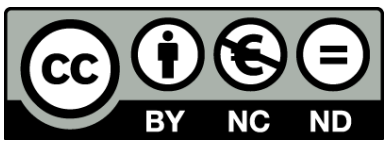
UNIVERSITAT DE BARCELONA

Role of the sigma-1 receptor in the sensory-discriminative and affective-motivational components of acute and chronic pain

Beatriz de la Puente Robles



Role of the sigma-1 receptor in the sensory-discriminative and affective-motivational components of acute and chronic pain

Doctoral Thesis

Beatriz de la Puente Robles

2015

UNIVERSITAT DE BARCELONA

UNIVERSITAT DE BARCELONA

Facultat de Farmàcia

Departament de Bioquímica i Biologia Molecular

Programa de Doctorat en Biotecnologia

ESTEVE

Drug Discovery & Preclinical Development

Department of Pharmacology

Role of the sigma-1 receptor in the sensory-discriminative and affective-motivational components of acute and chronic pain

Memoria presentada por Beatriz de la Puente Robles para optar al título de Doctor por la Universidad de Barcelona.

Trabajo realizado en el Departamento de Farmacología, Área de Descubrimiento de Fármacos y Desarrollo Preclínico de ESTEVE.

Dr. Enrique Portillo Salido
Director

Dr. Daniel Zamanillo Castanedo
Director

Dra. Josefa Badia Palacín
Tutora

Beatriz de la Puente Robles
Doctoranda

A Sergio

y a nuestra hija Ana

Agradecimientos

Muchas personas han contribuido, de una u otra forma, a la realización de este trabajo que ha llegado a su fin. Por ello, les quiero mostrar mi más sincera y profunda gratitud.

Al Dr. José Miguel Vela Hernández, por haber confiado en mí para formar parte de su grupo de investigación, hace ya muchos años, y haberme dado la oportunidad de realizar la Tesis Doctoral en ESTEVE.

Al Dr. Manuel Merlos Roca, por su interés e implicación en el desarrollo del presente trabajo y sin cuyo apoyo no hubiera sido posible la finalización de esta Tesis Doctoral.

A mis directores de Tesis, el Dr. Enrique Portillo Salido y el Dr. Daniel Zamanillo Castanedo, por haberme hecho partícipe de su excelencia científica y transmitirme su experiencia y conocimientos. Por enseñarme a pensar y poner negro sobre blanco cientos de ideas descolocadas, tarea ardua y difícil. Les agradezco su entusiasmo, entrega y todo el esfuerzo y tiempo que han invertido para que este trabajo llegara a su fin. Y sobre todo, les agradezco su amistad y los buenos momentos que hemos compartido durante la realización de esta Tesis Doctoral.

A la Dra. Josefa Badia Palacín, por su respaldo constante en estos años de formación. Por todos sus buenos consejos, amabilidad, buen humor y por hacer que todo fuera más fácil.

A la Dra. Luz Romero Alonso por su visión científica y sus consejos siempre imprescindibles. Su capacidad de transformar la complejidad científica en razonamientos sencillos me ha ayudado a poder englobar fácilmente los resultados obtenidos. También por su gran corazón, comprensión y apoyo personal en los tropezones que nos da la vida.

Mi agradecimiento especial al Dr. Sergio Ovalle Fernández por su gran calidad científica, técnica y humana, por su trabajo exquisito y metódico, y su capacidad crítica, imprescindibles para realizar un buen trabajo científico.

Quiero agradecer también a Carme Segalés su permanente ánimo y empuje, por ser una gran compañera y sobre todo una gran persona. A Georgia Gris, con quien he compartido el largo camino de doctorado que no ha sido nada fácil y al resto de compañeros del Departamento de Farmacología: Bertrand Aubel, Alba Vidal, Jordi Buxens, Alicia Carceller, Alicia Pardo, Ricard Sánchez, Montse Jané, Àngels Fisas, Xavier Codony, Begoña Fernández y Ana Montero. Trabajar con ellos siempre es un placer. También a Mercé Olivet y Xavier Costea por compartir conmigo, siempre, su buen humor.

Mi agradecimiento también a los trabajadores de la PRAAL (PCB), especialmente a Rosa Colomer, por tener siempre todo el material a punto.

Por último, a mis padres que, aunque lejos, me infunden la confianza que necesito y a mis hermanos, Álvaro y Javier, por creer en mí.

Y sobre todo gracias a mi marido, Sergio, y a mi hija, Ana, porque sin su comprensión, soporte y paciencia infinita hubiera sido imposible llegar hasta el final. Este trabajo es por y para vosotros.

XXIX

Caminante, son tus huellas
el camino, y nada más;
caminante, no hay camino:
se hace camino al andar.
Al andar se hace camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante, no hay camino,
sino estelas en la mar.

Antonio Machado

PROVERBIOS Y CANTARES

Presentación

El dolor es una experiencia sensorial y emocional desagradable. Es un mecanismo muy complejo determinado por dos componentes: el componente sensorial-discriminativo, que corresponde a los mecanismos neurofisiológicos de la nocicepción y que informa de las características del dolor (naturaleza, duración, intensidad,…) y el componente afectivo-motivacional, que expresa la connotación desagradable relacionada con la percepción del dolor y que conlleva consecuencias emocionales que afectan al estado de ánimo y al bienestar del individuo. En la presente Tesis Doctoral se estudian estos dos componentes que conforman el dolor, evaluando respuestas de comportamiento en dos modelos animales de dolor, un modelo de dolor agudo visceral y un modelo de dolor crónico de origen neuropático. Además, se estudia el papel del receptor sigma-1 (σ_1R) en cada modelo animal de dolor, evaluando su posible implicación tanto en el componente sensorial como en el afectivo. Para ello, se pusieron en práctica dos estrategias experimentales. Por un lado, se utilizaron ratones modificados genéticamente a los que se les ha eliminado el gen del receptor del sigma-1 (ratones *knock-out* σ_1R) y por otro lado, se realizó un tratamiento farmacológico sistémico en ratones salvajes (*wild-type*) con el E-52862 (S1RA), un antagonista del receptor sigma-1 altamente selectivo. El E-52862 es un compuesto que ha sido desarrollado por ESTEVE y que tras su primera evaluación en seres humanos ha demostrado un buen perfil de seguridad y tolerabilidad (estudios clínicos de Fase I). Actualmente continúa siendo evaluado en subsecuentes estudios clínicos como indicación para el dolor neuropático de diferentes etiologías (estudios de Fase II).

Los resultados de esta Tesis Doctoral proporcionan nuevos conocimientos sobre el receptor sigma-1 que apoyan el desarrollo clínico del antagonista selectivo para este receptor, el E-52862, como una opción terapéutica adecuada para el tratamiento del dolor neuropático y las comorbilidades afectivas asociadas a esta patología.

Summary

The present Doctoral Thesis focuses on the study of the sigma-1 receptor (σ_1R) in the field of pain. This research has been a part of the preclinical σ_1R project focusing on drug discovery of σ_1R ligands for the treatment of pain at the pharmaceutical company ESTEVE. The overall purpose of this Doctoral Thesis was to explore the role of σ_1R in the sensory-discriminative and affective-motivational components of pain. To this end, acetic acid-induced visceral pain and partial sciatic nerve ligation in mice were used to model an acute and chronic pain state, respectively. The sensory discriminative and affective-motivational components of pain in these models were infered by changes in reflexive and non-reflexive behavioural outcomes, respectively. Abdominal contractions (writhing) and paw behavioural hypersensitivity to mechanical and thermal stimuli were analysed as reflexive outcomes. On the other hand, pain-related changes of sweet preference, locomotor activity and reward-seeking behaviour were analysed as non-reflexive outcomes. These models and behavioural outcomes were used to evaluate the effect of pharmacological and genetic blockade of σ_1R and also the effects of some reference compounds. Furthermore, electrophysiological and molecular studies in knock-out ($\sigma_1R^{-/-}$) mice were used to understand the role of σ_1R in the sensory-discriminative component of chronic pain.

Taken together, the results of this Doctoral Thesis provide new knowledge about σ_1R and support the clinical development of selective σ_1R antagonists as a suitable therapeutic intervention to treat neuropathic pain and its mood-related comorbidities.

List of Abbreviations and symbols

%: percentage
ºC: degree Celsius
5HT: 5-hydroxytryptamine, serotonin
8-OH-DPAT: 8-hydroxy-2-(di-n-propylamino)tetralin
am: *ante meridiem*; from Latin, "before midday"
AA: acetic acid
ACC: anterior cingulate cortex
ad libitum: from Latin, "at liberty"
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMY: amygdala
ANOVA: analysis of variance
ATP: adenosine triphosphate
AUC: area under the curve
BG: basal ganglia
BiP: binding immunoglobulin protein
Ca^{2+}: calcium
CARR: carrageenan
CC: cingulate cortex
CCI: chronic constriction injury
CCS: constriction of the saphenous nerve
CFA: complete Freund's adjuvant
CNS: central nervous system
Δ^9-THC: Delta 9-tetrahydrocannabinol
d.f.: degrees of freedom
DRG: dorsal root ganglion
ED_{50}: median effective dose
EEG: electroencephalography
e.g.: *exempli gratia*; from Latin, "for example"
$E_{max:}$ maximum effect
ER: endoplasmic reticulum
ERK: extracellular signal-regulated protein kinase
Fig.: figure
fMRI: functional magnetic resonance imaging
g: gram(s)
h: hour(s)
H_2O: water
HPMC: hydroxypropylmethylcellulose
i.e.: *id est*; from Latin, "that is"
i.p./IP: intraperitoneal
IASP: International Association for the Study of Pain
Ibu: ibuprofen
kg: kilogram(s)
LMA: locomotor activity
LPS: lipopolysaccharide
MAM: mitochondrial-associated endoplasmic reticulum membrane
MAPK: mitogen-activated protein kinase

