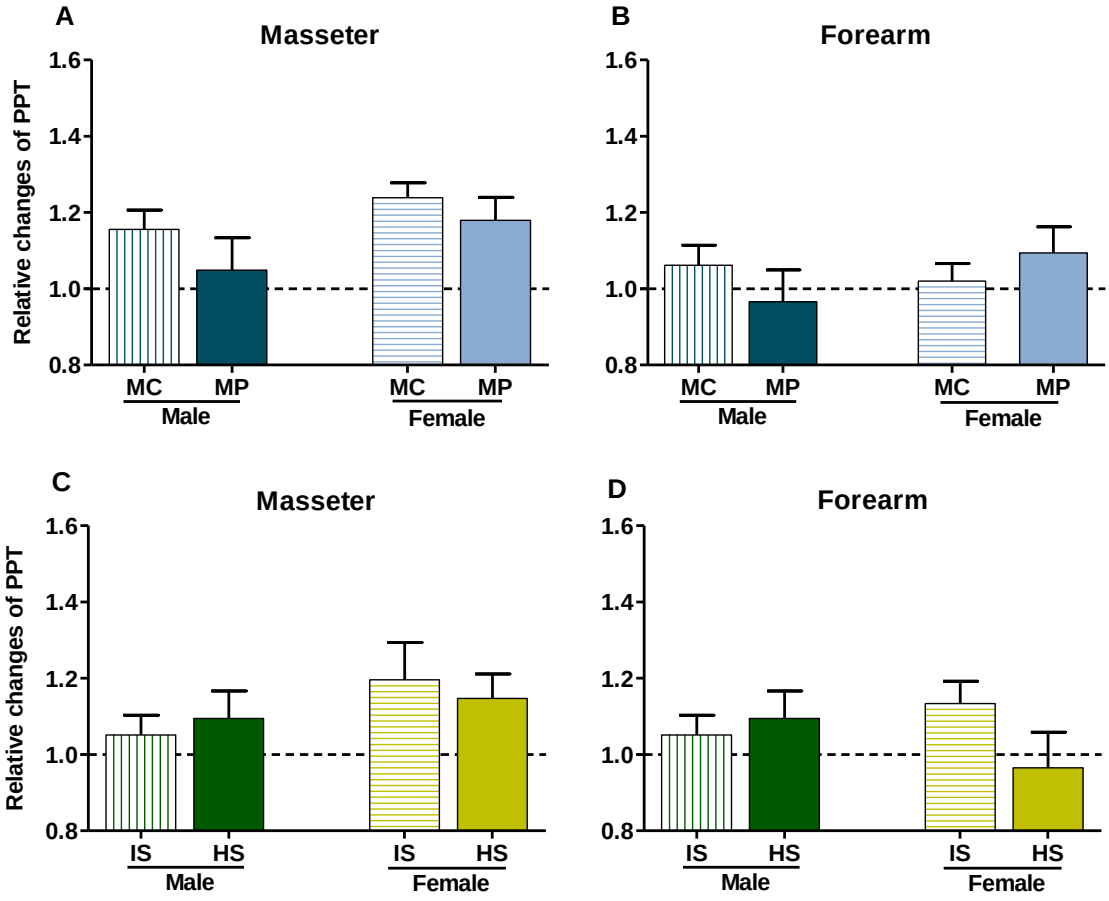


Figure 45. Variation in male and female masseter (A) and forearm (B) pressure pain thresholds after mechanical pain (MP) session compared to the mechanical control one (MC) (n=11). And variation in male and female masseter (C) and forearm (D) pressure pain thresholds (PPT) after hypertonic saline (HS) session compared to the isotonic saline one (IS) (males: n=12, females n=12). Bars represent relative changes of PPT (normalized values to baseline).

4.3.3 PPTol

Finally, PPTol did not vary either at any of the sites tested when comparing the pain sessions with their respective controls. PPTol did not vary in females or males (Table 23).

		IS	HS	MC	MP
Males	Masseter	1,0±0,04	1,1±0,08	1,1±0,06	1,1±0,09
	Forearm	1,0±0,05	1,0±0,07	1,0±0,06	1,0±0,09
Females	Masseter	1,2±0,1	1,2±0,1	1,3±0,1	1,3±0,1
	Forearm	1,0±0,05	1,0±0,07	1,1±0,09	1,0±0,07

Table 23. Variation of PPTol at masseter and forearm after hypertonic saline (HS), mechanical pain (MP) or their respective controls: isotonic saline (IS) and mechanical control (MC) in each gender (n=12). Data are shown as mean relative changes of PPTol (normalized values to baseline) ± SEM.

A		MC *vs* MP		IS *vs* HS	
		Females	Males	Females	Males
DURING PAIN	Masseter	ns	***↓	**↓	ns
	Forearm	*↑	↓(p=0.05)	*↓	ns
AFTER PAIN	Masseter	ns	ns	ns	ns
	Forearm	ns	ns	ns	ns

Table 24. Variation in masseter and forearm PPT in females and males during and after pain. The arrows represent how PPT vary when comparing the pain session (mechanical pain (MP) and hypertonic saline (HS)) with their respective controls (mechanical control (MC) and isotonic saline (IS)). ↑ means variation in PPT are increased in the pain session when compared to the control session, ↓ means variation in PPT are decreased in the pain session when compared to the control session. *p<0.05, **p<0.01, ***p<0.001 vs the control session.

B		MALES *vs* FEMALES			
		MC	MP	IS	HS
DURING PAIN	masseter	ns	ns	ns	ns
	forearm	ns	ns	ns	ns

Table 25. Gender differences in masseter and forearm during pain in the mechanical control (MC), mechanical pain (MP), isotonic saline (IS) and hypertonic saline (HS) sessions. ↑ means variation in PPT are increased in females vs males, *p<0.05 vs males

.

DISCUSSION

This study aimed to:

1. Analyze the response of each gender to the conditioning stimuli and baseline pain thresholds to each of the test stimuli.

2. Study if pain induced by two different conditioning stimuli, MP and HS, applied at the temporalis muscle (trigeminal innervation) alter pain thresholds at two different sites: a trigeminally innervated area (masseter) and a spinally innervated one (forearm) in male and female volunteers. Variation of pain thresholds were assessed at different time points: baseline, during pain and after pain.

1. BASELINE PAIN THRESHOLDS

This study showed that females have lower PPT than males at the masseter and forearm and also for thermal stimuli at the masseter, but no differences were found for HPT in the forearm.

These results are in agreement with most of the studies performed up to date, where males have found to have higher pain thresholds than females for masseter PPTs (Fillingim et al., 2005b; Komiyama and De Laat, 2005; Matos et al., 2011), forearm PPTs (Oono et al., 2012), masseter HPT (Sonnesen and Svensson, 2011). For forearm HPT some studies show no differences in pain thresholds between males and females as in this study (Fillingim and Maixner, 1996; Fillingim et al., 1998; Jones et al., 2003; Jensen and Petersen, 2006) and other studies show higher pain thresholds in males (Edwards and Fillingim, 1999; Edwards et al., 2004; Fillingim et al., 2005a; Fillingim et al., 2005b), possibly the lack of statistical significance may be due to a reduced sample size, as the studies with more participants do obtain higher heat pain thresholds in the forearm of males.

Also as in previous experiments females rated as more painful HS injection than males (Ge et al., 2004; Ge et al., 2006; Falla et al., 2008).

2. PAIN THRESHOLD VARIATION DURING PAIN INDUCED BY CONDITIONING STIMULI

Under our experimental conditions we found that females and males seemed to experience increased PPT during the control sessions in a different way depending on the control stimulus. Also the conditioning stimuli failed to induce a DNIC effect

except for the mechanical compressing device, which did increase PPT in the forearm of females.

A curious finding was the fact that the number of female responders was higher during the IS session than that of males It has previously been found that stress and distraction can reduce pain in an independent way, where stress induced by a nonpainful stimulus evokes hypoalgesia more effectively in females than males (Rhudy and Meagher, 2001; Sternberg et al., 2001) and state anxiety has been found to have higher scores in females before experimental sessions (Granot et al., 2008).

On the other the opposite happened with males: during the MC session more males were found as responders than females. Distraction seems to be a more effective method in reducing pain in males than females (Unrod et al., 2004; Quiton and Greenspan, 2007).

The weight over the head of the mechanical compressing device could have acted as a distracting factor, and the injection as a stressing factor. We did not include anxiety or distraction ratings, so the effect which anxiety and distraction could have had in this study cannot be excluded. Very little literature exists that permits a comparison of our results with previous ones.

During the pain sessions PPT were significantly lower than the control sessions but within baseline values, except for PPT in the forearm of females which increased. These results vary with previous ones where the same or similar compressing devices have been used, in these studies results show increases in male and female masseter PPT (Wang et al., 2010b; Oono et al., 2011; Sowman et al., 2011). The main difference of these studies with the present one is that in previous ones pressure was also exerted over the occiput and forehead whilst in this one pressure was only performed over the temporalis muscles. It has been shown that enlarging the area of noxious stimulation causes an increase in pain intensity (Price et al., 1989; Douglass et al., 1992; Nielsen and ArendtNielsen, 1997) and, although there is controversy on this, some studies have shown that increasing intensities induce a greater conditioned pain modulation (Lautenbacher et al., 2008; Oono et al., 2011). On the other hand, subjects in this study reported pain induced by the conditioning stimuli to be in a more localized area (Fig. 42), whilst in previous ones the mechanically compressing device induced pain in a more widespread area (Sowman et al., 2011). Previous authors have

found that DNIC effects occur after bilateral but not unilateral injection of HS, where more referred pain is found (Ge et al., 2003); this could be a possible explanation for the fact that we did not find a DNIC effect.

Only mechanical pain was capable of inducing a DNIC effect in females but not in males. This finding is very surprising because most studies indicate a more effective DNIC in males (Serrao et al., 2004; Goodin et al., 2009) or no sex differences (Tousignant-Laflamme et al., 2008; Weissman-Fogel et al., 2008). But in a systematic review performed by Popescu et al. (Popescu et al., 2010) where they retrieved data from different studies and analysed sex differences in DNIC modulation, they found several studies where the DNIC effect was higher in females then in males: they divided the different studies depending on how the pain thresholds of the test stimulus had been measured: when measuring with a VAS scale one out of 10 studies rated a higher DNIC effect for females (Quiton and Greenspan, 2007), 2 out of 5 rated a higher percentage of DNIC effect for females when test stimulus was analyzed with pain thresholds as we have done in this study (Martikainen et al., 2004; Rosen et al., 2008) and finally using nociceptive flexion reflex 2 out of 3 showed a higher percentage in DNIC for females (France and Suchowiecki, 1999; France and Suchowiecki, 2001).

To conclude, pain modulation has shown to depend on numerous variables as gender, anxiety, distraction or type of stimulation. The way each of these variables interact to finally result in pain perception is not yet clear. Future experiments are needed to elucidate how psychological factors and different types of pain stimulation affect endogenous pain modulation.

CONCLUSIONS

1. CONCLUSIONS DERIVED FROM ANIMAL STUDIES

1. A model to study spinally innervated muscle pain was developed. This model allows the comparison with the masseter pain model already described in the literature
2. Opioid and cannabinoids have an antinociceptive effect in the models of acute muscle pain used in this thesis, although the participation of central and peripheral receptors differ depending on the muscle tested:
   a. In the masseter both, peripheral and central opioid receptors participate in the antinociceptive effect of opioids, whilst in the spinally innervated muscles only central receptors are involved.
   b. The antinociceptive effect of cannabinoids is mediated by different cannabinoid receptors depending on the muscle and type of administration.
      - In the masseter: both, CB_1 and CB_2 receptors, are involved when cannabinoids are systemically and locally administered
      - In the gastrocnemius:
        - CB_1 receptors are involved when cannabinoids are systemically administered
        - Both receptors participate when cannabinoids are administered locally

The differences found in the antinociceptive involvement in the receptors in the different muscles may open opportunities to develop selective treatments.

2. CONCLUSIONS DERIVED FROM HUMAN STUDIES

3. Healthy females have lower pain thresholds to different stimuli than males.
4. The application of painful and non-painful stimuli can modulate the perception of pain in a different manner in male and female volunteers.