MCC: midcingulate cortex
MIA: monoiodoacetate
min: minute(s)
mL: millilitre(s)
mRNA: messenger ribonucleic acid
N.D.: not determined
NAc: nucleus accumbens
NMDA: N-methyl-D-aspartate
no.: number
NSAID: nonsteroidal anti-inflammatory drug
N.T.: not tested
P: probability
PAG: periaqueductal grey
PB: parabrachial nucleus
pERK: phosphorylated extracellular signal-regulated kinase
pm: *post meridiem*; from Latin,"after midday"
PFC: prefrontal cortex
post hoc: from Latin, "after this"
PSNL: partial sciatic nerve ligation
RM: repeated measures
RSB: reward seeking behaviour
RVM: rostroventral medulla
S1: primary somatosensory cortex
S2: secondary somatosensory cortex
SE: standard error
s: second(s)
SEM: standard error of the mean
SNI: spared nerve injury
SNL: spinal nerve ligation
SNT: spinal nerve transection
SPL: superior parietal lobe
σ_1R: sigma-1 receptor
σ_2R: sigma-2 receptor
$\sigma_1R^{+/-}$: sigma-1 receptor knock-out heterozygous
$\sigma_1R^{-/-}$: sigma-1 receptor knock-out homozygous
TNF-α: tumour necrosis factor α
TNT: tibial nerve transection
v/v: concentration, volume solute/volume of solution
VTA: ventral tegmental area
WO: wash-out
WT: wild-type

Contents

I. Introduction

1. The experience of pain

The ability to detect noxious stimuli is a normal sensation triggered in the nervous system as an alert to the possibility of injury and the need for rest and recuperation. Pain is an adaptive response because it improves the survival value and wellbeing and therefore enhances the probability of the body to survive and reproduce. The perception of pain facilitates fast withdrawal from a damaging situation, safeguards the affected body part while it heals, and provides a strong teaching signal that enables learning to avoid similar harmful situations in the future. Pain also has motor responses that lead to escape because it is part of the body's defense system and acts like a protective mechanism.

In addition, pain is considered to be a homeostatic emotion (Craig, 2003a), or primordial emotion (like thirst, hunger or fatigue), because it reflects an adverse condition in the body that requires a behavioural response, and also has autonomic/homeostatic responses (drinking, eating or resting in the above examples) aimed at maintaining the body's internal milieu in its ideal state. Homeostasis, as elucidated by Cannon (Cannon, 1939), is a dynamic and ongoing process comprising many integrated mechanisms that maintain an optimal balance in the physiological condition of the body for the purpose of survival, including autonomic, neuroendocrine and behavioural mechanisms. Pain contributes to the monitoring and maintenance of homeostasis. Neural basis for these integrated homeostatic emotional behaviours must be evolutionarily ancient because all vertebrates respond similarly to noxious stimuli (Craig, 2003a).

From the evolutionary perspective, the pain system is embedded within extensive circuitry (mediating emotions, reward/anti-reward, and motivation) that represents a neural network indispensable to preserve individuals and species, that promotes behaviours necessary for survival (food, water and sex), and that prevents behaviours that endanger wellbeing (pain and fear) through learning, conditioning and influence on decision making (Elman *et al*., 2013).

The International Association for the Study of Pain (**IASP**) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey, 1986). This definition segregates pain in two dimensions: sensory and emotional. The **sensory-discriminative component** of pain (i.e. nociception or sensory pain) provides information about the location, modality and intensity of painful stimuli, and the **affective-motivation component** of pain comprises the emotional dimension (the unpleasant character of pain perception) (Craig, 2003b) and cognitive aspects of the experience of pain (Casey, 1982) that are involved in the attention, anticipation and memory of past experiences; this component can interact with the other component, thus giving rise to pain modulation (Valet *et al*., 2004). Interestingly, this affective dimension of pain relies on neurophysiologic systems that are at least partly anatomically distinct from those involved in the sensory perception of pain (Duquette *et al.*, 2007).

Both experimental and clinical studies support the distinction between two separate but parallel neural systems. The **neuroanatomical basis** of pain has been conceptualized as involving "lateral" and "medial" subsystems (Albe-Fessard *et al.*, 1985). The "lateral" system (including thalamocortical afferents to somatosensory cortices and posterior insula) subserves the sensory-discriminative processing of pain inputs, whereas the

"medial" system (with projections to the anterior cingulate and prefrontal cortices) would be preferentially involved in the motor, cognitive/evaluative and emotional pain components. Over the last fifteen years solid evidence was generated indicating that multiple cortical and subcortical structures are involved in human pain perception. Human brain imaging studies have revealed consistent cortical and subcortical networks that are activated by pain (Baliki and Apkarian, 2015). The brain areas most commonly activated by noxious stimuli are primary somatosensory cortex (S1), secondary somatosensory cortex (S2), anterior cingulate cortex (ACC), insula, prefrontal cortex (PFC), thalamus, and cerebellum (Apkarian *et al.* 2005).

The role of the cortex in human pain perception remained controversial until the advent of non-invasive brain imaging technologies. The cortical perception of pain is intimately linked with areas of the brain involved in autonomic and neuroendocrine regulation (amygdala, hypothalamus, thalamic reticular nucleus, ventral tegmental area, locus coeruleus, laterodorsal tegmental nucleus) in a way that the perception of pain manifests physically with changes in blood pressure, heightened reflexes, and skin galvanomic response, among others (Blackburn-Munro, 2004). Moreover, the cortico-limbic pathway is known to integrate nociceptive inputs with information about the overall status of the body, in turn regulating (and sometimes amplifying) the effect attributed to pain (Price, 2002).

This **integrated system** might indicate that pain is not only part of an afferent system in which the final product is a change in behaviour, but that the perception of pain itself may be part of a complex two-way afferent-efferent system (Fregni *et al*., 2007; Zaghi *et al.*, 2009). Moreover, a new view suggests that specific pain centers exist which consist of a distinct "physical" sensation (represented in the parieto-insular cortex) and

an "emotional" component (represented in the anterior cingulate), which have evolved from a primitive system of the brain that controls the health of the body. The overlap between these areas and the emotion-processing regions of the brain could explain the peculiarly human subjective qualities of pain (Craig, 2003c).

2. The sensory-discriminative component of pain

The sensory-discriminative component allows localizing and feeling specific attributes of pain, and can be considered as a sensory modality similar to vision or olfaction. It has become increasingly evident that it is subserved by its own apparatus up to the cortical level.

2.1. Neuroanatomical basis

The conscious appreciation of pain is a complex series of mechanisms whereby the noxious stimulus is encoded and progressively transmitted to and processed by higher centers of the nervous system until it is perceived as pain. The perception of pain is thought to occur in the cortical structures (Fine and Ashburn, 1998). However, the exact location in the brain where pain becomes a conscious experience and the reasons individuals vary in their subjective experience of pain are unclear. Signals arriving in the central nervous system are the result of the activation of specialized sensory receptors called nociceptors that provide information about tissue damage. **Nociception** is the term used to describe normal pain transmission and sensation.

The peripheral nerve endings innervate the skin and viscera that receive either the high threshold internal (inflammatory soups) or external (heat, acid, and pressure) signals. The cellular process by which noxious stimuli are changed into the electrical energy necessary to transmit pain is defined as ***transduction*** (Fine and Ashburn, 1998). When transduction is complete, the ***transmission*** of the pain impulse along the nociceptor fibres (nerve axons) begins. Nociceptor fibres respond to mechanical, thermal or chemical noxious stimuli. Tissue damage occurs when tissues are exposed in sufficient quantity to these stimuli and induces the release of a number of substances. Nociceptors

are excitatory neurons and release glutamate as their primary neurotransmitter as well as other components including peptides (e.g., substance P, calcitonin gene-related peptide, somatostatin) that are important in both central synaptic signaling and efferent signaling in the skin (Basbaum *et al.*, 2009) that facilitate the transmission of pain from the periphery to the spinal cord (Pasero *et al.*, 1999).

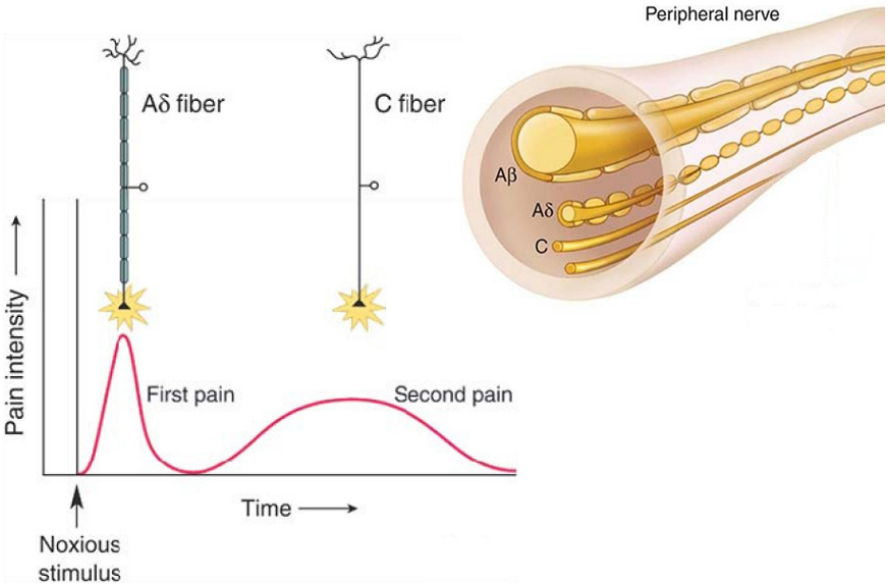


Fig. 1. Sensory neuron fibre types. Primary afferents are classified by their diameter, degree of myelination, and conduction velocity. The largest diameter afferent fibres, A-beta (Aβ), respond maximally to light touch and/or moving stimuli. There are two other classes of primary afferent nerve fibres: the small diameter myelinated A-delta (Aδ) and the unmyelinated (C) axons. The speed of transmission is directly correlated to the diameter of axons of sensory neurons, whether or not they are myelinated. C-fibres have a small diameter (Woolf and Ma, 2007) are bundled in fascicles surrounded by Schwann cells, and support conduction velocities of 0.4-1.4 m/s (Djouhri and Lawson, 2004). Initial fast-onset pain is mediated by A-fibre nociceptors whose axons are myelinated and support conduction velocities of approximately 5-30 m/s, mostly in the slower Aδ range, although a substantial proportion of A-fibre nociceptors conduct in the Aβ conduction velocity range (Djouhri and Lawson, 2004). *Available from: <http://www.1pain.com/textbook/Chapter1.html>* and *<http://clinicalgate.com/back-and-neck-pain>*

There are two major classes of nociceptors that transmit pain from the site of transduction (periphery) to the spinal cord, A-fibres and C-fibres (Meyer *et al.*, 2008) (Fig. 1). A-fibres are thinly myelinated, large-diameter, fast-conducting fibres that transmit well-localized sharp pain. A-fibres include medium diameter myelinated (Aδ) afferents that mediate acute, well-localized "first" or fast pain. These myelinated afferents differ considerably from the larger diameter and rapidly conducting Aβ fibres that respond to innocuous mechanical stimulation (i.e., light touch). C-fibres are unmyelinated, small-diameter, slow-conducting fibres that transmit poorly localized, dull and aching pain. During the first segment of transmission, the impulse is carried along nociceptor fibres in an ascending fashion to the dorsal horn of the spinal cord.

Nociceptors, like other primary somatosensory neurons, are pseudounipolar with a single process emanates from the cell body located in the dorsal root ganglion (DRG) (Fig. 2) —or trigeminal ganglion in the cephalic area— and bifurcates, sending a peripheral axon to innervate the skin and a central axon to synapse on second-order neurons in the dorsal horn of the spinal cord —or the trigeminal subnucleus caudalis, respectively—. In this way, the propagation of electrical signals between periphery and spinal cord (or brainstem) follows a direct axonal pathway, thus reducing the risk of conduction failure (Amir and Devor, 2003). The central axon of DRG neurons enters the spinal cord via the dorsal root and sprouts branches that terminate predominantly in laminae I, II, IV and V of the dorsal horn on relay neurons and local interneurons important for signal modification (Millan, 1999; Basbaum *et al.*, 2009; Alvarez-Leefmans, 2009). Relay neurons project to the medulla, mesencephalon and thalamus, which in turn project to somatosensory and anterior cingulate cortices to drive sensory-discriminative and affective-motivational aspects of pain, respectively (Millan,

1999). The impulse is followed by transmission from the spinal cord to the brainstem and thalamus. The thalamus, acting as a relay station, sends the impulse to the cortex where it can be processed (Pasero *et al.*,1999) (Fig. 3).

The sensory-discriminative component of pain results in preferential activation of the lateral thalamus and somatosensory cortices (S1 and S2) (Ohara *et al.*, 2005), as well as the posterior insular cortex (Moisset and Bouhassira, 2007). The somatosensory cortices (S1 and S2) encode information about sensory features, such as the location and duration of pain (Kenshalo and Isensee, 1983; Kenshalo *et al*., 1988; Chudler *et al*., 1990; Greenspan *et al*., 1999; Ploner *et al*., 1999).

As revealed by neuroimaging studies in experimental and clinical pain states, sensitization to noxious stimuli has specific correlates in cerebral pain processing (Apkarian *et al.*, 2005; Borsook *et al.*, 2007a; Moisset and Bouhassira, 2007; Tracey and Mantyh, 2007; Seifert and Maihöfner, 2009). As a main mechanism there is an increased activity in pain processing brain areas (S1, S2, insula, ACC, PFC) and an additional recruitment of other brain areas mainly reflecting cognitive, autonomic and motor processing or modulatory networks (Apkarian *et al.*, 2005; Maihöfner and Handwerker, 2005; Borsook *et al.*, 2007b; Moisset and Bouhassira, 2007; Seifert *et al.*, 2008). Together, these processes convey encoded nociceptive information to pathways of ascending hierarchical processing in the spinal cord and brain. Damage to the anterolateral (spinothalamic) pain system makes it difficult to localize or describe the physical characteristics of pain; however an unpleasant experience persists (Ploner *et al.*, 1999).

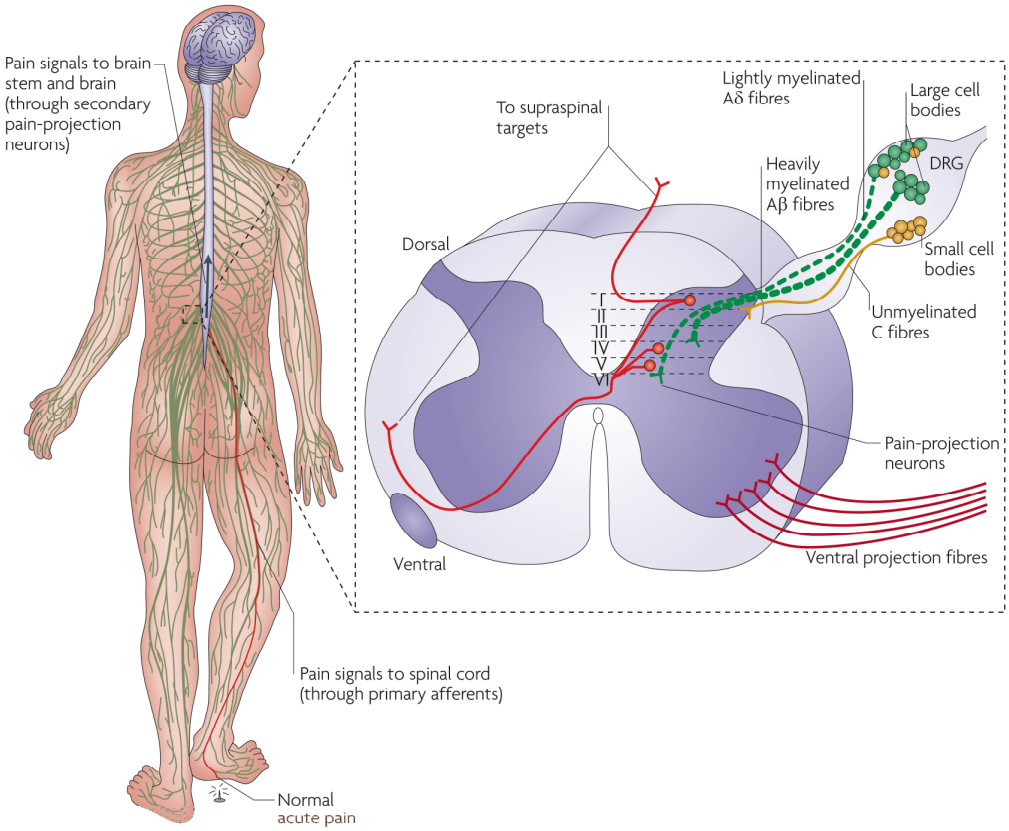


Fig. 2. Perception of pain (I). Normal pain signaling in the body is transmitted to the spinal cord dorsal horn through nociceptors. Nociceptive pain leads to the release of pain transmitters from primary afferent terminals that project onto pain-projection neurons, primarily laminae I, IV and V in the spinal cord dorsal horns. Nociceptive signaling from the dorsal root ganglia (DRG) is relayed to the dorsal spinal cord, brain stem and brain, where the experience of pain occurs. The DRG contain pseudounipolar sensory neurons —so called because they give rise to a single axon that bifurcates, with one part projecting to the periphery and the other projecting to the dorsal horn of the spinal cord. The cell bodies of nociceptive neurons in the DRG are broadly classified into large and small types. Immunohistochemical staining studies have shown that slowly conducting C and Aδ fibres have small cell bodies, whereas faster-conducting Aβ fibres tend to have larger cell bodies. The inset shows a cross section of spinal cord including the spinal cord dorsal horn and the DRG. *From: Milligan ED and Watkins LR. Pathological and protective roles of glia in chronic pain. Nature Reviews Neuroscience. 2009; 10:23-36.*