RESUMEN

1. INTRODUCCIÓN

1.1 FISIOLOGÍA DEL DOLOR

En la actualidad La Asociación Internacional para el Estudio de Dolor (IASP) define el dolor como: "una experiencia sensorial o emocional desagradable, asociada a daño tisular real o potencial, o bien descrita en términos de tal daño". Es importante distinguir este concepto de él de nocicepción que se define como: "proceso neuronal mediante el que se codifican y procesan los estímulos potencialmente dañinos para los tejidos"

Para que la señal nociceptiva se convierta en sensación dolorosa, deben cumplirse los siguientes pasos (Kitahata, 1993):

1. Los estímulos potencialmente lesivos deben ser convertidos en señales electroquímicas (**Transducción**).
2. Estas señales viajan desde donde han sido generadas hasta el sistema nervioso central (**Transmisión**).
3. En el trayecto, el estímulo nociceptivo puede sufrir modificaciones, principalmente a nivel del asta dorsal de la médula espinal (**Modulación**).
4. Finalmente, estos procesos interactúan con factores individuales creando la sensación subjetiva y experiencia emocional llamada "dolor" (**Percepción**).

Así, los dos primeros pasos son de conducción ascendente del estímulo nociceptivo, pero el sistema nervioso central también tiene la capacidad de modular la señal nociceptiva que entra en este sistema.

En el organismo se pueden distinguir dos sistemas sensoriales diferentes:

- El sistema espinal que inerva todo el organismo a excepción de la región orofacial.
- El sistema trigeminal que recoge la sensibilidad de la mayor parte de la región orofacial.

Estos dos sistemas tienen ciertas similitudes pero también diferencias que hacen que no sea posible extrapolar los hallazgos en un sistema directamente al otro. Estas diferencias han sido revisadas por Sessle (Sessle, 2005), entre las que se

encuentran diferencias en inervación, transmisión del estímulo nociceptivo así como condiciones patológicas específicas de la región orofacial.

1.2 DOLOR MUSCULAR

El primer abordaje hacia el estudio del dolor muscular fue publicado con los trabajos pioneros de Lewis y Kellgren, de esta manera introdujeron el concepto de dolor muscular experimental (Kellgren, 1938). En las dos últimas décadas el estudio de este tipo de dolor ha despertado un gran interés, debido a que es una de las principales causas de incapacidad, problemas sanitarios y supone un gran coste económico en el mundo. De aquí la necesidad de estudiar su fisiopatología y nuevas dianas farmacológicas para su tratamiento (Badley et al., 1994).

Hasta el momento el tejido mejor estudiado es la piel, pero numerosas diferencias han sido encontradas entre piel y músculo. Estas diferencias han sido revisadas por Walder y Sluka (Walder and Sluka, 2008). Así, estas diferencias se traducen en una necesidad de esclarecer la fisiopatología subyacente para así poder desarrollar mejores herramientas diagnósticas y dianas farmacológicas que mejoren el tratamiento de distintas condiciones patológicas (Arendt-Nielsen and Graven-Nielsen, 2008).

Para estudiar el dolor en animales y voluntarios sanos, diferentes modelos y tests de nocicepción pueden ser empleados. Básicamente consisten en la aplicación de un estímulo nocivo, ya sea térmico, mecánico, eléctrico o químico. Cuando los estudios se realizan en animales se estudia una conducta desarrollada tras la aplicación del estímulo nocivo, es decir se registran resultados objetivos. En humanos, debido a que es posible una comunicación verbal, también los parámetros subjetivos de dolor pueden ser registrados. La intensidad del dolor se mide, generalmente, mediante una escala visual analógica y para recoger la calidad y localización del dolor existen numerosos cuestionarios (Graven-Nielsen and Arendt-Nielsen, 2008).

Para el estudio del dolor muscular se ha empleado frecuentemente la administración intramuscular de sustancias algógenas. Entre estas una de las más utilizadas es el suero hipertónico (SH), tanto en animales como humanos, ya que no tiene efectos secundarios, ni se ha descrito ningún efecto tóxico tras su

administración. La inyección puede realizarse mediante un bolo o por infusión continua (para desencadenar un dolor mantenido en el tiempo).

Según el tipo de dolor que se quiera estudiar otros algógenos, como por ejemplo la formalina o la carragenina, pueden ser utilizados. La desventaja que presentan algunos de ellos es que, debido a sus secuelas, no pueden ser empleados en humanos.

1.3 OPIOIDES

El opio es el jugo que se extrae de las cápsulas de la adormidera (*Papaver somniferum*).

En los dos últimos siglos el conocimiento acerca del sistema opioide ha avanzado considerablemente con el aislamiento de la morfina (Sertürner, 1806), la demostración de la existencia de receptores específicos para estos compuestos en el sistema nervioso central (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973) y el descubrimiento del primer opioide endógeno (Hughes et al., 1975).

Los péptidos endógenos, los receptores a los que se les unen estos péptidos y las enzimas que los sintetizan y degradan se conocen como "sistema opioide endógeno". Este sistema participa en numerosas acciones fisiológicas y farmacológicas en el organismo.

Hasta la fecha, se conocen tres tipos de receptores opioides diferentes (μ, δ y κ) que se localizan tanto en el sistema nervioso central como periférico. La localización de los receptores opioides ha sido resumida por Lesniak y Lipkowski (Lesniak and Lipkowski, 2011) (Tabla 1).

LIGANDOS OPIOIDES

Al igual que los opioides endógenos, aquellos opioides administrados de manera exógena actúan sobre los receptores opioides. Los opioides pueden ser clasificados como agonistas o antagonistas.

Agonistas: son aquellos ligandos que al unirse al receptor lo activan e inducen un efecto similar que la molécula endógena.

Antagonistas: son aquellos ligandos que al unirse al receptor previenen la acción del agonista sobre el receptor o la respuesta subsiguiente, pero no tienen efecto por si solos.

En investigación, los agonistas y antagonistas opioides sirven como herramientas farmacológicas. En esta tesis se han empleado para determinar la participación de los receptores centrales y periféricos en el efecto antinociceptivo de los opioides. Por tanto aquí se describirán las características de aquellos que han sido empleados.

Agonistas de receptores opioides

Morfina: se une principalmente al receptor μ, aunque también a los receptores δ y κ. Se considera el fármaco de referencia cuando se están estudiando fármacos nuevos analgésicos. Debido a que atraviesa barrera hematoencefálica actúa tanto en sistema nervioso periférico como central.

Loperamida**:** se une principalmente a receptores de tipo μ. La diferencia fundamental con la morfina es que, tras absorberse bien en el tracto gastrointestinal, se metaboliza casi completamente en el hígado y sus metabolitos se excretan en la bilis. Debido a esto, la loperamida apenas pasa a la circulación sistémica y consecuentemente no alcanza el sistema nervioso central y por tanto se utiliza cuando se quieren evitar efectos centrales y, en investigación básica, se utiliza con frecuencia como herramienta para estudiar los efectos periféricos de los opioides (Guan et al., 2008).

Antagonistas de receptores opioides

Naloxona: es un antagonista, principalmente del receptor opioide μ, aunque a altas concentraciones puede unirse a los tres receptores. Atraviesa la barrera hematoencefálica, por lo que antagoniza los efectos centrales y periféricos de los opioides.

Naloxona metiodada: es un derivado cuaternario de la naloxona. Se une principalmente a receptores μ periféricos ya que no cruza barrera hematoencefálica y, por tanto, se utiliza para estudiar los efectos periféricos de los opioides (Brown and Goldberg, 1985; Goicoechea et al., 2008).

EFECTOS FARMACOLÓGICOS DE LOS OPIOIDES

La morfina y la mayoría de los agonistas opioides usados clínicamente ejercen sus efectos a través del receptor opioide μ, por lo que estos fármacos afectan a diversas funciones fisiológicas desencadenando distintos efectos como:

- Analgesia
- Efectos psicomiméticos
- Efectos respiratorios
- Efectos cardiovasculares
- Efectos genitourinarios
- Efectos gastrointestinales
- Efectos inmunológicos
- Tolerancia y dependencia

Por desgracia, con la unión de los opioides a sus receptores no sólo se puede obtener un efecto analgésico deseado, sino también varios efectos secundarios que pueden dificultar o incluso impedir su uso clínico. Entre los efectos secundarios los más frecuentes son: náuseas y vómitos, constipación, confusión o somnolencia. La mayoría de estos efectos secundarios se debe a la unión de los opioides a sus receptores en el sistema nervioso central. Así, los agonistas de los receptores opioides periféricos pueden tener importantes ventajas tales como la disminución de estos efectos secundarios y reducir la adicción a la vez que se mantiene el efecto analgésico.

OPIOIDES Y ANALGESIA

El efecto analgésico de los opioides se debe a la presencia de los receptores opioides en las vías que transmiten el dolor. Estos receptores no solo se encuentran en las estructuras localizadas en el sistema nervioso central, sino también en la periferia.

Mecanismos centrales de analgesia opioide

La activación de receptores opioides a nivel supraspinal puede aliviar el dolor a través de tres mecanismos diferentes:

- Activación de los sistemas inhibitorios de dolor descendentes.
- Modulación del componente afectivo y emocional del dolor.
- Inhibición de la transmisión ascendente de dolor.

La analgesia opioide espinal está directamente ligada a la supraespinal a través de los sistemas inhibitorios descendentes, de manera que ambos mecanismos no pueden actuar de una manera independiente. Se ha visto que los tres receptores opioides principales están presentes en el asta dorsal (Gouarderes et al., 1993).

En varios modelos experimentales de dolor en animales se ha demostrado que la morfina administrada de manera sistémica reduce la conducta nociceptiva, como por ejemplo en modelos de dolor inflamatorio (Sevostianova et al., 2005; Burgos et al., 2010) o en modelos de dolor neuropático (Erichsen et al., 2005).

Sin embargo, apenas existen estudios que demuestren el efecto antinociceptivo de los opioides en dolor muscular; la administración intratecal de morfina aumenta el umbral de dolor a la estimulación mecánica tras la administración repetida de suero ácido en el gemelo (Sluka et al., 2002) y el levorfanol revierte la hiperalgesia mecánica tras la inyección de carragenina en el tríceps (Kehl et al., 2000).

Mecanismos periféricos de analgesia opioide

A diferencia de la visión clásica de que la analgesia opioide está únicamente mediada por receptores en el sistema nervioso central, se ha demostrado que los receptores periféricos también median el efecto antinociceptivo cuando los agonistas opioides son administrados de forma local. Este efecto es mayor en condiciones de inflamación y ha sido demostrado tanto en humanos como en animales (Barber and Gottschlich, 1992; Stein and Lang, 2009).

Además de la administración local de fármacos, también se puede lograr el efecto analgésico deseado, y evitar los efectos secundarios, mediante la administración de agonistas opioides que no crucen barrera hematoencefálica. Como resultado se ha observado que agonistas que no cruzan la barrera hematoencefálica son beneficiosos en pacientes con dolor neuropático (Wallace et al., 2006) y visceral (Mangel et al., 2008). Uno de los primeros abordajes que abalaron esta posibilidad fue la administración intrarticular de morfina tras la cirugía de rodilla (Heine et al., 1994).

En estudios animales la administración local de morfina también ha demostrado prevenir la alodinia mecánica secundaria en un modelo inflamatorio (Ambriz-Tututi et al., 2009). Por otro lado, la administración de loperamida reduce la alodinia mecánica e hiperalgesia al calor en un modelo de dolor neuropático (Guan et al., 2008).

Opioides y dolor orofacial

Los primeros autores en demostrar la presencia de receptores opioides en el sistema trigeminal fueron Jessell e Iversen en 1977; demostraron que el tratamiento

con opioides inhibía la liberación de sustancia P en el cuerpo de las fibras aferentes primarias en rata (Jessell and Iversen, 1977). Posteriormente, se ha demostrado la existencia de ARNm para los tres tipos de receptores en el núcleo espinal del trigémino (Mansour et al., 1994). Unos años más tarde se encontraron receptores μ y δ co-expresados con sustancia P y con el péptido relacionado con el gen de la calcitonina (CGRP) (Li et al., 1998; Ichikawa et al., 2005).

En estudios de conducta, los opioides han sido estudiados por su efecto antinociceptivo tanto central como periférico. La morfina, administrada de forma sistémica, reduce las conductas nociceptivas en modelos de dolor inflamatorio (test de la formalina en la articulación temporomandibular) (Eisenberg et al., 1996; Burgos et al., 2010), en modelos de dolor muscular orofacial (Ro et al., 2003) y previene la alodinia mecánica (Deseure et al., 2002) e hiperalgesia térmica (Chichorro et al., 2009) en modelos de dolor neuropático.

Por otro lado, la administración local de morfina reduce la conducta nociceptiva en un modelo de dolor muscular (Han et al., 2008) y en ambas fases del test de la formalina (Eisenberg et al., 1996).