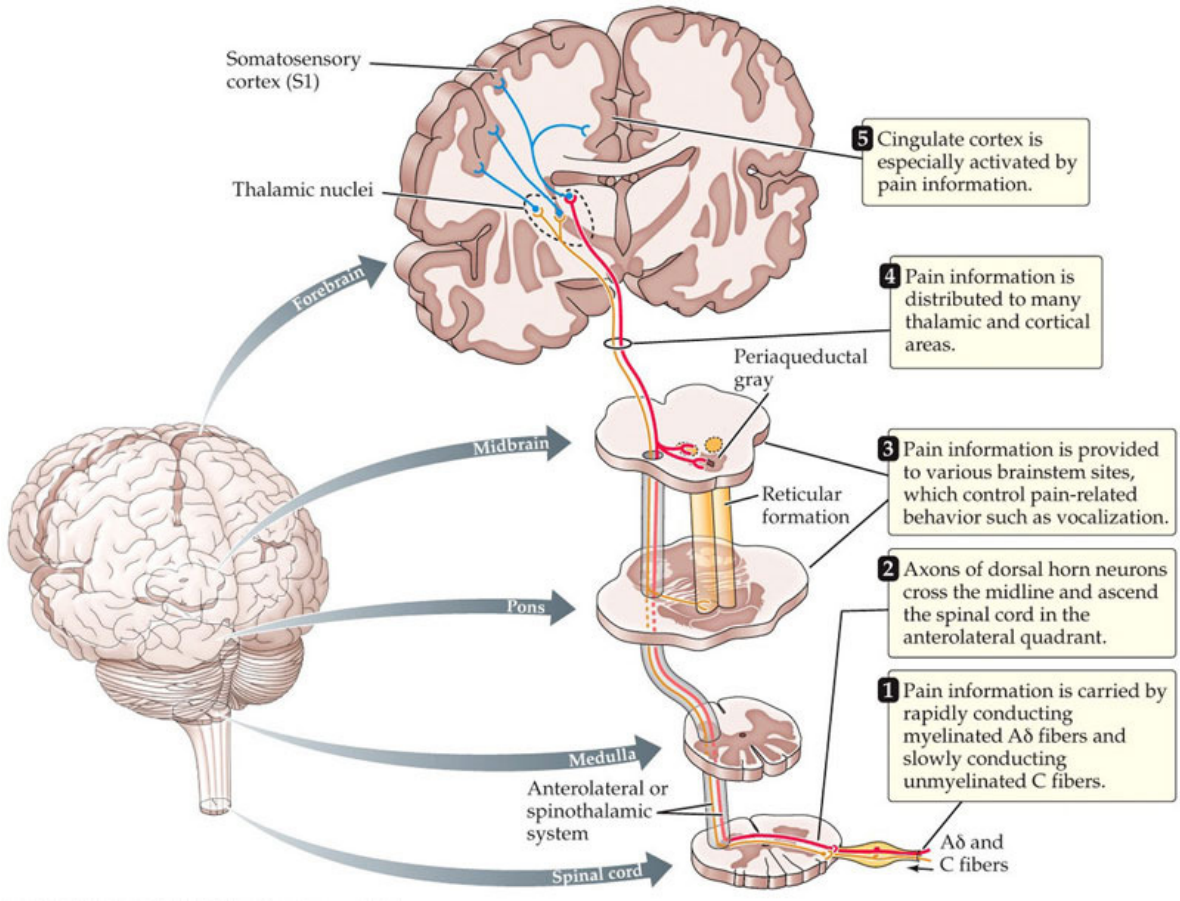


Fig. 3. Perception of pain (II). Pain information enters the spinal cord, crosses the midline, and ascends through the anterolateral (spinothalamic) system to the brain. The lateral pain system, which supports the sensory-discriminative dimension of pain, has axons that ascend laterally within the spinothalamic tract of the spinal cord, synapse within lateral nuclei of the thalamus, and ultimately project to the primary somatosensory cortex. *From: Breedlove SM and Watson NV. Chapter 8: General Principles of Sensory Processing, Touch, and Pain. Available from: <http://7e.biopsychology.com/vs08.html>*

Moreover, descending pathways from the brainstem, cortex or other central nervous system (CNS) regions affect pain processing by facilitating or inhibiting the information ascending in nociceptive pathways. ***Modulation*** (inhibition and alteration) of pain transmission occurs at several locations within the central nervous system (Fine and Ashburn, 1998). The neuronal pathways involved in modulation are often referred to as

the descending pain system because they originate in the brain stem and descend to the dorsal horn of the spinal cord (Portenoy and Kanner, 1996). These descending pathways release substances, such as endogenous opioids, serotonin and norepinephrine, which can inhibit the transmission of noxious stimuli and produce analgesia (Pasero *et al.*, 1999). These descending inhibitory and facilitatory pathways originating in the brain, as well as local inhibitory and excitatory interneurons in the dorsal horn, modulate the transmission of nociceptive signals, thus contributing to the prioritization of pain perception relative to other competing behavioural needs and homeostatic demands (Heinricher *et al.*, 2009).

2.2. Sensory consequences of pain

The detection of pain facilitated by the sensory-discriminative component draws attention to an environmental problem that threatens damage, or is causing damage, to bodily tissues. It motivates a behaviour that would tend to remedy the problem. Pain perception, in a first instance, interrupts and demands attention, and triggers a series of rapid behavioural reactions in an attempt to end such unpleasant sensation (Eccleston and Crombez, 1999). These behaviours are known as "protective behaviours" and include any actions oriented to escape from the pain-related stimulus or pain relevant environment (**withdrawal reflexes** and **escape behaviours**) and any behaviours that prevent or alleviate pain, including actions oriented towards protecting or caring for an injury (guarded postures, disabled behaviour). These protective behaviours are highly conserved among species. Nociceptive pain normally subsides with time; when noxious stimulation ceases, pain also ceases (Backonja, 2003).

However **the experience of chronic pain** is entirely different from acute pain. The transition from acute to chronic pain involves a time dependent neural reorganization that initiates a series of events that potentiate one pathway at the cost of the other, thus resulting in an abnormal processing of sensory inputs (Pasero *et al*., 1999; Dworkin *et al.* 2003). Persistent pain arises from increases in excitability, or sensitization, of one or more peripheral or central components of pain transduction pathways (Carrasquillo and Gereau, 2008; Costigan *et al.*, 2009; Woolf, 2011). Due to this plasticity, pain is no longer coupled, as acute nociceptive pain is, to the presence, intensity or duration of noxious peripheral stimuli. Instead, the injury-related plasticity produces pain hypersensitivity by changing the sensory response elicited by normal inputs, including those that usually evoke innocuous sensations which can be present in the nervous system for weeks, months, even years beyond the expected period of healing or resolution of the source of pain. This pain-related sensitization due to injury or inflammation are present both at the peripheral and central level.

Peripheral sensitization results from a reduction in the firing threshold and an increase in responsiveness of the peripheral nociceptors, which can result initially from local exposure to neurogenic inflammatory factors such as calcitonin gene-related peptide, substance P and serotonin, and later to a welter of noxious chemicals known as "inflammatory soup" (Basbaum *et al.*, 2009). A partial list of these chemicals includes noradrenaline, bradykinin, histamine, prostaglandins, potassium, cytokines, and neuropeptides. These cellular mediators act to sensitize nociceptors to further neural input. This produces changes in the number and location of ion channels —especially sodium ion channels— in injured nociceptor nerve fibres and their dorsal root ganglia. As a result, the threshold for depolarization is lowered, and spontaneous discharges

known as ectopic discharges can occur in abnormal locations. Consequently, the response of nociceptors to thermal and mechanical stimuli is increased. Nociceptor hyperactivity induces secondary changes in processing neurons in the spinal cord and brain, so that inputs from mechanoreceptive A-fibres are perceived as pain. Neuroplastic changes in the central pain modulatory systems can lead to further hyperexcitability. As a consequence of peripheral nociceptor hyperactivity, dramatic secondary changes in the spinal cord dorsal horn can lead to central sensitization (Basbaum *et al.*, 2009).

Central sensitization occurs as nociceptive neurons of the dorsal horn of the spinal cord become persistently hyperexcitable. The term was first described by Woolf (Woolf, 1983) and nowadays is defined as the enhanced nociceptive synaptic transmission within the spinal cord following persistent pain states, or more precisely, the abnormal hyperexcitability of central nociceptor neurons.

Several excitatory amino acids and neuropeptides within the dorsal horn of the spinal cord released following the peripheral nociception hyperactivity lead to postsynaptic changes of second-order nociceptive neurons, such as phosphorylation of NMDA and AMPA receptors (Ultenius *et al.*, 2006) or expression of voltage-gated sodium channels (Hains *et al.*, 2004). Increasing levels of mRNA for voltage-gated sodium channels seem to correlate with ectopic activity (i.e., spontaneous depolarization), and an increased expression of sodium channels in lesioned and intact fibres might lower the action potential threshold until ectopic activity takes place (Lai *et al.*, 2003; Black *et al.*, 2008; Siqueira *et al.*, 2009). In addition to voltage-gated sodium channels, several other ion channels probably undergo alterations after a nerve lesion, such as voltage-gated potassium channels (Bahia *et al.*, 2005), which might also contribute to changes in the

membrane excitability of nociceptive nerves. These changes induce neuronal hyperexcitability that enables low-threshold mechanosensitive Aβ and Aδ afferent fibres to activate second-order nociceptive neurons. Mechanistically, central sensitization can be driven by pre- and postsynaptic changes as well as by increases in the postsynaptic membrane excitability (Latremoliere and Woolf, 2009). Long-term alterations in neuronal excitability are not limited to the spinal cord; indeed, a major component of persistent pain is now thought to arise from adaptive changes in structure and function of a number of central brain regions both directly and secondarily implicated in higher processing of pain-related sensory information (Woolf, 2011). Collectively, these changes result in a phenomenon known as "wind-up", i.e., an increased excitability and sensitivity of spinal cord neurons (Seal *et al.*, 2009; Pfau *et al.*, 2011; Woolf, 2011) evoked by repetitive stimulation of afferent C-fibres, and stands for a spinal amplification of the message coming from peripheral nociceptors (Dickenson and Sullivan, 1987; Herrero *et al.*, 2000). **Wind up** is a progressive frequency-dependent facilitation of the responses of nociceptive neurons observed in the application of repetitive (usually electrical) stimuli of constant intensity (Herrero *et al.*, 2000).

Central sensitization, together with peripheral changes, is believed to account for the sensory symptoms of persistent pain (Fig. 4). **Allodynia** is a clinical term that describes pain due to a stimulus that does not normally provoke pain. Mechanical dynamic and mechanical static allodynia describe a state in which pain is evoked by light moving touch or light pressure, respectively. Accordingly, cold and heat allodynia is evoked by normally non-painful cold and warm stimuli (Nickel *et al*., 2012). Normally, A-fibres produce the sensation of touch when stimulated; however, these same fibres can

produce the sensation of intense pain in some individuals with pathophysiological pain (Dworkin, 2002). **Hyperalgesia** is defined as an increased sensation of pain in response to a normally painful stimulus. In mechanical hyperalgesia, normally slightly painful pinprick stimulation is perceived as more painful. Peripheral sensitization, mediated through C-fibres, is the mechanism responsible for hyperalgesia (Byers and Bonica, 2001). Two types of hyperalgesia are frequently described: primary hyperalgesia, which is increased pain and sensitivity at the site of injury; and secondary hyperalgesia, which is increased sensitivity and pain in uninjured tissue that surrounds the site of injury. Whereas primary hyperalgesia is thought to be the result of peripheral changes that occur after tissue damage, secondary hyperalgesia, like allodynia, is thought to result from events that occur within the dorsal horn of the spinal cord after injury (Siddall and Cousins, 1997; Byers and Bonica, 2001).

Other clinical symptoms associated to central sensitization, such as sensations of burning pain, may be the result of continuous discharge in C-fibres, whereas dysesthesia (unpleasant abnormal sensations) and paresthesia (abnormal sensations) may arise from intermittent spontaneous discharges in Aδ or Aβ fibres (Rowbotham and Petersen, 2001). Sensing ongoing spontaneous pain and paroxysmal shooting pain in the absence of any external stimulus is caused by ectopic impulse generation within the nociceptive pathways. Several clinical conditions involving pain, such as fibromyalgia, irritable bowel syndrome, chronic fatigue syndrome, endometriosis and migraine, have all been linked to central sensitization (Tietjen *et al.*, 2009; Woolf, 2011).

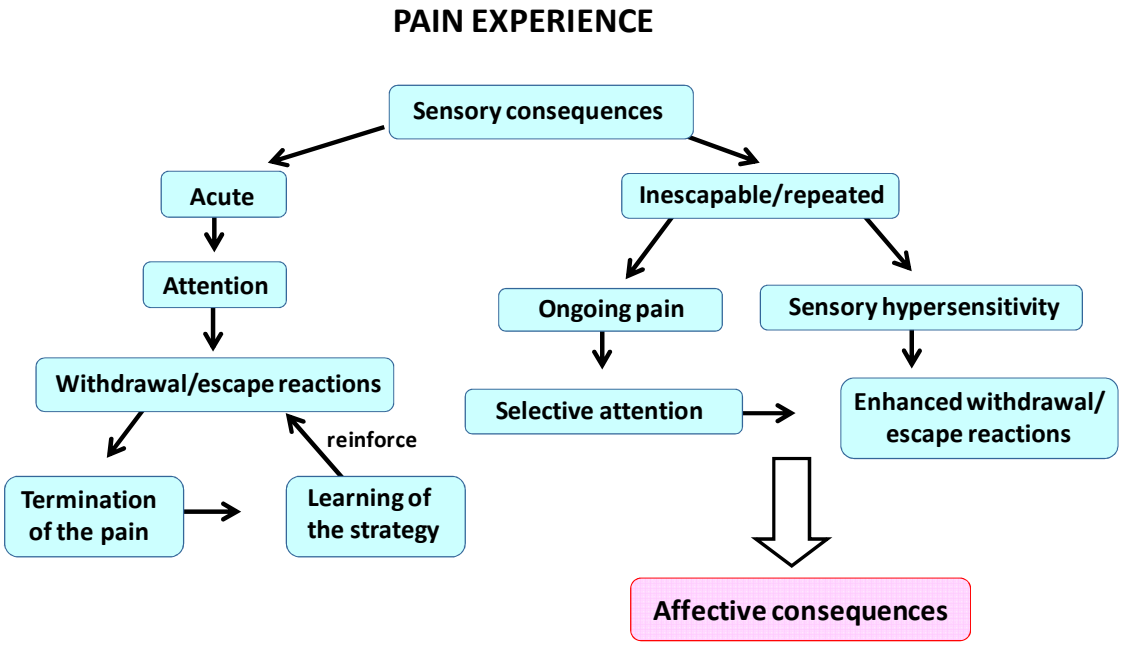


Fig. 4. **Schematic representation of sensory consequences of acute and chronic pain.** In acute pain conditions, noxious stimuli detected evoke a reflex response that rapidly moves the entire animal, or the affected part of its body, away from the source of the stimulus. With pain persisting past the injury, defined as chronic pain, the affected person has symptoms of somatosensory hypersensitivity (i.e., allodynia, hyperalgesia) and ongoing pain (i.e., stimulus-independent pain). These are the conditions for which patients repeatedly seek medical help, and there are no adequate treatments for the vast majority of them. When pain is more persistent it becomes clinically more relevant.

2.3. Preclinical measurement of the sensory-discriminative component

A large number of **animal models of pain,** both acute and chronic, have been developed to better understand pain (Wall *et al.*, 1979; Lombard *et al.*, 1979; Bennett and Xie, 1988; Diehl *et al.*, 1988) and its pharmacological and non-pharmacological modulation (Woolf, 1983; Coderre, 1992; Basbaum *et al.*, 2009; Woolf, 2011). Some of these animal models attempt to mimic inflammatory, osteoarthritic, neuropathic, cancer and postoperative pain in rodents. Inflammation can be induced experimentally by several agents, including carrageenan (CARR), complete Freund's adjuvant (CFA) or

bacterial lipopolysaccharide (LPS). Other inflammatory pain models involve inflammation of the viscera, such as cyclophosphamide-induced cystitis. Osteoarthritis can be induced experimentally by several methods, including the injection of the chondrocyte glycolytic inhibitor monoiodoacetate (MIA) or by surgical destabilization of the medial meniscus. The most widely used models of traumatic nerve injury include chronic constriction injury (CCI), constriction of the saphenous nerve (CCS), partial sciatic nerve ligation (PSNL), L5/L6 spinal nerve ligation (SNL), tibial nerve transection (TNT), L5 spinal nerve transection (SNT), and spared nerve injury (SNI). Peripheral neuropathy can also be induced experimentally (and clinically) by antineoplastics, including paclitaxel, vincristin, cisplatin or oxaliplatin. In addition to peripheral neuropathic pain models, neuropathy can also be induced by damaging the central nervous system by mechanical contusion or electrolytic lesions, among others. Cancer pain is usually induced in rodents by injecting tumor cells into the bone marrow (femur, calcaneus or tibia), which is accompanied by bone destruction. To model postoperative pain, the first model proposed was plantar incision. More recently, more realistic surgical procedures have been used, such as laparotomy or thoracotomy. These different injuries attempt to model clinical pain states in order to study their pathophysiological mechanisms and test new candidate analgesics. These animal models undoubtedly more closely mimic relevant human pain conditions as compared to the evaluation of acute pain (Cobos and Portillo-Salido, 2013).

The commonly used **outcomes used to assess the sensory component of pain** in these models have mainly focused on the appearance of reflexive movements upon the presentation of a noxious stimulus. The noxious stimulus may be thermal, mechanical, electrical or chemical, and is applied to a convenient body part, usually hindpaws, tail or

abdomen. Common examples include withdrawal responses from stimuli that can be escaped (e.g. paw withdrawal from a mechanical or thermal stimulus) or stretching/flinching responses from stimuli that cannot be escaped (e.g. stretching responses elicited by intraperitoneal injection of noxious chemical stimuli). Some of these behaviours provide the animal with control of the intensity or duration of the stimulus because the behaviour results in escape from the aversive stimulus.

In studies of acute nociception, pain is mainly assessed as the latency to the appearance of reflexive movements of the stimulated area (the paw or tail for the hot plate or tail-flick tests, respectively). Similarly, in tonic pain models, either formalin injected into the hindpaw or the intraperitoneal injection of chemical algogens are also able to elicit pain-induced reflexive responses (flinching/licking of the affected paw or twitching of the body in the **formalin** and the **writhing tests**, respectively). Pathological pain models focus on evaluating the enhancement of pain-like responses evoked by either mechanical (von Frey and Randall-Selitto tests) or thermal (Hargreaves and acetone drop tests) stimuli applied acutely. The **von Frey test** uses calibrated plastic hairs of different diameters (intensities of stimulation) which are applied sequentially to the target area of the animal to estimate the mechanical threshold for reflex withdrawal. The **Randall-Selitto test** consists in applying increasing pressure via blunt mechanical stimulation to the hindpaw until a withdrawal reflex, struggling or vocalization appears. Recent modifications of this test use fixed pressure to the paw and record the latency to the first pain-like response. The **Hargreaves test** uses a controlled light beam directed to the paw from the bottom of a glass surface where the animal is placed, and the outcome measure is latency to the paw withdrawal reflex. Finally, the **acetone drop test** is based on the sensation of cold produced by the evaporation of acetone placed on the

paw, which might elicit pain-like responses under pathological conditions. The outcome measures vary from the number or frequency of brisk foot withdrawals to the time spent reacting by licking or shaking the stimulated paw. Alterations in these outcomes illustrate the sensory hypersensitivity (hyperalgesia and allodynia) which often accompanies a chronic pain condition, and constitute the standard measure of pain in preclinical pain research laboratories worldwide (Cobos and Portillo-Salido, 2013).

3. The affective-motivational component of pain

Pain includes a strong emotional component that makes it aversive, and there is a reciprocal relationship between pain and affective states (Fig. 5). The affective-motivational component is close to what may be considered as "suffering from pain", and is clearly related to aspects of emotion, arousal and behavioural programming. The affective-motivational component of pain (i.e., the moment-by-moment unpleasantness of pain) refers to the **emotional responses** elicited by a painful stimulus —painful stimuli invoke feelings of suffering, fear, exhaustion, disgust, sadness and anxiety, among others, which motivate the individual to escape from or reduce the source of pain. Interestingly, this affective dimension of pain relies on neurophysiological systems that are at least partly anatomically different from those involved in the sensory perception of pain (Duquette *et al.*, 2007).