Así pues, a pesar de los estudios clásicos en los que se pensaba que eran los receptores opioides centrales los que mediaban el efecto antinociceptivo de los opioides, en los últimos años hay un creciente número de investigaciones dirigidas a estudiar los receptores periféricos como diana terapéutica. La ventaja que presentan estos últimos es que no participan en la mayor parte de los efectos secundarios derivados del tratamiento con opioides, lo que supone una gran ventaja a la hora de su prescripción.

1.4 CANNABINOIDES

Cannabis sativa, comúnmente conocido como marihuana, es una planta que pertenece a la familia de Cannabaceae.

Debido a los efectos psicoactivos del cannabis y la existencia de otros fármacos analgésicos (como la morfina), no ha sido hasta la segunda mitad del siglo XIX cuando realmente se ha profundizado en la investigación de sus posibles aplicaciones terapéuticas. Uno de los avances más importantes fue la caracterización del Δ^9-

Tetrahidrocannabinol (THC), que es el principal responsable de los efectos psicoactivos del cannabis (Gaoni and Mechoulam, 1964).

Actualmente se conoce la existencia de un sistema cannabinoide endógeno, que, al igual que el sistema opioide, está formado por receptores, cannabinoides endógenos y enzimas que regulan la concentración de los componentes de este sistema.

Hasta la fecha existen dos receptores identificados, CB_1 Y CB_2. Ambos pertenecen a la superfamilia de receptores acoplados a proteínas G. Entre ellos se diferencian, principalmente, en la secuencia de aminoácidos, en el mecanismo de transducción de señales al interior de la célula y en su distribución en los tejidos (Howlett et al., 2002).

Receptor CB_1:

Fue el primer receptor en ser clonado de la corteza cerebral de rata (Matsuda y col., 1990) poco después en humano y ratón (Gérard y col 1991; Chakrabarti y col., 1995). Presenta una homología en la secuencia de aminoácidos del 97-99% en ratón, rata y humano.

La distribución de los receptores en todas las especies es similar, aunque existen algunas diferencias como una mayor expresión de estos receptores en amígdala y córtex cingulado en humanos en comparación con rata y mono (Herkenham y col., 1990).

Este receptor está principalmente expresado en el sistema nervioso central y, en menor medida, en el periférico (Galiegue et al., 1995). En el sistema nervioso central es el receptor acoplado a proteína G más abundante, con niveles de concentración especialmente altos en el cuerpo estriado, cerebelo, ganglio basal, corteza cerebral e hipocampo (Herkenham et al., 1990; Herkenham et al., 1991).

Receptor CB_2:

Tres años después de que se descubriera el receptor CB_1 se clonó, a partir de células promielocíticas, un segundo receptor para cannabinoides denominado CB_2 (Munro et al., 1993). Este receptor en humanos presenta una homología en la secuencia de aminoácidos del 81 y 82% con ratón y rata, respectivamente (Shire et al., 1996; Griffin et al., 2000).

A diferencia del receptor CB_1, los receptores CB_2 están expresados principalmente en tejidos periféricos y especialmente en los tejidos del sistema inmune (Galiegue et al, 1995; Howlett et al, 2004).

LIGANDOS CANNABINOIDES

Al igual que los opioides, los fármacos cannabinoides pueden ser utilizados en investigación como herramientas farmacológicas. En esta tesis se han empleado los siguientes agonistas y antagonistas:

Agonistas:

WIN 55, 212-2: agonista no selectivo de los receptores cannabinoides CB_1 y CB_2.

ACEA: agonista selectivo del receptor cannabinoide CB_1.

JWH015: agonista selectivo del receptor cannabinoide CB_2.

Antagonistas:

AM251: antagonista selectivo del receptor cannabinoide CB_1.

AM630: antagonista selectivo del receptor cannabinoide CB_2.

En este caso no disponemos de fármacos que ejerzan su efecto a nivel preferentemente periférico, por lo que para analizar esta posibilidad hay que recurrir a la administración tópica de pequeñas cantidades de agonistas o antagonistas.

EFECTOS FARMACOLÓGICOS DE LOS CANNABINOIDES

Debido a la localización tan heterogénea de los receptores cannabinoides sus agonistas juegan un papel importante en la fisiología del organismo, así los cannabinoides juegan un papel importante en numerosos procesos y causan:

- Analgesia
- Cambios de humor, sensación de euforia, sedación y relajación
- Alteración de la percepción del tiempo y la memoria a corto plazo
- Actividad orexígena y antiemética
- Efectos sobre el tono muscular y la coordinación motora
- Reducción de la presión intraocular
- Hipotermia
- Broncodilatación
- Efectos cardiovasculares: hipotensión y taquicardia

- Efectos neuroendocrinos: inhibición de la liberación de hormonas relacionadas con el sexo y el aumento de la liberación de las hormonas relacionadas con el estrés
- Efectos inmunomoduladores: inmunoestimulación a dosis bajas e inmunoestimulación a dosis más altas
- Efectos antiproliferativos

La gran variedad de efectos fisiológicos de los cannabinoides hace que sean posibles "candidatos" en el tratamiento de numerosas patologías. Hasta la fecha ha habido pocos fármacos cannabinoides aprobados para uso clínico en España: Nabilona y Dronabinol, análogos estructurales de la molécula de THC, se prescriben como antieméticos en la terapia oncológica. Muy recientemente, se ha aprobado el Sativex® (cannabidiol y THC) para el tratamiento del dolor y la espasticidad relacionada con la esclerosis múltiple (Sastre-Garriga et al., 2011).

CANNABINOIDES Y ANALGESIA

Al igual que ocurre con el sistema opioide los agonistas cannabinoides tienen un efecto antinociceptivo debido a la presencia de este sistema en las estructuras que transmiten y modulan el dolor tanto a nivel central como periférico.

Mecanismos centrales de analgesia cannabinoide

A nivel supraspinal La presencia del receptor CB_1 es moderada o alta en aquellas estructuras involucradas en la transmisión y modulación de la señal nociceptiva como: el ganglio dorsal de la médula espinal, médula espinal, tálamo, sustancia gris periacueductal, amígdala y región rostroventromedial del bulbo raquídeo (Tsou et al., 1998).

Los primeros estudios indicaban que los receptores CB_2 no estaban presentes en las neuronas del sistema nervioso central, aunque recientemente se han localizado en varias estructuras del sistema nervioso central: se ha encontrado ARNm en la corteza, hipocampo, cerebelo y tronco cerebral (Van Sickle et al., 2005a). Una revisión más detallada sobre la distribución de los receptores CB_1 y CB_2 en el sistema nervioso central ha sido realizada por Svizenska y colaboradores (Svizenska et al., 2008).

En la médula espinal la mayoría de receptores CB_1 se han localizado en las capas superficiales de la médula espinal (capas que reciben la señal nociceptiva) (Hohmann and Herkenham, 1999). Varios estudios han demostrado que el efecto

antinociceptivo de los cannabinoides se debe a la interacción de agonistas directamente con receptores espinales (Lichtman and Martin, 1991; Welch and Stevens, 1992; Drew et al., 2000; Kelly and Chapman, 2001).

Por otro lado, recientemente se ha localizado receptores CB_2 en médula espinal (Beltramo et al., 2006). Los estudios demuestran que este receptor esta sobreexpresado en condiciones de sensibilización, mientras que está en el límite para su detección en animales sanos (Elmes et al., 2004; Nackley et al., 2004).

En estudios animales los cannabinoides han demostrado tener un importante efecto antinociceptivo en múltiples modelos, lo que ha sido revisado por Guindon y Hohman (Guindon and Hohmann, 2009). En modelos de dolor muscular tan solo un estudio ha analizado este efecto; donde el WIN 55,212-2 es capaz de mejorar la fuerza de agarre de la rata en un modelo inflamatorio en triceps (carragenina) y en un modelo de cáncer (Kehl et al., 2003).

Mecanismos periféricos de analgesia cannabinoide

Los receptores cannabinoides no solo han sido localizados en la médula espinal sino también en el ganglio dorsal de la médula espinal donde estos son sintetizados y transportados hacia las terminaciones de las fibras aferentes primarias.

Los dos tipos de receptores, CB_1 y CB_2, han sido localizados en las aferencias de las neuronas nociceptivas en el ganglio dorsal de la médula espinal de rata (Hohmann and Herkenham, 1999; Ahluwalia et al., 2000; Salio et al., 2002) y en fibras nerviosas cutáneas humanas mielinizadas y no mielinizadas (Stander et al., 2005). El receptor CB_1 ha sido localizado en el ganglio del trigémino (Price et al., 2003) y en músculo esquelético de humanos (Eckardt et al., 2009; Guindon and Hohmann, 2009).

Para evitar los efectos psicoactivos de los cannabinoides, agonistas CB_1 o no selectivos han sido administrados de forma local y han mostrado tener un efecto antinociceptivo en modelos de dolor agudo (Johanek and Simone, 2004), inflamatorio (Richardson et al., 1998; Gutierrez et al., 2007) y neuropático (Fox et al., 2001). Recientemente, se han sintetizado y estudiado nuevos agonistas CB_1 con poca capacidad para atravesar barrera hematoencefálica (Yu et al., 2010)(Cumella et al., 2012).

Debido a que los efectos psicoactivos son mediados por el receptor CB_1 los agonistas CB_2 podrían ser una buena alternativa como analgésicos, evitando los efectos secundarios. Varios estudios han demostrado su efecto antinociceptivo en modelos de dolor agudo (Malan et al., 2001), neuropático (Kinsey et al., 2011) e inflamatorio (Nackley et al., 2003; Gutierrez et al., 2007; Kinsey et al., 2011). También sería una alternativa los agonistas CB_1 con acciones fundamentalemente mediadas por efectos periféricos

Cannabinoides y dolor orofacial

El primer trabajo que estudió la participación del sistema cannabinoide en el dolor orofacial demostró la existencia de receptores CB_1 en el ganglio del trigémino, pero se vio que la mayor parte de estos receptores estaban asociados a neuronas no nociceptivas. En este mismo estudio no se encontraron evidencias del receptor CB_2 (Price et al., 2003). Hasta la fecha no se conoce con exactitud los mecanismos por los que los cannabinoides ejercen un efecto antinociceptivo a nivel trigeminal.

Dos estudios han demostrado el efecto antinociceptivo del WIN55,212-2 en dos modelos de dolor orofacial; uno neuropático (Liang et al., 2007) y otro inflamatorio (test de la formalina) (Burgos et al., 2010). Este efecto parece estar mediado a través de los receptores CB_1. Recientemente, se ha demostrado el efecto antinociceptivo de un agonista selectivo CB_1 que no atraviesa barrera hematoencefálica en un modelo de dolor muscular (Cumella et al., 2012).

En resumen: al igual que en el caso de opioides, actualmente se están sintetizando y estudiando nuevos agonistas del receptor CB_2 o agonistas del receptor CB_1 que no atraviesen barrera hematoencefálica, con el fin de obtener un efecto analgésico mediante la activación de receptores cannabinoides CB_1 periféricos o CB_2. De esta manera, seria posible que el paciente tuviese un tratamiento analgésico que a su vez careciera de los efectos secundarios que empeoran su calidad de vida.

1.5 MODULACIÓN CONDICIONADA DEL DOLOR

La mayor parte de las neuronas de amplio rango dinámico y algunas nociceptivas específicas pueden ser inhibidas mediante estímulos nocivos que se inducen fuera de su campo receptivo. Este fenómeno se traslada a la clínica como una reducción en la percepción de dolor frente a un estímulo cuando se induce dolor

mediante otro estímulo condicionante. Se ha demostrado que este fenómeno se da tanto en el asta dorsal de la médula como en el núcleo del trigémino. Se ha propuesto que el nivel de supresión de dolor, mientras se induce dolor a través del estímulo condicionante, refleja la eficacia del sistema de control inhibitorio difuso.

Este sistema lo describió Le Bars et al. (Lebars et al., 1979) por primera vez. Hoy se sabe que consiste en un "circuito" que involucra a la médula y bulbo, aunque existen publicaciones recientes que demuestran que estructuras corticales pueden influir en la respuesta de este sistema, lo que explicaría el por qué factores psicológicos pueden modificarlo (Goffaux et al., 2007).

Varias investigaciones han demostrado que este sistema está alterado en algunas condiciones patológicas crónicas en humanos (Pielsticker et al., 2005; Leonard et al., 2009) y juega un papel en el mantenimiento de la sensibilización central e hiperalgesia generalizada en modelos animales de dolor (Porreca et al., 2002; Tillu et al., 2008).

A pesar de los numerosos estudios que hay sobre este tema, todavía quedan numerosos aspectos por esclarecer, ya que los resultados son contradictorios. Algunos de estos aspectos son: si el sistema de control inhibitorio difuso es más eficaz en un género u otro, si el estímulo condicionado necesita ser doloroso o el tamaño del área estimulada para inducir el efecto inhibitorio.

Parte de los ensayos experimentales de esta tesis doctoral están relacionados con el sistema de control inhibitorio difuso en voluntarios sanos. Estos ensayos se realizaron durante una estancia breve de tres meses en el Laboratorio de Dolor Orofacial, en Sensory Motor Interaction Center, Aalborg, Dinamarca.

2. OBJETIVOS

Los objetivos de esta tesis se dividen en dos grandes grupos:

1. Estudiar el efecto antinociceptivo de fármacos opioides y cannabinoides en un modelo de dolor muscular orofacial *vs* un modelo de dolor muscular de inervación espinal.
    a. Desarrollar un modelo de dolor muscular de inervación espinal que permita la comparación con el dolor muscular orofacial.
    b. Estudiar la participación de receptores opioides y cannabinoides, centrales y periféricos, en estos modelos.

2. Estudiar la modulación de la percepción de dolor en dos modelos de dolor muscular orofacial en voluntarios sanos.

Los experimentos del primer objetivo se realizaron en el Departamento de Farmacología y Nutrición de la Universidad Rey Juan Carlos, Madrid España. Los experimentos correspondientes al segundo objetivo fueron realizados en The Sensory Motor Interaction Center, Alborg, Dinamarca.

EFECTO ANTINOCICEPTIVO DE OPIOIDES Y CANNABINOIDES EN EL DOLOR MUSCULAR

3. MATERIAL Y MÉTODOS

3.1 ANIMALES

Para este trabajo se han empleado ratas macho Wistar (250-300 g). Los animales fueron suministrados por la Unidad Veterinaria de la Universidad Rey Juan Carlos y se mantuvieron en el animalario de esta misma Universidad. Durante su estabulación se les administró agua y bebida "ad libitum", se mantuvieron a una temperatura constante de 23±1ºC y bajo ciclos de luz/oscuridad de 12 horas. A todos los animales se les permitió un periodo de adaptación de al menos dos días antes de comenzar los experimentos. Todos los experimentos fueron revisados y aprobados por el Comité de Ética de la Universidad antes de su realización.

3.2 FÁRMACOS

Los siguientes opioides y cannabinoides fueron empleados para los experimentos de esta tesis doctoral:

OPIOIDES

Agonistas:

- Morfina (Sigma-Aldrich Química, Madrid, España).
- Loperamida (Alcaliber, Madrid, España).

Antagonistas:

- Naloxona (Sigma-Aldrich Química, Madrid, España).
- Naloxona metiodada (Sigma-Aldrich Química, Madrid, España).

CANNABINOIDES

Agonistas:

- WIN 55,212-2 (WIN)(Sigma-Aldrich Madrid, España).
- ACEA (TOCRIS, Biogen Científica S.L. Madrid, España).
- JWH015 (Sigma-Aldrich Madrid, España).

Antagonistas

- AM251 (TOCRIS, Biogen Científica S.L. Madrid, España).
- AM630 (TOCRIS, Biogen Científica S.L. Madrid, España).

Los fármacos fueron disueltos de la siguiente manera:

La morfina, naloxona y naloxona metiodada en solución salina (NaCl 0.9%)

La loperamida en cremophor 20% (Dehaven-Hudkins et al., 1999).

Todos los cannabinoides, excepto el JWH015, en etanol 1 mg: 1 ml y posteriormente en etanol y Tween 80 (1:2) (TE), posteriormente el etanol se evaporó y finalmente se añadió salino hasta llegar a la concentración final requerida (Pertwee et al., 1992).

El JWH015 fue disuelto en Tocrisolve (TOCRIS, Biogen Científica S.L. Madrid, España), 1mg/50 µl, las dosis de 3 mg/kg y mayores tuvieron que ser sonicadas para poder ser disueltas.

MODELOS ANIMALES DE DOLOR

El algógeno usado en todos los modelos de dolor muscular de esta tesis ha sido el suero hipertónico (NaCl 5%) (SH). El SH ha sido inyectado sobre tres músculos: 1 de inervación trigeminal (masetero) y dos de inervación espinal (tríceps y gemelo).

MODELO DE DOLOR DE MASETERO

Este modelo se ha llevado a cabo tal y como lo describió Ro y col (Ro et al., 2003), a excepción de la metodología para anestesiar a las ratas, para lo que se administró equitesín (i.p.); para mantener el nivel de anestesia deseado se administró 0.2 ml de Equitesin cada 30 min. Este modelo consiste en inyectar SH en el masetero de ratas que están ligeramente anestesiadas a través de una cánula. La administración i.m. de SH provoca una conducta nociceptiva que consiste en sacudidas rápidas de la pata trasera ipsilateral, lo que se considera índice nociceptivo. En estos experimentos contamos el número de sacudidas durante 2.5 minutos.

Antes de comenzar el experimento se comprobó que el animal estuviese en estado de anestesia superficial mediante la valoración de la conservación del reflejo de retirada de la pata trasera y contracción abdominal.

Los primeros experimentos fueron llevados a cabo con el fin de poner en marcha el modelo de dolor, para lo que se administraron diferentes volúmenes de SH (50 o 100 µl) en distintos grupos de ratas.

El segundo grupo de experimentos se llevó a cabo para determinar si esta conducta era reproducible bajo nuestras condiciones experimentales, para ello se realizaron dos infusiones de SH separadas entre si por 30 min.

MODELO DE DOLOR DE GEMELO Y TRICEPS

Para poner en marcha el modelo, la primera prueba fue inyectar SH en el gemelo de rata superficialmente anestesiada, pero esto no indujo ninguna conducta cuantificable. Sin embargo, esta misma inyección, en rata despierta indujo una conducta de protección en la que la rata permaneció con la pata levantada o mal apoyada. Se cronometró durante 5 min el tiempo que la rata permaneció en esta postura y esta conducta se consideró índice nociceptivo.

Con el fin de validar el modelo se realizaron tres grupos experimentales distintos, tanto en gemelo como triceps:

1. Grupo sham: en el que el animal recibió el pinchazo con la jeringa de insulina pero sin administrar ninguna sustancia.
2. Grupo control: al que se le administró 0.5 ml de SH en el músculo.
3. Grupos experimentales: a 3 grupos distintos de ratas se les inyectó un volumen distinto de SH (0.1, 0.25, 0.5 ml) en el músculo.

Finalmente se valoró si este modelo era reproducible, para lo que se inyectó tres veces SH en el mismo músculo, en intervalos de 30 min.

3.3 PROTOCOLO

Cada grupo experimental está compuesto por al menos 6 ratas y cada animal tan solo se empleó para un experimento.

Antes de comenzar con los grupos experimentales de fármacos se realizaron los siguientes controles:

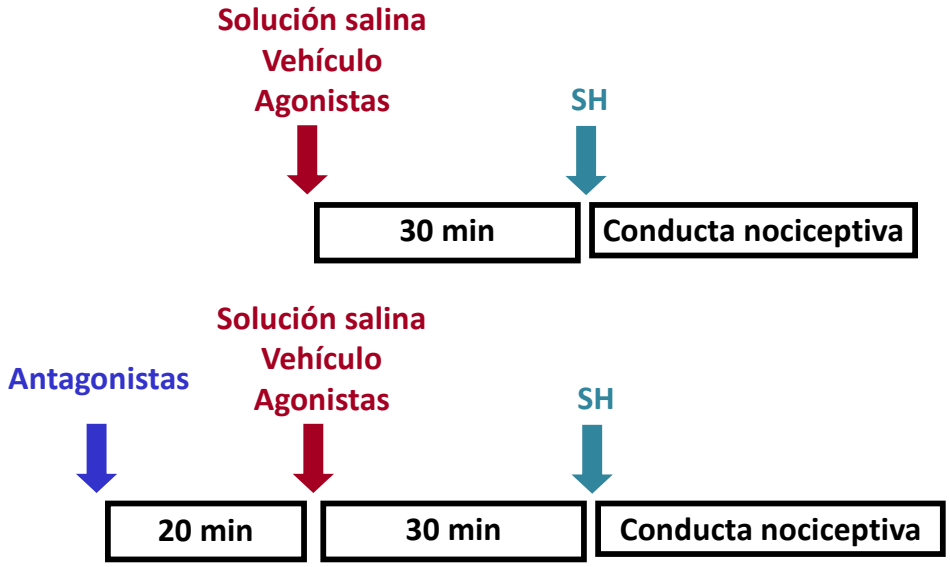
1. Administración de solución salina en cada músculo, con el fin de asegurar que la conducta nociceptiva es debida al SH y no a la manipulación animal
2. Administración de vehículos i.p. o i.m. para comprobar que no modifican la respuesta al SH.

Todos los fármacos administrados por via i.p. se administraron en un volumen de 1,5 ml/kg. Los fármacos administrados por via i.m. se administraron en un volumen de 30 µl (masetero) o 100 µl (gemelo).

EVALUACIÓN DEL EFECTO ANTINOCICEPTIVO DE OPIOIDES

Modelos de dolor en masetero, gemelo y triceps

Para llevar a cabo la evaluación del efecto antinociceptivo de los opioides en los modelos de dolor muscular, todos los fármacos fueron administrados por vía i.p. siguiendo el siguiente protocolo experimental:



Las dosis de opioides de cada grupo experimental están recogidas en las siguientes tablas:

MASETERO		
TRATAMIENTO OPIOIDE		**DOSIS** (mg/kg)
Agonistas	Morfina	0.6, 1.25, 2.5
	Loperamida	0.6, 1.25, 2.5
Antagonistas	Naloxona	1
	Naloxona metiodada	1
Antagonistas + Agonistas	Naloxona + Morfina	0.5 + 1.25
		1 + 1.25
		2 + 1.25
	Naloxona + Loperamida	0.5 + 1.25
		1 + 1.25
	Naloxona metiodada + Morfina	0.5 + 1.25
		1 + 1.25
		2 + 1.25
	Naloxona metiodada + Loperamida	0.5 + 1.25
		1 + 1.25

		GEMELO	**TRICEPS**
TRATAMIENTO OPIOIDE		**DOSIS** (mg/kg)	**DOSIS** (mg/kg)
Agonistas	Morfina	2.5, 5, 10	2.5, 5, 10
	Loperamida	5, 10	5, 10
Antagonistas	Naloxona	1	1
	Naloxona metiodada	1	1
Antagonistas + Agonistas	Naloxona + Morfina	1 + 10	1 + 10
	Naloxona metiodada + Morfina	1 + 10	1 + 10

Test de retirada de la cola ("Tail-flick")

Este test consiste en la aplicación de un estímulo térmico de alta intensidad en la cola de la rata, lo que provoca un reflejo de retirada. Para este test se utilizó un aparato estandarizado (Analgesic-Meter LI7106, Letica Scientific Instruments). La intensidad de la fuente de calor se ajustó para inducir el reflejo de retirada de la cola en un tiempo entre 2-4 s, y el tiempo de corte se estableció de 10 s. Los resultados obtenidos se expresan como el porcentaje del máximo efecto posible (% M.E.P):

% M.E.P =(test-basal)/(tiempo de corte-basal)*100

Test: tiempo de respuesta tras el tratamiento con opioides

Basal: tiempo de respuesta basal (antes de la administración de opioides)

Este test se realizó tanto en ratas despiertas como en ratas superficialmente anestesiadas.