3.1. Neuroanatomical basis

The affective-motivational dimension of pain is supported by the medial system, which arises from neurons whose axons project medially within the spinothalamic tract in the cord and brainstem, and synapse within medial thalamic nuclei before projecting to a number of regions, including the cingulate cortex and the limbic system. People whose pain is treated by destroying part of the medial pain system (e.g. the cingulate cortex or the medial thalamus) report alleviation of the affective component of pain, but no loss of sensory discrimination. Such observations have influenced clinical practice. For example, destroying the cortical and thalamic components of the medial pain system to treat intractable pain regained some popularity (Romanelli *et al.*, 2004).

The brain circuitry interfacing acute pain representation and diverse chronic pain conditions involves areas commonly thought to be essential in emotional learning and memory, and in reward and addictive behaviour (Apkarian, 2008).

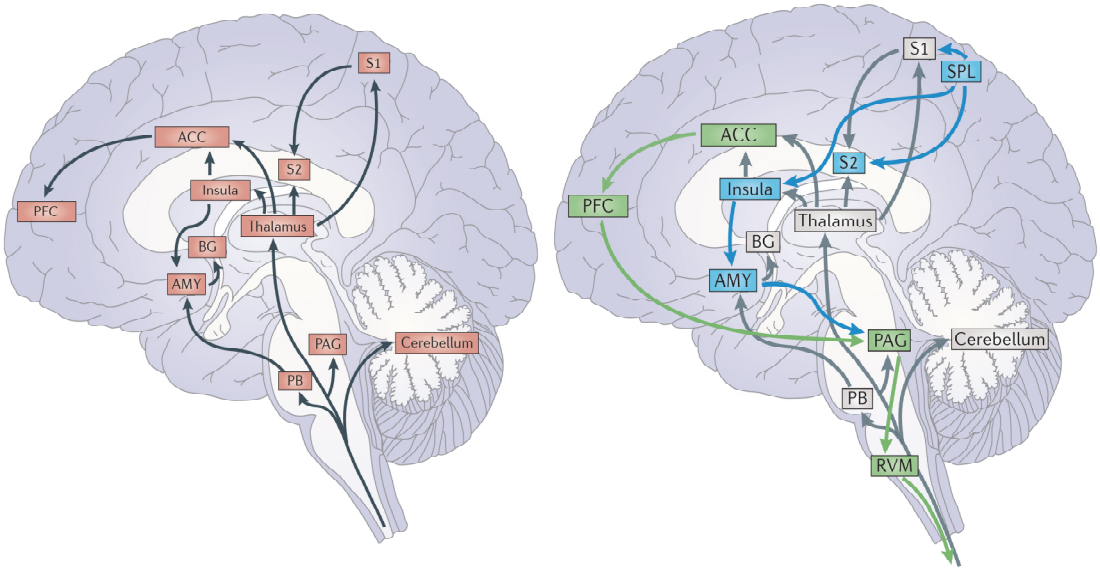


Fig. 5. Brain regions involved in the experience of pain. ***Left panel*** shows afferent pain pathways projecting to multiple brain regions involved in affective states. Afferent nociceptive information enters the brain from the spinal cord. Nociceptive information from the thalamus is projected to the insula, anterior cingulate cortex (ACC), primary somatosensory cortex (S1) and secondary somatosensory cortex (S2), whereas information from the amygdala (AMY) is projected to the basal ganglia (BG). ***Right panel*** shows how attentional and emotional factors modulate pain perception via different pathways. Attention and emotion alter pain via different descending modulatory systems. Emotions activate circuitry involving the anterior cingulate cortex (ACC), prefrontal cortex (PFC) and periaqueductal grey (PAG) (shown in green), whereas attention activates circuitry involving projections from the superior parietal lobe (SPL) to the primary somatosensory cortex (S1) and insula (shown in blue). Grey regions show parts of the ascending pain pathways depicted in left panel. PB, parabrachial nucleus; RVM, rostroventral medulla. *From: Bushnell MC, Ceko M and Low LA. Cognitive and emotional control of pain and its disruption in chronic pain. Nature Reviews Neuroscience 2013;14:502-11.*

The **prefrontal cortex (PFC)** is associated with high-order cognitive and emotional functions including attention and working memory (Gusnard *et al.*, 2001, Phelps *et al.*, 2004). In humans, different sub regions of the PFC have a role in acute pain; the **medial prefrontal cortex (mPFC)** was found to be involved in signalling the unpleasantness of pain (Lorenz *et al.,* 2002). The mPFC plays an important role in cognitive functions, such as decision making, avoidance of risky choices, and goal-directed behaviours in animals and humans. The mPFC is implicated in controlling our so called motivational behaviours (how we act to meet our "needs").

Pain has been associated with functional and structural abnormalities in prefrontal cortical areas (Apkarian *et al.,* 2004b; Metz *et al.,* 2009) and can impair emotion-based decision making in humans (Apkarian *et al.,* 2004a) and animals (Pais-Vieira *et al.,* 2009; Ji *et al.,* 2010). Functional MRI (fMRI) studies in patients with chronic pain have shown that chronic pain disrupts emotional and cognitive functions that are typically associated with the mPFC in primates, thus suggesting that this disruption may involve mPFC reorganization (Baliki *et al.*, 2006; Schweinhardt *et al.*, 2006). Additionally, studies in humans with chronic back pain show impaired performance on emotional decision-making tasks (Apkarian *et al.*, 2004b), which implies mPFC involvement. Thus, the emerging picture is that the higher the intensity of chronic pain, the higher the activity of mPFC (Metz *et al*., 2009). Finally, magnetic resonance studies show that chronic pain is associated with decreased grey matter density in various PFC regions (Apkarian *et al.*, 2004b; Kuchinad *et al.*, 2007).

The **cingulate cortex (CC)** is a critical area for the cortical processing of nociceptive inputs. Its anterior and supracallosal part (Brodmann's area 24) receives direct afferent fibres from several thalamic nuclei (midline, intralaminar, and mediodorsal nuclei) involved in pain and limbic circuitry (Vogt, 2005). One of the CC anatomical subdivisions proposed by Vogt and co-workers (Vogt *et al.*, 1993, 2005), the **anterior cingulate cortex (ACC)**, is a key cortical region for pain perception (Zhuo, 2008; Apkarian *et al.*, 2005; Vogt, 2005; Johansen and Fields, 2004). The ACC is a brain region that plays important roles in cognition and other executive functions. The ACC consists of different layers of pyramidal cells and local interneurons. Anatomic and functional studies reveal that ACC pyramidal cells receive sensory inputs projecting from the thalamus as well as other subcortical structures (Fig. 5). For the output projections of the ACC, pyramidal cells project to sensory related brain areas, including the motor cortex, amygdala, midbrain areas, brainstem, and spinal cord (Zhuo, 2007). The ACC mediates the affective component of pain responses (Rainville *et al.*, 1997) and the placebo effect (Wager *et al.,* 2004), and anticipation of pain is positively correlated with activity in both the ACC and mPFC (Porro *et al.*, 2002). Numerous functional neuroimaging studies and microelectrode exploration of the human brain have shown that the **midcingulate cortex (MCC)**, another anatomical subdivision, is also activated by pain stimuli (Peyron *et al.,* 2000; Jones *et al.,* 2002; Hutchinson *et al.*, 1999).

The **insular cortex** is anatomically positioned to serve as one potential interface between afferent processing mechanisms and more cognitively-oriented modulatory systems. Afferent nociceptive information can be transmitted rostrally from S2 to the posterior insula and then to the anterior insula (Fig. 5). The reciprocal connections of

the insula with the PFC, ACC, amygdala, parahippocampal gyrus and S2 can allow afferent nociceptive information to be integrated with information related to working memory, affection, and attention (Mufson *et al.*, 1981; Mesulam and Mufson, 1982; Mufson and Mesulam, 1982; Friedman and Murray, 1986; Friedman *et al.*, 1986).

The ACC and the **insula**, which have long been considered components of the limbic (emotional) part of the brain (Penfield and Boldrey, 1937; MacLean, 1949), are more important for encoding the emotional and motivational aspects of pain. The insula not only receives inputs from the sympathetic and parasympathetic systems, but also from pain pathways, and sends them to other cortical areas. As mentioned above, the insula is connected to the ACC, an important cortical area by itself, in connection with the PFC, and involved in consciousness and behaviour. The insula may be well positioned to utilize cognitive information to modulate connected brain areas involved in the processing of the sensory-discriminative and affective-motivational components of pain. Accordingly, insula lesions would be expected to lead to a complex pattern of altered experiences of pain evoked by suprathreshold noxious stimuli. Such changes could even include increased pain sensitivity due to a loss of modulation of the brain areas involved in various aspects of nociceptive processing (Starr *et al*., 2009).

The insula is also connected to the **amygdala**, the brain area responsible for fear responses and that is itself connected with the ACC (Medford and Critchley, 2010). The amygdala is an almond-shaped structure located in the temporal lobe of mammals and thought to be involved in assigning emotional significance to environmental information, and triggering adapted physiological, behavioural and affective responses. The amygdala is considered to provide an emotional value —either positive or negative— to sensory information, thus leading to adapted behavioural and affective

responses, and contributing to emotional memory (Veinante *et al.*, 2013). Pain-related hyperactivity in the amygdala leads to mPFC deactivation and decision-making deficit (Ji *et al*., 2010). These mechanisms of pain-related inhibition of the mPFC are not yet known.

In general, the influence of cognitive aspects and emotions on the processing of sensory information is mediated by extensive neuroanatomical networks with a pivotal role of the insular cortex and ACC (Seminowicz *et al.*, 2004). The affective-motivational aspect of pain preferentially activates the ACC and the anterior insular cortex, which in turn activate other components of the limbic system (i.e. areas of executive processing such as the medial and dorsolateral PFC) (Moisset and Bouhassira, 2007), which play an integral role in focusing attention to salient stimuli.