El protocolo de administración de fármacos en este test fue igual que para los modelos de dolor muscular. Las dosis de opioides de cada grupo experimental están recogidas en las siguientes tablas:

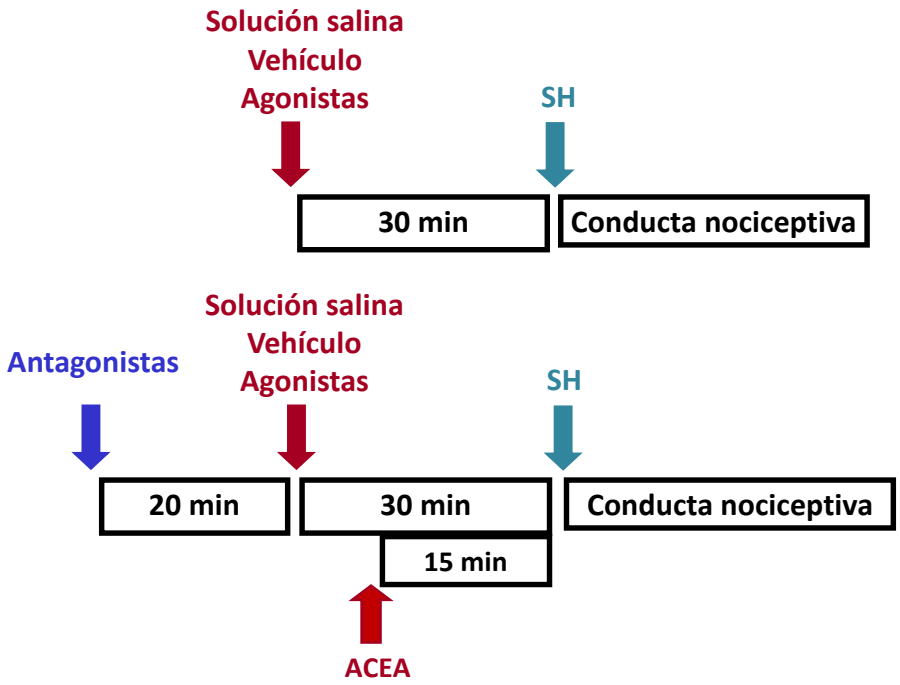
		RATAS CONSCIENTES	**RATAS ANESTESIADAS**
TRATAMIENTO OPIOIDE		**DOSIS (mg/kg)**	**DOSIS (mg/kg)**
Agonistas	Morfina	2.5, 5, 10	2.5, 5, 10
	Loperamida	5, 10	5, 10
Antagonistas + Agonistas	Naloxona + Morfina	0.5 + 10 1 + 10	1 + 10
	Naloxona metiodada + Morfina	1 + 10 2 + 10	1 + 10

EVALUACIÓN DEL EFECTO ANTINOCICEPTIVO DE CANNABINOIDES

La evaluación del efecto antinociceptivo de cannabinoides se realizó en el modelo de dolor de masetero y gemelo. Para estudiar la participación de los receptores centrales y periféricos los cannabinoides se administraron por dos vías distintas: sistémica (i.p.) y local (i.m.).

Administración sistémica de cannabinoides

La siguiente figura muestra el protocolo de administración de los fármacos cannabinoides en los dos modelos de dolor muscular:



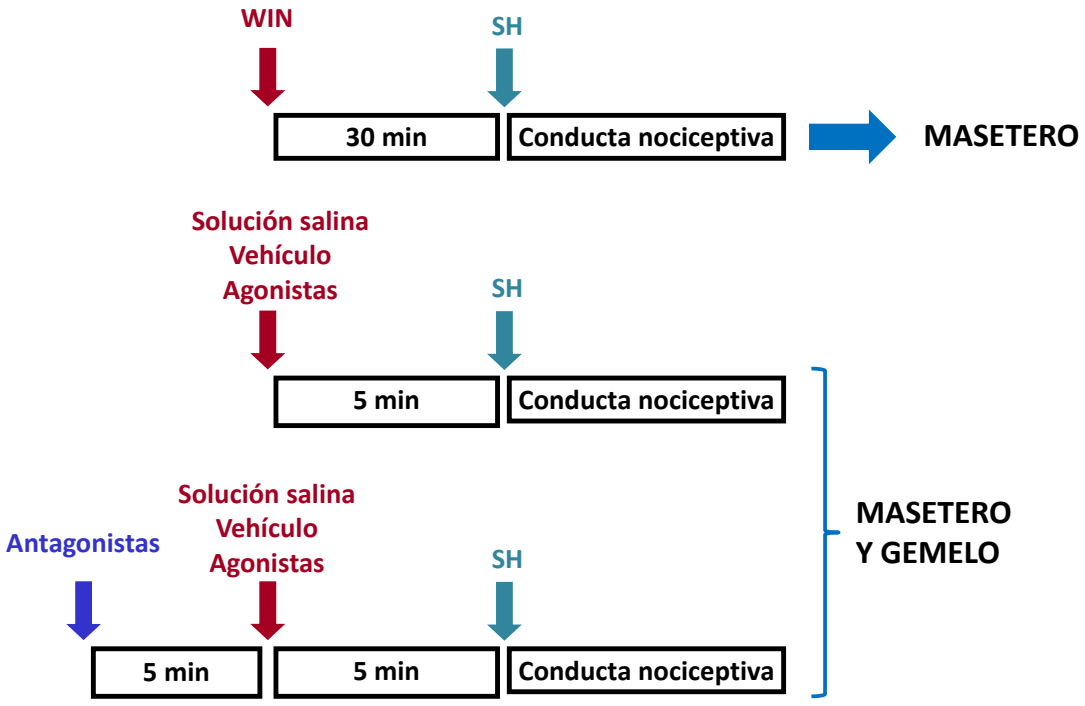
Las dosis de cannabinoides de cada grupo experimental están recogidas en las siguientes tablas:

MASETERO		
TRATAMIENTO CANNABINOIDE		**DOSIS (mg/kg)**
Agonistas	WIN 55,212-2	0.25, 0.5, 1, 3
	ACEA	0.125, 0.5, 1, 3
	JWH015	1, 3, 5
Antagonistas	AM251	0.5
	AM630	0.5
Antagonistas + Agonistas	AM251 + WIN 55,212-2	0.5 + 1
	AM251 + ACEA	0.5 + 1
	AM630 + WIN	0.5 + 1
	AM630 +JWH015	0.5 + 5

GEMELO		
TRATAMIENTO CANNABINOIDE		**DOSIS (mg/kg)**
Agonistas	WIN 55,212-2	0.5, 1, 3
	ACEA	0.5, 1
	JWH015	3, 5
Antagonistas	AM251	0.5
	AM630	0.5
Antagonistas + Agonistas	AM251 + WIN 55,212-2	0.5 + 1
	AM251 + ACEA	0.5 + 0.5
	AM630 + WIN 55,212-2	0.5 + 1

Administración local de cannabinoides

La siguiente figura muestra el protocolo de administración de los fármacos cannabinoides en los modelos de dolor muscular. En masetero, el efecto del WIN fue estudiado administrándolo 30 y 5 min antes de la inyección de SH, en gemelo tan solo se administró 5 min antes:



Las dosis de cannabinoides de cada grupo experimental están recogidas en las siguientes tablas:

MASETERO		
TRATAMIENTO CANNABINOIDE		**DOSIS (mg/kg)**
Agonistas	WIN 55,212-2	0.05, 0.1, 0.2 (30 min)
		0.0125, 0.01285, 0.025, 0.05 (5 min)
	ACEA	0.00625, 0.0125, 0.025
	JWH015	0.025, 0.05
Antagonistas	AM251	0.02
	AM630	0.02
Antagonistas + Agonistas	AM251 + WIN 55,212-2	0.01+0.0185
		0.02 + 0.0185
	AM251 + ACEA	0.02 + 0.0125
		0.04 + 0.0125
		0.5 (i.p.) + 0.0125
	AM630 + WIN	0.02 + 0.0185
	AM630 + JWH015	0.02 + 0.05

GEMELO		
TRATAMIENTO CANNABINOIDE		**DOSIS (mg/kg)**
Agonistas	WIN 55,212-2	0.0125, 0.05, 0.1
	ACEA	0.0125, 0.025, 0.05
	JWH015	0.0125, 0.05
Antagonistas	AM251	0.02
	AM630	0.04
Antagonistas + Agonistas	AM251 + WIN 55,212-2	0.02 + 0.05
	AM251 + ACEA	0.02 + 0.0125
	AM630 + WIN 55,212-2	0.02 + 0.05
		0.04 + 0.05
	AM630 + JWH015	0.02 + 0.0125
		0.04 + 0.0125

Tetrada cannabinoide

Para evaluar el posible efecto psicoactivo de los agonistas cannabinoides se realizó la tetrada cannabinoide 20 min tras la administración de WIN 0.5-1 mg/kg. Los siguientes parámetros fueron evaluados:

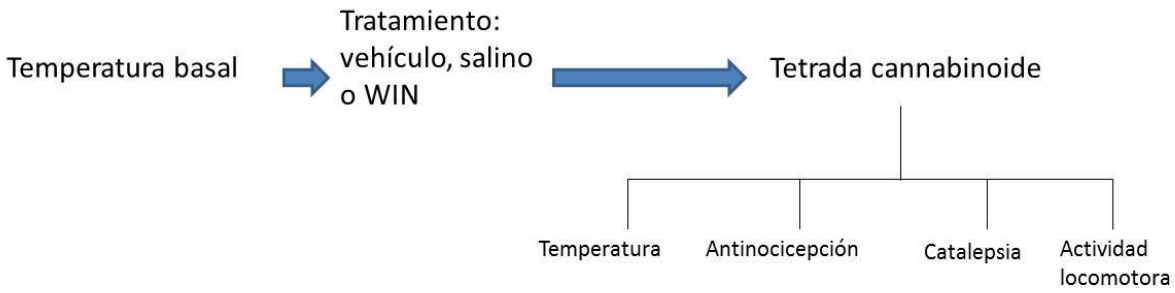
Temperatura rectal: se midió antes (temperatura basal) y 20 min después del tratamiento con WIN usando un termómetro P6 (Cibertec, España)

Antinocicepción: se utilizó el aparato Randall-Selitto (Analgesic-Meter LE7306, Letica Scientific Instruments). Los umbrales de dolor a la presión en el gemelo fueron medidos como describieron Schafers et al. (Schafers et al., 2003). Se realizaron 3 medidas por rata y se consideró la media de los valores obtenidos como el umbral de respuesta.

Catalepsia: se evaluó según lo descrito por Fox y col (Fox et al., 2001). Se realizaron 3 medidas por rata y se consideró la media de los valores obtenidos como la medida de catalepsia.

Actividad locomotora: la evaluación de la actividad espontánea se realizó utilizando una jaula dotada con células fotoeléctricas (CIBERTEC, España). Con este sistema se registra el número de interrupciones debidas al movimiento del animal durante 30 min.

Estos cuatro parámetros se analizaron en grupos control (salino y tween etanol) y en dos grupos tratados: WIN 0.5 mg/kg y WIN 1 mg/kg. La secuencia y protocolo viene ilustrada en la siguiente figura:



3.4 ANÁLISIS ESTADÍSTICO

El análisis estadístico se realizó mediante el test de ANOVA de una via y el análisis posthoc empleado fue Newman-Keuls. Para esto se utilizó el programa Prism© (GraphPad software). En todos los análisis se consideró una diferencia estadísticamente significativa a partir de una $p<0.05$.

4. RESULTADOS

4.1 PUESTA EN MARCHA DE LOS MODELOS DE DOLOR

MODELO DE DOLOR MUSCULAR DE MASETERO

Se utilizaron dos grupos experimentales a los que se les administró dos volúmenes distintos de suero hipertónico (SH), 50 y 100 µl. La administración de ambos volúmenes indujeron una conducta nociceptiva semejante (Fig. 12). Para posteriores experimentos se eligió un volumen de 100 µl como se ha descrito previamente en la literatura.

Para determinar si esta conducta era reproducible, bajo nuestras condiciones experimentales, se inyectó SH dos veces, cada inyección separada de la otra por un intervalo de 30 min. No se observaron diferencias estadísticamente significativas al comparar las respuestas inducidas por las dos administraciones de SH (Fig. 13).

MODELOS DE DOLOR MUSCULAR EN GEMELO Y TRICEPS

Para desarrollar un modelo de dolor muscular de la manera más parecida al modelo de dolor en masetero, se inyectó SH en el gemelo y tríceps de rata anestesiada. Esto no indujo ninguna respuesta así que lo siguiente fue probar la inyección de SH en rata despierta; la administración i.m. de 0.5 ml de SH indujo que la rata se mantuviera con la pata levantada o mal apoyada. El tiempo que la rata permaneció así se cronometró durante 5 min y se consideró como índice nociceptivo. Finalmente, la administración de diferentes volúmenes de SH (0.1, 0.25, 0.5 ml) provocaron esta conducta de forma volumen dependiente (Fig. 14).

A continuación se probó si este modelo era reproducible; se realizaron 3 administraciones de SH separadas entre si por 30 min. No se observaron diferencias estadísticamente significativas entre las conductas nociceptivas inducidas por las distintas administraciones de SH (Tabla 10).

El resto de experimentos se llevaron a cabo administrando 0.5 ml de SH.

4.2 EFECTO ANTINOCICEPTIVO DE LOS OPIOIDES

A continuación se expondrán los resultados obtenidos de estos experimentos:

Modelo de dolor muscular en masetero

- La administración sistémica de morfina y loperamida redujo el número de sacudidas inducidas por la administración de SH de manera dosis dependiente (Fig. 15).
- El efecto de la morfina fue antagonizado por la naloxona y naloxona metiodada mientras que el efecto de la loperamida tan solo fue antagonizado por la naloxona metiodada (Fig. 16).
- La administración de antagonistas o vehículo no modificó la respuesta inducida por el SH (Tabla 11).

Modelo de dolor muscular en gemelo y triceps

- La morfina tuvo efecto antinociceptivo tanto en el modelo de dolor muscular en gemelo como tríceps, mientras que la loperamida no modificó el tiempo que la rata se mantuvo con la pata levantada o mal apoyada, tras la inyección de SH, al compararlo con el grupo control (Fig. 17).
- La administración de naloxona revirtió el efecto antinociceptivo de la morfina en ambos modelos, sin embargo, la administración de naloxona metiodada no antagonizó este efecto de la morfina (Fig. 18).
- La administración de antagonistas o vehículo no modificaron la respuesta inducida por el SH (Tabla 12).

Test de retirada de la cola

- Tanto en ratas despiertas como superficialmente anestesiadas, la morfina tuvo un efecto antinociceptivo mientras que la loperamida no modificó la respuesta de ninguno de los dos grupos experimentales en el test de retirada de la cola (Fig. 19).
- La administración de naloxona revirtió el efecto antinociceptivo de la morfina en este test. Sin embargo, al igual que sucedió en los modelos de dolor muscular en gemelo y tríceps, la administración de naloxona metiodada no antagonizó el efecto de la morfina (Fig. 20).

Como conclusión: en el efecto antinociceptivo de los opioides, en el moselo de dolor de masetero, participan tanto los receptores centrales como periféricos,

mientras que en los modelos musculares de inervación espinal tan solo parecen participar los receptores del sistema nervioso central.

4.3 EFECTO ANTINOCICEPTIVO DE LOS CANNABINOIDES

Modelo de dolor muscular de masetero

Administración sistémica

- La administración de WIN indujo un efecto antinociceptivo de forma dosis dependiente (Fig. 21), este efecto se antagonizó con los antagonistas selectivos delos receptores CB_1 y CB_2 (Fig. 22).
- Los agonistas selectivos, ACEA y JWH015, también redujeron el número de sacudidas inducidas por la administración i.m. de SH (Fig. 23). Este efecto se antagonizó por la administración previa por sus antagonistas selectivos, AM251 y AM630 respectivamente (Fig. 24).

Administración local

El estudio del efecto antinociceptivo de los cannabinoides mediante su administración local, produjo resultados semejantes a los que se obtuvieron con su administración sistémica:

- La administración de WIN indujo un efecto antinociceptivo de forma dosis dependiente tanto si se administra 30 como 5 min antes que el SH (Fig. 25). Esta respuesta se antagonizó con los antagonistas selectivos de los receptores CB_1 y CB_2 (Fig. 26).
- De la misma manera que el WIN, los agonistas selectivos, ACEA y JWH015, también indujeron un efecto antinociceptivo en este modelo (Fig. 27) que fue antagonizado por sus antagonistas selectivos, AM251 y AM630 respectivamente (Fig. 28).
- La administración de los antagonistas y vehículos (tanto por vía sistémica como intramuscular) no modificó la respuesta nociceptiva inducida por la administración de SH (Tablas 13 y 14).

Modelo de dolor muscular en gemelo

Administración sistémica

- La administración sistémica de WIN indujo un efecto antinociceptivo (Fig. 29) que fue antagonizado por el antagonista selectivo CB_1 pero no por el CB_2 (Fig. 30).

- En línea con los resultados anteriores, la administración de ACEA tuvo un efecto antinociceptivo, estadísticamente significativo, mientras que esto no se observó con la administración de JWH015 (Fig. 31). El antagonista AM251 revirtió el efecto del ACEA (Fig. 32).

Administración local

- La administración de WIN indujo un efecto antinociceptivo (Fig. 33). Esta respuesta se antagonizó con los antagonistas selectivos CB_1 y CB_2 (Fig. 34).
- A su vez, los agonistas selectivos, ACEA y JWH015, también indujeron un efecto antinociceptivo en este modelo (Fig. 35) que fue antagonizado por sus antagonistas selectivos, AM251 y AM630 respectivamente (Fig. 36).

Para concluir: En el modelo de dolor muscular de masetero, el efecto antinociceptivo de los cannabinoides, administrados por vía sistémica e intramuscular, está mediado por ambos receptores cannabinoides (CB_1 y CB_2) tanto en sistema nervioso periférico como central.

En el modelo de dolor muscular tan solo participa el receptor CB_1 en la administración sistémica, mientras que en la administración local participan ambos receptores.

Tetrada cannabinoide

La administración sistémica de WIN 0.5-1 mg/kg no modificó la temperatura corporal ni indujo catalepsia. Ambas dosis indujeron un efecto antinociceptivo de manera dosis dependiente y con la dosis de 1 mg/kg se observó una reducción en la actividad locomotora (Fig. 37).

5. DISCUSIÓN

Como ya se ha descrito en la introducción de esta tesis, el sistema trigeminal tiene determinadas características que lo diferencian del sistema espinal. Teniendo esto en cuenta no se puede asumir que los descubrimientos farmacológicos que se dan en un sistema se puedan aplicar al otro de forma directa.

Recientemente se ha descrito un nuevo modelo de dolor muscular orofacial (Ro et al., 2003). Este modelo consiste en la administración, intramuscular, de sustancias algógenas en el masetero de la rata, esta administración induce una respuesta nociceptiva que consiste en numerosas sacudidas de la pata trasera ipsilateral. Este modelo permite una administración local de fármacos más fácil, a la vez que la manipulación de la rata es más sencilla, ya que se desarrolla en ratas ligeramente anestesiadas.

Entre todos los algógenos utilizados en investigación clínica y preclínica el SH es de los más usados, esto es debido a que reproduce las características del dolor muscular clínico y no presenta efectos secundarios (Stohler and Lund, 1994). Además otra de sus ventajas es que su efecto nociceptivo en el masetero de rata es fácilmente reproducible (Ro et al., 2003).

Para poder comparar los resultados obtenidos en el masetero de rata (inervación trigeminal) con un músculo de inervación espinal es conveniente que el algógeno que induce el efecto nociceptivo sea el mismo. En el momento de iniciar esta tesis no existía ningún modelo de dolor en músculo espinal en el que se empleara el SH como algógeno. Por tanto, nuestro primer objetivo fue desarrollar un modelo de dolor muscular que permitiese esta comparación y elegimos los músculos gemelo y triceps.

Con el fin de reproducir el modelo de Ro et al. de la manera más semejante en músculos de inervación espinal primero se administró el SH en el gemelo o tríceps de ratas superficialmente anestesiadas para ver qué efecto producía, pero no indujo ninguna conducta nociceptiva. El mismo procedimiento se repitió en ratas despiertas y observamos que el SH producía la retirada de la pata o mal apoyo de ésta tras inyectar el SH en el músculo; esta conducta se consideró índice nociceptivo y se valoró durante 5 min. Además, pudimos comprobar que la administración de distintos volúmenes de SH en gemelo o tríceps indujo una respuesta nociceptiva volumen dependiente.

Estos dos modelos han sido utilizados en los experimentos de esta tesis doctoral como un primer abordaje para comparar los efectos antinociceptivos de opioides y cannabinoides en estos dos sistemas. No obstante, una vez finalizados los experimentos en modelos agudos, estudios en modelos de dolor crónico podrán ser más interesantes debido a su repercusión clínica.

5.1 EFECTOS CENTRALES Y PERIFÉRICOS DE LOS OPIOIDES EN DOLOR MUSCULAR AGUDO

El objetivo del primer grupo de experimentos de esta tesis fue estudiar el efecto antinociceptivo de fármacos opioides y la participación de receptores opioides centrales y periféricos en dicho efecto, en tres modelos de dolor muscular agudo; uno de inervación trigeminal (masetero) y dos de inervación espinal (gemelo y tríceps).
Como era de esperar, la administración sistémica de morfina redujo la conducta nociceptiva inducida por la inyección de SH en los tres músculos estudiados. Además, este efecto se antagonizó mediante la administración previa de naloxona. La administración de naloxona metiodada (fármaco de acción periférica) previa a la morfina, en los tres modelos de dolor, revirtió el efecto de la morfina tan solo en el masetero, lo que hace pensar que solo en este tipo de dolor participan receptores opioides periféricos.

Con el fin de contrastar estos resultados, se realizaron los mismos experimentos con loperamida (agonista opioide periférico); la loperamida indujo un efecto antinociceptivo únicamente en el masetero, el cual se bloqueó tanto con naloxona como con naloxona metiodada. La loperamida no modificó la respuesta inducida por SH ni en gemelo ni en tríceps. Por tanto, estos resultados confirman la participación de receptores opioides periféricos en el modelo de dolor muscular orofacial.

A pesar de que se puede considerar que 10 mg/kg de morfina es una dosis elevada, el efecto antinociceptivo observado en el dolor de gemelo o tríceps con esta dosis no puede ser atribuido a una alteración en la locomoción ya que estudios previos en nuestro laboratorio han descartado esta posibilidad (Burgos et al., 2010).

Para comprobar si la diferencia encontrada en la participación de receptores opioides centrales y periféricos pudiera deberse a la diferencia en la metodología utilizada en los modelos de dolor muscular (en masetero se usan animales anestesiados y en gemelo y tríceps, animales conscientes), decidimos evaluar el efecto antinociceptivo de estos mismos opioides en el test de la retirada de la cola (D'amour and Smith, 1941), tanto en ratas despiertas como anestesiadas. En este test los opioides ejercen su efecto principalmente por su unión a receptores espinales (Le Bars et al., 2001). Los resultados obtenidos fueron semejantes a los de los experimentos realizados en los modelos de dolor muscular de gemelo y tríceps; tan solo la morfina indujo un efecto antinociceptivo que se revirtió únicamente por la administración previa de naloxona, mientras que la loperamida no produjo ningún efecto antinociceptivo. Estos resultados fueron iguales tanto en ratas conscientes como anestesiadas, lo que elimina la posibilidad de que la anestesia pueda ser la responsable de las diferencias encontradas entre los distintos músculos.

Los resultados obtenidos en el modelo de masetero coinciden con estudios realizados por otros autores, que demuestran que, la administración intramuscular de morfina reduce la conducta nociceptiva inducida por la administración de aceite de mostaza en el masetero de rata (Ro et al., 2003; Han et al., 2008). Además, se ha demostrado que la administración intramuscular de DAMGO (agonista selectivo para el receptor μ) reduce la conducta nociceptiva tras la inyección de SH en el masetero de rata y que, en condiciones de inflamación, este receptor está sobreexpresado en el ganglio del trigémino (Nunez et al., 2007).

También se ha demostrado el efecto antinociceptivo de opioides periféricos en otros modelos de dolor orofacial (Eisenberg et al, 1996; Pelissier et al, 2002) y en tejido visceral mediante el test del ácido acético (Labuz et al., 2007).

Los resultados obtenidos bajo nuestras condiciones experimentales, sugieren que los receptores opioides periféricos no participan en el efecto antinociceptivo de la morfina en los modelos de dolor muscular de tríceps y gemelo. Estudios previos en estructuras de inervación espinal han demostrado que los opioides, administrados de forma local, no tienen un efecto antinociceptivo cuando son administrados en tejidos no inflamados (Stein et al., 1989). Por el contrario, los receptores opioides periféricos participan en el control de la información nociceptiva en varios modelos de dolor

inflamatorio (Sevostianova et al, 2005; (Mecs et al., 2009; Pereira Santos et al., 2009). Además se ha demostrado, en el test de la formalina (modelo de dolor inflamatorio), que la analgesia periférica está asociada con un incremento en el transporte de receptores opioides hacia la periferia, aumento de la transcripción de ARNm y una mayor densidad de receptores opioides en el ganglio dorsal de la médula espinal (Obara et al., 2009).

Además, en nuestros experimentos las dosis de fármaco necesarias para inducir un efecto antinociceptivo en el dolor de masetero son menores que para obtener este mismo efecto en tríceps y gemelo, lo que confirma también que el efecto analgésico de los opioides en este tipo de dolor esté mediado por receptores periféricos. Esto podría presentar una ventaja importante en el tratamiento del dolor orofacial, ya que dosis menores de fármaco implican también menos efectos secundarios.