3.2. Affective consequences of pain

Animal studies of persistent neuropathic and inflammatory pain conditions suggest an elaborate reorganization of the peripheral and spinal cord properties. Depending on the type of injury, the details of the reorganization seem to impact distinct cell types and receptor types in the periphery as well as the spinal cord, giving rise to enhanced nociceptive transmission cephalad probably through distinct pathways (Hunt and Mantyh, 2001; Woolf and Salter, 2006). During persistent inflammatory, neuropathic or visceral pain, a long-lasting functional plasticity of brain activity contributes to an enhancement of the pain experience, including affective anxiety-like states (Fig. 6). Functional brain imaging studies in patients with visceral pain have provided evidence of an exaggerated activation of a vigilance network (i.e., the prefrontal cortex) and a failure to activate regions involved in pain inhibition (e.g., anterior cingulate cortex)

(Chang, 2005; Derbyshire, 2003; Piche *et al.*, 2010). Additionally, these functional brain imaging studies revealed that negative emotions of anxiety, anger and stress correlated negatively with anticipatory downregulation within the dorsal pons, amygdala and anterior cingulate cortex during visceral pain (Berman *et al.*, 2008).

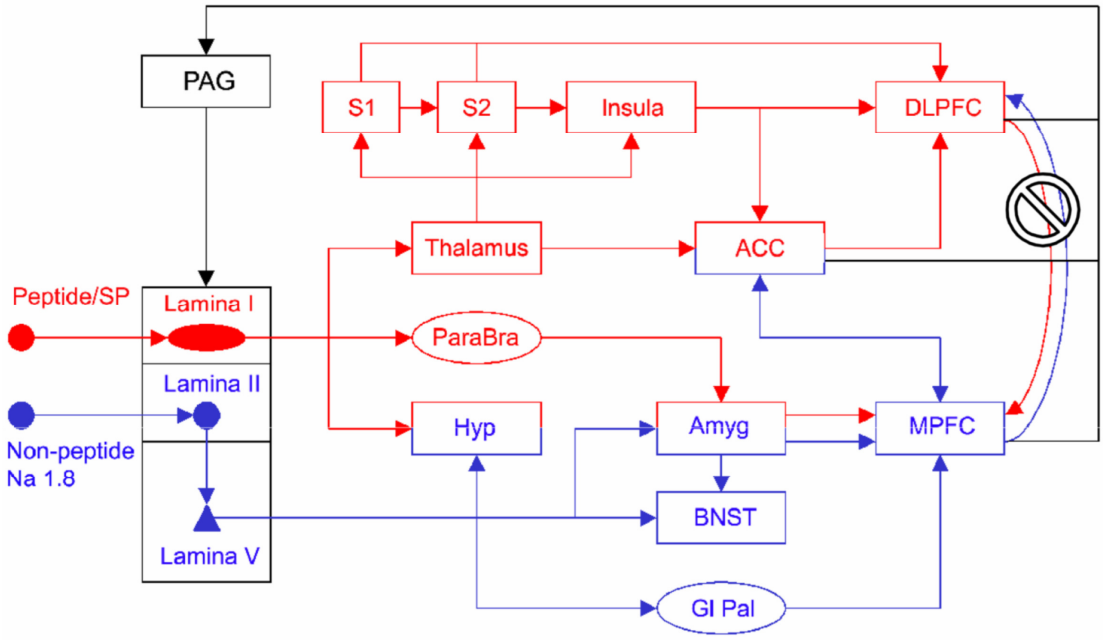


Fig. 6. Interactions between sensory-discriminative and affective-motivational components of pain. A simplified theoretical diagram of the main interactions between both components of pain. The interaction between basal ganglia (Gl Pal), amygdala (Amyg), and medial prefrontal cortex (MPFC) constitutes the emotional, motivational and hedonic components, which hypothetically influence the quality of perceived pain and also modulate nociceptive processing at the spinal cord level through descending pathways. **Blue pathways** engage motivation, hedonics, and affection. **Red pathways** are more involved in sensory coding, including inputs to the thalamus, primary and secondary somatosensory cortices (S1 and S2), and insula. The interaction between MPFC and dorsolateral prefrontal cortex (DLPFC) is mutually inhibitory. Authors hypothesize that blue pathways are strengthened in chronic neuropathic pain and red pathways are more involved in acute pain. Descending pathway indicated through the periaqueductal gray (PAG). The specific interactions between ascending and descending modulations would determine the specific brain activity patterns identified in distinct chronic pain conditions, which would reflect peripheral as well as learned central reorganization. *From: Apkarian AV, Baliki MN and Geha PY. Towards a theory of chronic pain. Progress in neurobiology 2009;87:81-97.*

***Depressive disorders are a widespread condition in chronic pain*.** There is a substantial body of human research supporting the association of chronic pain with high levels of emotional distress, particularly depression and anxiety (Gatchel *et al.*, 2007). This is not surprising because prolonged pain can be conceptualized as a complex form of stress (Blackburn-Munro and Blackburn-Munro, 2001, Chapman *et al.*, 2008) and stress plays a pivotal role in the development of both depression and anxiety (Taylor *et al.*, 2006; Gotlib *et al.*, 2008; Andrews and Thomson, 2009; El Hage *et al.*, 2009; Nugent *et al.*, 2011). Persistent pain, together with physical impairment often associated with pain conditions, are likely to produce high levels of stress which greatly contribute to anxiety and depression processes (Romera *et al.*, 2011). In addition, depression and anxiety in human patients are often comorbid and involve sleep disturbances (Drake *et al.*, 2003; Batmaz *et al.*, 2013). This has been repeatedly observed in human pain patients (Pilowsky *et al.*, 1985; Wilson *et al.*, 2002; Batmaz *et al.*, 2013, Li *et al.*, 2012). Sleep disturbances have been associated with pain severity (Wilson *et al.*, 2002; Li *et al.*, 2012), nocturnal pain episodes (Li *et al.*, 2012), and physical disability and subsequent psychological disturbances (Pilowsky *et al.*, 1985: Batmaz *et al.*, 2013, Li *et al.*, 2012). Altogether, these problems have a substantial negative impact on the patients' daily activities.

The convergence of depression and pain is reflected in the circuitry of the nervous system. In the experience of pain, communication between body and brain goes both ways. Normally, the brain diverts signals of physical discomfort so that the individual can concentrate on the external world. When this shut-off mechanism is impaired, physical sensations, including pain, are more likely to become the centre of attention. Brain pathways that handle the reception of pain signals, including the seat of emotions

in the limbic region, use some of the same neurotransmitters involved in mood regulation, particularly serotonin and norepinephrine. When regulation fails, pain is intensified along with sadness, hopelessness and anxiety. Chronic pain, like chronic depression, can alter the functioning of the nervous system and perpetuate itself (Bair *et al*., 2003; Turk and Okifuji, 2002).

The reduction in the ability of chronic pain sufferers to enjoy everyday pleasures (i.e., anhedonia) might form part of a vicious cycle for patients, where both negative mood and lack of pleasure result in exacerbated pain, leading to more negative mood and anhedonia (Marbach and Lund 1981; Marbach *et al*., 1983). The reciprocal relationship between mood and anxiety disorders and pain, make experience of one precipitate or exacerbate the symptoms of the other. People with chronic pain have three times the average risk of developing psychiatric symptoms —usually mood or anxiety disorders— and depressed patients have three times the average risk of developing chronic pain. Mood and anxiety disorders have also been associated with an increased likelihood of developing chronic pain symptoms (Bair *et al.*, 2003; Gureje *et al.*, 2001; Magni *et al.*, 1994) and of inducing greater sensitivity to experimentally induced noxious stimulation (Rhudy and Meagher, 2000; Tang *et al.*, 2008). On the other hand, it may also reflect the fact that cognitive and affective symptoms associated with mood and anxiety disorders affect the perception of chronic and acute pain. Finally, pain slows recovery from depression and depression makes pain more difficult to treat; for example, it may cause patients to drop out of pain rehabilitation programs. Worse, both pain and depression feed on themselves, by changing both the brain function and behaviour. Depression leads to isolation and isolation leads to further depression; pain causes fear of movement, and immobility creates the conditions for further pain. When

depression is treated, pain often fades into the background and when pain goes away, so does much of the suffering that causes depression (Lesho, 2003).

Pain-induced disturbances of reward processing. Some current evidences suggest that long-term pain impairs several aspects of reward processing: 1) anhedonia (i.e. the inability to feel pleasure) is associated with chronic pain (Marbach and Lund, 1981; Marbach *et al.*, 1983); 2) decreased reward sensitivity and/or decreased motivation has been observed in rats with neuropathic pain (Ozaki *et al.*, 2002); 3) impaired decision-making based on reward and punishment has been reported in patients with chronic back pain and complex regional pain syndrome (Apkarian *et al.*, 2004a) as well as in an animal model of arthritic pain (Ji *et al.*, 2010; Pais-Vieira *et al*., 2009); and 4) impaired operant learning of pain sensitization and habituation has been found in fibromyalgia patients (Becker *et al.*, 2011).

Numerous neuroimaging studies show that several brain regions are implicated in both pain and reward processing, including the anterior and posterior insula, amygdala, ACC, dorsal and ventral striatum, and orbitofrontal cortex (Leknes and Tracey, 2008). The pain–reward interactions found in behavioural data are mirrored in anatomical and functional overlaps of cerebral systems that process pain and reward (Leknes and Tracey, 2008). Chronic pain seems to alter the brain systems that process pain and reward, thereby possibly affecting how pain and reward interact (Becker *et al*., 2012) but the way pain changes reward processing is currently unknown. More recent studies have emphasized the active role of the cortex in clinical, primarily chronic pain conditions, and suggest that distinct chronic pains may have unique associated brain activity, reorganize the brain in unique ways, and also have an impact on the modulation of information processing in specific ways (Apkarian, 2008).