Para resumir: nuestros resultados demuestran que la morfina y loperamida modulan de forma diferente la respuesta nociceptiva inducida por el SH según el músculo que se esté estudiando (masetero, gemelo o tríceps). Estos datos sugieren que pueden existir diferencias fisiopatológicas en el dolor muscular según el tipo de músculo afectado.

5.2 EFECTOS CENTRALES Y PERIFÉRICOS DE LOS CANNABINOIDES EN DOLOR MUSCULAR AGUDO

En la misma línea experimental, el objetivo del segundo grupo de experimentos fue estudiar el efecto antinociceptivo de fármacos cannabinoides y la participación de receptores periféricos y centrales, CB_1 y CB_2, en los músculos masetero y gemelo. Para este estudio se emplearon los mismos modelos descritos anteriormente.

Existen numerosos estudios que demuestran el efecto antinociceptivo y antihiperalgésico de los cannabinoides tanto en animales como humanos (Martin Fontelles and Goicoechea Garcia, 2008; Elikkottil et al., 2009; Karst et al., 2010; Thaler et al., 2011).

Los experimentos realizados en esta tesis demuestran que los agonistas cannabinoides son capaces de reducir el efecto nociceptivo inducido por la administración intramuscular de SH. Este efecto fue estudiado mediante la

administración sistémica (i.p.) y local (i.m.) de agonistas no selectivos y selectivos de los receptores CB_1 y CB_2.

Los datos obtenidos en el modelo de dolor muscular de masetero demuestran que el agonista no selectivo, WIN 55,212-2, y los agonistas selectivos para los receptores CB_1 y CB_2, ACEA y JWH015 respectivamente, tienen un efecto antinociceptivo cuando se administran tanto por vía sistémica como local. Además los antagonistas selectivos CB_1 y CB_2, AM251 y AM630, antagonizaron el efecto del WIN 55,212-2 y el de sus correspondientes agonistas selectivos. Estos resultados sugieren la participación de ambos tipos de receptores en este modelo de dolor orofacial.

La administración local (i.m.) de WIN 55,212-2 se realizó a dos tiempos diferentes; 5 y 30 min antes de la inyección de SH, para comprobar que el efecto local aparece antes que el sistémico. Como era de esperar, al administrar el WIN 55,212-2 5 min antes se necesitaron dosis menores de fármaco (que administrándolo 30 min antes). Por tanto, para todos los experimentos restantes el agonista cannabinoide se inyectó 5 min antes que el SH.

Existen estudios previos que sugieren que los agonistas cannabinoides podrían ser efectivos en el tratamiento del dolor orofacial (Papanastassiou et al., 2004). Los resultados de experimentos previos, realizados también en la región orofacial, sugieren que este efecto antinociceptivo se debe principalmente a la unión de los agonistas al receptor CB_1, mientras que nuestros estudios indican que, tanto los receptores CB_1 como CB_2, están implicados. Estás diferencias podrían ser atribuidas a los diferentes modelos y tejidos en los que han sido investigados: un modelo de dolor inflamatorio en la articulación temporomandibular (test de la formalina) (Burgos et al., 2010) y la ligadura del nervio infraorbitario (Liang et al., 2007), a pesar de que la inervación es trigeminal en todos los casos.

En el modelo de dolor muscular de gemelo la administración sistémica de WIN 55,212-2 indujo un ligero efecto antinociceptivo que, tan solo fue revertido por el antagonista selectivo del receptor CB_1.

Para descartar que este efecto analgésico reducido del WIN 55,212-2 se debiera a sus posibles efectos psicoactivos llevamos a cabo la tétrada cannabinoide, en la que se estudia la antinocicepción, catalepsia, temperatura corporal y alteración de la locomoción tras la administración de agonistas cannabinoides (Pertwee, 1972). A las

dosis de WIN 55,212-2 evaluadas no se observó catalepsia ni hipotermia, pero sí analgesia e hipolocomoción tras la administración de la dosis de 1 mg/kg. Esta disminución en la actividad locomotora podría explicar el reducido efecto analgésico del WIN 55,212-2 observado en el modelo de dolor muscular del gemelo.

Cuando se administraron los agonistas cannabinoides por vía intramuscular en el gemelo, tanto el WIN 55,212-2 como los agonistas selectivos indujeron un efecto antinociceptivo, que a su vez fue antagonizado por los antagonistas selectivos. Esto sugiere la participación de ambos tipos de receptores, CB_1 y CB_2, en este modelo de dolor.

Sin embargo, al realizar la administración sistémica de los agonistas cannabinoides el receptor CB_2 no parece participar en el efecto antinociceptivo observado, pero no se puede descartar completamente su participación, ya que sí participa en este efecto al administrar los fármacos por vía intramuscular. Además, previamente se ha demostrado que ambos receptores están implicados en el efecto antihiperalgésico del WIN 55,212-2 en un modelo de dolor inflamatorio en el tríceps de rata (Kehl et al., 2003), y la expresión de ambos receptores en el músculo esquelético de rata y humano (Cavuoto et al., 2007).

Al comparar las dosis necesarias para obtener un efecto analgésico en ambos músculos, se observó que en masetero son necesarias dosis menores que en el gemelo. Estos resultados coinciden con los obtenidos en el estudio realizado por Burgos et al., en un modelo de dolor inflamatorio (Burgos et al., 2010) y confirma la existencia de las diferencias entre los sistemas trigeminal y espinal (Sessle, 2005; Takemura et al., 2006).

Existe una creciente evidencia de que la unión de agonistas cannabinoides a receptores periféricos induce un efecto antinociceptivo, evitando los efectos secundarios derivados de su unión a receptores centrales. Así, diversos agonistas cannabinoides no selectivos han demostrado modular la nocicepción periférica en modelos animales de dolor por inflamación, por estímulos térmicos y por neuropatías (Guindon et al., 2007; Kress and Kuner, 2009) y en modelos humanos de dolor agudo (Dvorak et al., 2003; Rukwied et al., 2003). Recientemente, se ha demostrado la eficacia analgésica de agonistas cannabinoides aplicados de manera tópica en el

tratamiento del dolor de la neuralgia postherpética, a la vez que son bien tolerados (Phan et al., 2010).

A pesar de los estudios del efecto analgésico periférico realizados hasta el momento, no existen datos acerca del posible efecto antinociceptivo tras la administración local de cannabinoides en músculo. Nuestros resultados demuestran que ambos tipos de receptores cannabinoides periféricos modulan el efecto antinociceptivo de los agonistas cannabinoides en el dolor muscular. Además varios estudios han demostrado el efecto antinociceptivo de los agonistas selectivos de los receptores CB_2 en modelos de dolor inflamatorio, neuropático y visceral (Valenzano et al., 2005; Cheng and Hitchcock, 2007; Anand et al., 2010). El uso de agonistas selectivos del receptor CB_2 podría ser interesante, desde un punto de vista clínico, ya que carecen de los efectos psicoactivos y por tanto, mejorarían la calidad del tratamiento (Guindon and Hohmann, 2008).

Las conclusiones más relevantes de los experimentos de esta tesis son la demostración de la participación de receptores periféricos en el efecto antinociceptivo de fármacos opioides y cannabinoides en el dolor agudo muscular orofacial, así como de los receptores cannabinoides periféricos en el modelo de dolor muscular de inervación espinal.

A pesar de que sería necesario conocer si las diferencias encontradas entre el sistema trigeminal y espinal también existen en humanos, se puede sugerir que los receptores opioides periféricos podrían ser posibles dianas terapéuticas en el tratamiento del dolor muscular orofacial, y los receptores cannabinoides periféricos en el tratamiento del dolor muscular orofacial y de inervación espinal. Así se obtendría el efecto analgésico deseado evitando los efectos secundarios derivados de la activación de los receptores centrales, que a menudo dificultan su prescripción clínica.

MODULACIÓN CONDICIONADA DEL DOLOR MUSCULAR INDUCIDO POR COMPRESIÓN MECÁNICA Y ADMINISTRACIÓN DE SUERO HIPERTÓNICO

6. MATERIAL Y MÉTODOS

6.1 PARTICIPANTES

En el estudio participaron doce hombres y doce mujeres, sin diferencias de edad entre los dos grupos (edad media±EEM 24.75±0.89). Los criterios de exclusión fueron: estar en tratamiento con analgésicos o padecer enfermedades o lesiones que pudiesen afectar el funcionamiento normal del sistema somatosensorial. Todos los experimentos se realizaron en el laboratorio de dolor orofacial del "Center for Sensory-Motor Interaction" de la Universidad de Aalbor.

6.2 TESTS DE ESTIMULACIÓN NOCICEPTIVA

Todos los test de estimulación nociceptiva se realizaron en el masetero derecho y en el antebrazo izquierdo.

TESTS TÉRMICOS

Mediante este test se determinó los umbrales de dolor al frio y calor (TPT), para ello se utilizó el aparato sensorial "TSA 2001 II" (CHEPS-MEDOC). La temperatura basal se estableció en +32ºC y las temperaturas de corte en 0ºC y + 50ºC. Primero se evaluó el umbral de dolor al frío y después al calor; para ello, se pidió a cada voluntario que apretase el botón cuando la temperatura empezara a sentirse dolorosa y en ese momento la temperatura volvía a sus niveles basales. Se realizaron 3 medidas por estimulación fría y tres por estimulación caliente y se consideró la media de los valores obtenidos como el umbral de respuesta.

TEST DE PRESIÓN

Con este test se midieron los umbrales de dolor a la presiónr (PPT) y tolerancia a la presión (PPTol). Para ello se utilizó algómetro manual (SomedicAB, Suecia). La presión se aplicó perpendicularmente a las zonas estudiadas con una intensidad creciente y constante de 30 kPa/s. La presión de corte se estableció en 2200 kPa.

A los participantes se les pidió que pulsaran el botón cuando la presión se sintiera ligeramente dolorosa (umbral de dolor o PPT) o cuando sintieran el máximo dolor tolerable (PPTol). Al igual que los umbrales de dolor térmico, se realizaron 3 medidas de dolor a la presión y se consideró la media de los valores obtenidos como el

umbral de respuesta. Para hallar la tolerancia de dolor a la presión tan solo se realizó una medida.

6.3 TESTS NOCICEPTIVOS CONDICIONANTES

Dos tipos de estimulación condicionante fueron experimentados:

1. Dolor mecánico (MP): el dolor se indujo mediante un aparato que ya ha sido descrito en la literatura (Sowman et al., 2011). Consiste en un aparato que se coloca sobre la cabeza y, a través de cuatro dispositivos, se puede aplicar presión sobre los músculos temporales, región frontal y occipital (Fig. 38). Una vez que el aparato estaba correctamente colocado sobre la cabeza se aplicó una presión semejante sobre los dos músculos temporales, de forma creciente, hasta que el voluntario indicaba sentir una intensidad de dolor graduado en 6, sobre una escala visual analógica del 0-10, o se alcanzaba la presión de corte (60 N).
2. El segundo estímulo condicionante consistió en una inyección de 1 ml de suero hipertónico 0.5% (SH) en el músculo temporal derecho.

Una vez terminada la estimulación condicionante se pidió al sujeto que dibujase la localización del dolor en un esquema de la cabeza.

6.4 VALORACIÓN DE LA INTENSIDAD DEL DOLOR CONDICIONANTE

Para que los participantes pudieran aportar una retroalimentación constante sobre el dolor inducido por el estímulo condicionante, se les proporcionó una escala visual analógica electrónica. El registro de esta información se realizó mediante un ordenador. El voluntario evaluó la intensidad del dolor desde el comienzo de la aplicación del dolor condicionante o control, hasta que la sensación del dolor desapareció.

6.5 PROTOCOLO

Al inicio de cada sesión se midieron los umbrales de dolor térmicos (TPT) seguido de los umbrales de dolor a la presión (PPT) y finalmente los umbrales de tolerancia a la presión (PPTol). A continuación, se indujo el dolor condicionante o control, y de nuevo se evaluaron en el mismo orden TPT, PPT y PPTol. Tras estas

valoraciones y una vez que el sujeto no sentía dolor los PPTs se evaluaron cada dos minutos durante 20 min. Una vez finalizados estos 20 min de nuevo TPT, PPT y PPTol fueron evaluados. Las mediciones se realizaron siempre en el masetero y el antebrazo El protocolo que se siguió durante las 4 sesiones queda recogido en la siguiente tabla:

<table>
<tr><td colspan="2">Basal</td><td>TPT
PPT
PPTol</td></tr>
<tr><td colspan="2">Durante la aplicación de dolor condicionante</td><td>TPT
PPT
PPTol</td></tr>
<tr><td rowspan="2">Después de la aplicación de dolor condicionante</td><td>0 min Post1
2 min Post2
4 min Post3
6 min Post4
8 min Post5
10 min Post6
12 min Post7
14 min Post8
16 min Post9
18 min Post10
20 min Post11</td><td>PPT</td></tr>
<tr><td>22 min Post12</td><td>TPT
PPT
PPTol</td></tr>
</table>

6.6 ANALISIS ESTADÍSTICO

Los umbrales de dolor a los distintos estímulos fueron normalizados para su posterior análisis.

Para analizar si el estímulo condicionante afectaba a los umbrales de dolor mientras el voluntario sentía dolor, se utilizó un test de Student. Para analizar si el estímulo condicionante afectaba a los umbrales de dolor una vez que el voluntario

había dejado de sentir dolor se realizó un test de ANOVA de dos vías (las dos variables fueron: tiempo y condición (dolor o control). El test posthoc utilizado fue Bonferroni.
Si en estos análisis se observaron diferencias, estas se analizaron comparando el máximo efecto posible mediante un test de Student.

La existencia de significación estadística se estableció cuando $p<0.05$.

7. RESULTADOS

Los resultados obtenidos en los experimentos realizados en voluntarios sanos se exponen a continuación.

7.1 UMBRALES BASALES DE DOLOR A ESTÍMULOS TÉRMICOS DE PRESIÓN

- Los hombres presentaron mayores umbrales de dolor que las mujeres:
  - A la presión en el antebrazo y en el masetero.
  - A la tolerancia a la presión en el antebrazo y en el masetero.
  - Al calor en el masetero
- No se encontraron diferencias en los umbrales de dolor al calor en el antebrazo entre hombres y mujeres (Fig. 39).

7.2 INTENSIDAD DE DOLOR DEL ESTÍMULO CONDICIONANTE

Para determinar la intensidad del dolor inducido por el estímulo condicionante se halló el área bajo la curva (curva generada mediante el registro contínuo, por parte del voluntario, de la intensidad de dolor inducido por el estímulo condicionante).

- No se encontraron diferencias entre hombres y mujeres en el área bajo la curva correspondiente a las sesiones de dolor mecánico.
- El área bajo la curva obtenida en las sesiones de SH fue mayor en mujeres que en hombres (la intensidad del dolor inducido por el SH fue mayor en mujeres que en hombres) (Fig. 40).
- Tanto en hombres como en mujeres el área bajo la curva obtenida fue mayor en la sesión de dolor mecánico en comparación con el área bajo la curva obtenida en la sesión de SH (Fig. 41).

7.3 MAPAS DE DOLOR

El dolor se localizó en el lugar de la aplicación del estímulo doloroso condicionante y en algunos casos refirió a zonas adyacentes (Fig. 42).

7.4 EFECTOS DEL ESTÍMULO CONDICIONANTE SOBRE LOS UMBRALES DE DOLOR TÉRMICOS DE PRESIÓN

Los efectos del estímulo condicionante sobre los umbrales de dolor se midieron a tres tiempos distintos:

1. Mientras el voluntario sentía dolor; en este momento se midieron los umbrales térmicos y de presión.
2. Inmediatamente después de que el paciente dejase de sentir dolor; en este momento tan solo se midieron los umbrales de dolor a la presión.
3. 20 min después de que el paciente dejase de sentir dolor; en este momento se volvieron a medir los umbrales térmicos y de presión.

Mientras el voluntario sentía dolor

- Los hombres presentaron menores umbrales de dolor a la presión en el masetero durante la sesión de dolor mecánico con respecto a su sesión control (control mecánico) (Fig. 43).
- Las mujeres presentaron menores umbrales de dolor a la presión en el masetero durante la sesión de suero hipertónico al compararlos con su sesión control (suero salino) (Fig. 43).
- Las mujeres presentaron mayores umbrales de dolor a la presión en el antebrazo durante la sesión de dolor mecánico *vs* con su sesión control (control mecánico) (Fig. 43).
- No se encontraron diferencias en el resto de parámetros estudiados con respecto a su sesión control (Fig. 43, Tabla 18).

Después de que el paciente dejase de sentir dolor

En el resto de los análisis realizados, después de que el paciente dejase de sentir dolor, no se encontraron diferencias en las variaciones de los umbrales térmicos o a la presión o en los umbrales de tolerancia a la presión (Tablas 20-23, Fig. 45).

8. DISCUSIÓN

Los objetivos de este estudio fueron:

Evaluar si existen diferencias entre hombres y mujeres en los umbrales de dolor a diferentes estímulos.

Estudiar si el dolor inducido por dos estímulos condicionantes (presión y SH) aplicados en el músculo temporal modifican los umbrales de dolor en dos zonas distintas: en el masetero (inervación trigeminal) y antebrazo (inervación espinal) tanto en hombres como en mujeres.

8.1 UMBRALES BASALES DE DOLOR

Los resultados de este estudio demuestran que las mujeres tienen umbrales de dolor, a la presión en el masetero y antebrazo, y al calor en el masetero, menores que los hombres. Sin embargo, no se encontraron diferencias entre hombres y mujeres en los umbrales de dolor al calor en el antebrazo.

Estos resultados coinciden con la mayoría de los estudios publicados hasta el momento, donde se ha demostrado que los hombres tienen mayores umbrales de dolor a la presión en el masetero (Fillingim et al., 2005b; Komiyama and De Laat, 2005; Matos et al., 2011), en el antebrazo (Oono et al., 2012) y al calor en el masetero (Sonnesen and Svensson, 2011). Existe controversia acerca de las diferencias de género con respecto a los umbrales de dolor al calor en el antebrazo, así en algunos estudios no se encuentran diferencias (Fillingim and Maixner, 1996; Fillingim et al., 1998; Jones et al., 2003; Jensen and Petersen, 2006), mientras que en otros se ha encontrado que en hombres los umbrales son mayores (Edwards and Fillingim, 1999; Edwards et al., 2004; Fillingim et al., 2005a; Fillingim et al., 2005b). Es posible que en los estudios en los que no se han encontrado diferencias sea debido a que la muestra sea pequeña, ya que en los estudios con una muestra mayor si se han encontrado estas diferencias.

Finalmente, la intensidad del dolor inducido por el SH fue mayor en mujeres que en hombres, estos resultados coinciden con los resultados de estudios previos (Ge et al., 2004; Ge et al., 2006; Falla et al., 2008).

8.2 MODULACIÓN DE LOS UMBRALES DE DOLOR MEDIANTE UN ESTÍMULO CONDICIONANTE

Bajo nuestras condiciones experimentales, se modificaron los umbrales de dolor a la presión de manera diferente en los hombres y mujeres, dependiendo de la sesión. Curiosamente el estímulo condicionante doloroso no indujo un efecto inhibitorio, a excepción del dolor mecánico que incrementó los umbrales de dolor a la presión en el antebrazo de mujeres.

Un resultado sorprendente fue el hecho de que, durante la sesión de suero salino, hubiese un mayor porcentaje de mujeres respondedoras que hombres (es decir, que sus umbrales de dolor a la presión se incrementaron un 10% con respecto a los basales). Se ha demostrado que el estrés y la distracción pueden modular el dolor de manera independiente y que el estrés, provocado por un estímulo no doloroso, puede inducir hipoalgesia de manera más efectiva en mujeres que en hombres (Rhudy and Meagher, 2001; Sternberg et al., 2001); también se ha demostrado que las mujeres tienen mayores niveles de estrés que los hombres antes de las sesiones experimentales (Granot et al., 2008).

Sin embargo, sucedió lo contrario en hombres: durante la sesión de control mecánico hubo un mayor porcentaje de hombres respondedores que mujeres. La distracción parece ser una manera más efectiva para reducir el dolor en hombres que en mujeres (Unrod et al., 2004; Quiton and Greenspan, 2007).

El peso del aparato de compresión, colocado sobre la cabeza, podría haber actuado como un factor de distracción, mientras que la inyección de suero salino como un factor de estrés. En este estudio no se incluyeron escalas de estrés o distracción, por lo tanto no se puede descartar el efecto que estos factores pudieron jugar en este estudio.

Durante las sesiones de dolor condicionante, los umbrales de dolor a la presión fueron menores que en las sesiones control, pero en torno a los niveles basales, excepto cuando fueron medidos en el antebrazo de mujeres, en los que se incrementaron. Estos resultados difieren de estudios previos en los que se utilizó el mismo aparato compresivo, en estos estudios se observó un incremento de los umbrales de dolor a la presión, en el masetero, durante la aplicación del estímulo

condicionante (Wang et al., 2010b; Oono et al., 2011; Sowman et al., 2011). La principal diferencia de estos estudios con respecto a éste fue que el dolor fue inducido mediante la aplicación de presión no solo sobre los músculos temporales, sino también sobre la frente y región occipital. Se ha demostrado que al incrementar el área de dolor también se incrementa la intensidad del dolor percibido (Price et al., 1989; Douglass et al., 1992; Nielsen and ArendtNielsen, 1997). Algunos estudios han demostrado que incrementando la intensidad del dolor condicionante también se aumenta el efecto inhibitorio del estímulo condicionante (Lautenbacher et al., 2008; Oono et al., 2011). En nuestro estudio el área de dolor inducido por la compresión mecánica es mucho menor (Fig. 43) que en el caso de los estudios anteriores, donde el área de dolor es mucho mayor (Sowman et al., 2011). Además, previamente se ha demostrado que existe un efecto inhibitorio del dolor tras la inyección bilateral de SH y no tras una inyección unilateral (Ge et al., 2003).

Tan solo se encontró un efecto inhibitorio en el antebrazo de mujeres en la sesión de dolor mecánico. Este resultado fue sorprendente ya que la mayor parte de las investigaciones encuentran un mayor efecto inhibitorio en hombres (Serrao et al., 2004; Goodin et al., 2009) o no encuentran diferencias entre sexo (Tousignant-Laflamme et al., 2008; Weissman-Fogel et al., 2008). Recientemente, en una revisión sistemática (Popescu et al., 2010) se ha demostrado que en algunos estudios existe un efecto inhibitorio sobre el dolor mayor en mujeres que en hombres (Quiton and Greenspan, 2007); Martikainen et al., 2004; Rosen et al., 2008; (France and Suchowiecki, 1999; France and Suchowiecki, 2001).

Para concluir: se ha demostrado que la modulación endógena del dolor depende de numerosas variables como sexo, ansiedad, distracción o estimulación condicionante. La manera en la que estas variables interactúan para finalmente dar lugar a la sensación conocida como “dolor” no se conoce con exactitud. Más estudios son necesarios para conocer cómo factores psicológicos y físicos afectan a la modulación endógena del dolor.

9. CONCLUSIONES

9.1 CONCLUSIONES DERIVADAS DE LOS ESTUDIOS EN ANIMALES

1. Se ha desarrollado un modelo para estudiar el dolor agudo en dos músculos de inervación espinal gemelo y tríceps.
2. Los agonistas opioides y cannabinoides tienen un efecto antinociceptivo en los modelos de dolor muscular agudo estudiados. La participación de los receptores centrales y periféricos varía en función del músculo.
   a. En la analgesia opioide en masetero participan receptores centrales y periféricos y en los músculos de inervación espinal (gemelo y tríceps) tan solo receptores centrales.
   b. En la analgesia cannabinoide mediada por receptores CB_1 y CB_2, participan:
      - En el masetero: ambos receptores cannabinoides, tanto cuando los fármacos se administran por vía sistémica como intramuscular.
      - En el gemelo: la analgesia es mediada por CB_1 tras administración sistémica y por CB_1 y CB_2 cuando se administran por vía intramuscular.

Las diferencias encontradas en la participación en el efecto antinociceptivo de receptores centrales y periféricos en ambos músculos pueden abrir nuevas oportunidades en el desarrollo de tratamientos analgésicos selectivos para el tratamiento del dolor muscular.

9.2 CONCLUSIONES DERIVADAS DEL ESTUDIO EN HUMANOS

1. Las mujeres sanas tienen umbrales de dolor menores que los hombres sanos frente a estímulos térmicos y de presión.
2. La aplicación de un estímulo condicionante, doloroso o no doloroso, modifica de manera distinta la percepción del dolor a otro estímulo en hombres y mujeres sanos.

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