Some evidence indicates that prolonged pain and stress increase tonic dopamine release (Puglisi-Allegra *et al.*, 1991; Wood, 2006) impairing reward processing in the orbitofrontal cortex and ventral striatum, and dopamine activity. In line with this notion, fibromyalgia patients actually failed to show striatal dopamine release in response to an experimental pain stimulus (intramuscular hypertonic saline injection) as compared to healthy controls (Wood *et al.*, 2007). Further, dopamine release in the nucleus accumbens (NAc) after morphine treatment is markedly suppressed by sciatic nerve ligation (Ozaki *et al.*, 2003), a model of neuropathic pain. The striatum, particularly the ventral striatum, or NAc and amygdala, and related brain areas, are important in emotional memory, and could as a result mediate anhedonia, anxiety and reduced motivation predominantly found in many patients (Nestler *et al.*, 2002). Indeed, dysregulation or lesion of areas from these regions is associated with anhedonia (Miller *et al.*, 2006; Schlaepfer *et al.*, 2008).

3.3. Preclinical measurement of the affective-motivational component

The excessive overfocuses on reflex behaviors have been evaluated critically because the pain phenotype in humans involves complex behavioral alterations, including changes in daily living activities and psychological disturbances (the key affective component of pain phenomena) which are not reflected by reflexes. Recently, additional measures of supraspinal integration using non-reflexive pain behaviours have been increasingly pursued over the past few years (Cobos and Portillo-Salido, 2013). These behavioural outcomes, as a measure of the affective-motivational component of pain in several pain models, are summarized in Table 1.

Table 1. Pain model and behavioural responses used in preclinical research for the measurement of the sensory-discriminative (reflexive outcome) and affective-motivational (non-reflexive outcome) components of pain.

Pain state modeled	Experimental manipulation	Reflexive outcome	Nonreflexive outcome
Tonic pain	Formalin	Licking/flinching of the paw	Conditioned place aversion
	AA (i.p.)	Stretching of the body	Decreased exploratory activity Decreased wheel running Conditioned place aversion Anhedonia
Postoperative pain	Plantar incision	Thermal and mechanical hypersensitivity	Weight bearing asymmetry (if performed in the paw)
	Other surgical procedures	-	Decreased exploratory activity Increased analgesic self-administration
Inflammatory pain	Injection of inflammatory compound	Thermal and mechanical hypersensitivity	Weight bearing asymmetry Gait alterations Grip strength deficits Decreased exploratory activity Decreased burrowing behavior Decreased wheel running Decreased home cage activity Heat avoidance Analgesic self-administration Conditioned place aversion by pain Conditioned place preference by analgesia Increased anxiety and depression related behaviors? Sleep alterations?
Osteoarthritis	MIA	Thermal and mechanical hypersensitivity	Weight bearing asymmetry Grip strength deficits Decreased home cage activity Decreased exploratory activity Conditioned place preference by analgesia
Cancer pain		Thermal and mechanical hypersensitivity	Grip strength deficits
Peripheral neuropathy	Traumatic peripheral nerve injury	Thermal and mechanical hypersensitivity	Decreased burrowing behavior Cold avoidance Analgesic self-administration Conditioned place aversion by pain Conditioned place preference by analgesia Increased anxiety and depression related behaviors? Sleep alterations?
Central neuropathy	Central nervous system damage	Mechanical hypersensitivity	Decreased exploratory activity Conditioned place preference by analgesia

?: Unclear at the moment; AA: acetic acid; MIA: monosodium iodoacetate. From: Cobos EJ and Portillo-Salido E. "Bedside-to-Bench" Behavioral Outcomes in Animal Models of Pain: Beyond the Evaluation of Reflexes. Current neuropharmacology 2013; 11:560-91.

The methodology to quantify pain-related changes in behaviour can be categorized based on whether the primary outcome measure is reflexive or non-reflexive. These non-reflexive measures such as operant learning measures, spontaneous nocifensive behaviours, avoidance behaviours, physical activity levels or quality of life/function activity, involve cortical processing and decision making.

Non-reflexive measures that reflect more complicated consequences of pain in animals should help improve our understanding of mechanisms and diseases of co-morbidities associated to pain states and would provide a complete picture of the animal's experience of chronic pain (Blackburn-Munro, 2004). Further, in animal tests, studying the signs affected by pain which contribute to a reduced quality of life is important to understand the precise action of putative analgesic drugs and improve the relevance of these studies to humans. These include anxiety-, reward-, social- and despair-based behaviours as well as alterations in general locomotion, sleep, food and liquid intake.

4. Sensitivity of the sensory-discriminative and the affective-motivational components of pain to analgesics drugs

Despite the ever-expanding number of medications indicated for various pain conditions, there is still an unmet need for chronic pain management (Harden and Cohen, 2003). Pain management is limited by the inability to match a patient's condition —and pain mechanisms— to optimal treatment(s). Some evidences in **clinical population** point to the hypothesis that sensory and affective pain descriptor profiles exhibit an analgesic-specific response. For instance, lower doses of morphine result in statistically reliable reductions in the affective but not the sensory intensity responses, while highest dose of morphine reduce both sensory and affective responses to nociceptive stimuli (Price *et al*., 1985). In a comparative clinical study with post-orthopedic surgery patients decreases in the sensory component of pain were mostly associated with pain relief following ibuprofen, and decreases in the affective component of pain were mostly associated with pain relief following acetaminophen plus codeine (Heidrich *et al*., 1985). Roberts and co-workers showed a pharmacodynamic interaction of drugs in the affective, but not the sensory, dimension of pain in an experimental pain model involving thermal stimuli applied to normal subjects (Roberts *et al*., 2006). In this study, subjects received Δ^9-THC alone or in combination with morphine and rated the pain associated with the application of thermal stimuli to skin using two visual analog scales, one for the sensory and one for the affective aspects of pain. Among sensory responses, neither Δ^9-THC nor combination with morphine had a significant effect; however the combination of both drugs had a synergistic affective analgesic effect. Finally, exploratory analysis of data from a trial of

a gabapentin-morphine combination has also showed that sensory and affective pain descriptor profiles exhibit a treatment-specific response (Gilron *et al*., 2013).

All these studies suggest that the discrimination between the sensory and affective components of pain could be particularly useful when comparing analgesics of differing pharmacologic classes and when comparing the results of such assays in pain syndromes characterized by differing pain qualities. The investigations to evaluate treatment-specific effects on multiple sensory and affective pain descriptors, and prediction of treatment response by these descriptors, will advance efforts toward developing and implementing more effective individualized pain therapies.

Preclinical research has also shown that affective-motivational outcomes of pain have an extraordinary sensitivity to drug-induced analgesic-like effects in comparison to sensory-discriminative outcomes of pain (Cobos and Portillo-Salido, 2013).

Comparative results from preclinical studies summarized in Table 2 clearly show that mechanisms underlying the affective-motivational dimension of pain can be dissociated from the processing of sensory-discriminative component of pain.

The majority of evaluated drugs showed more effect or were more potent on the affective than the sensory component of pain. This enhanced sensitivity to drug-induced analgesia of the affective-motivational dimension of pain is seen for known analgesics of different classes, such as opioids and NSAIDs, and in a variety of outcomes. In fact, there are many drugs that have effects on the affective-motivational component of pain at doses which were inactive or minimally active in the sensory-discriminative component of pain, thus suggesting that these compounds might mediate their

antinociceptive actions by affecting exclusively the affective-motivational component of pain.

In many cases higher plasma levels of pain relievers in reflex based rodent models of pain are necessary in comparison to doses that relieve pain clinically. Thus, the doses that decrease the affective-motivational component of pain may be more relevant to the doses that are needed to relieve pain in the clinic. However, given the complex nature of pain perception in humans, both the affective-motivational and sensory aspects of pain must be important.

Only a few studies displayed in Table 2 reported a lower sensitivity to drug-induced analgesia in a nonreflexive outcome compared to a reflexive response. Curiously these studies used the locomotor and exploration activity (LMA) as nonreflexive/affective outcome. For instance, in the study by Stevenson (2009), morphine was shown to be 56-fold less potent in reversing the depression in locomotion induced by the intraperitoneal injection of acetic acid compared to acetic acid-induced writhing. These results suggest that LMA is not a particularly useful outcome to discriminate between the sensory and the affective components of pain.

In summary, results displayed in Table 2 clearly show that mechanisms underlying the affective-motivational dimension of pain can be clearly dissociated from the processing of sensory-discriminative component of pain and the majority of evaluated drugs showed more effect or were more sensitive on the affective than sensory component of pain suggesting that the high sensitivity of these outcomes to drug effects are an intrinsic quality of these measures.

Table 2. Comparison of drug effects on the sensory and affective component of pain. Compilation of published preclinical studies where different systemically administered drugs were reported with their analgesic potency or their analgesic effect on the components of pain, i.e., the sensory-discriminative (Sens) and the affective-motivational (Aff).

Drug type	Mechanism of action	Drug name	Efficacy	Potency	Pain model	Sensory endpoint	Affective endpoint	Animal specie	Reference
NSAIDs	COX-1/COX-2 inhibitors	aspirin	Sens << Aff	Sens << Aff	CARR	mechanical hyperalgesia	PEAP	rat	LaBuda and Funchs, 2001
		ibuprofen	Sens >> Aff	Sens > Aff		thermal hyperalgesia	LMA		Zhu *et al*., 2012
			N.D.	Sens << Aff	CFA	mechanical hypersensitivity	wheel-running	mouse	Cobos *et al*., 2012
		diclofenac	Sens < Aff	Sens < Aff			PEAP	rat	Boyce-Rustay *et al*., 2010
			Sens = Aff	Sens = Aff	CARR	thermal hyperalgesia	LMA		Zhu *et al*., 2012
		naproxen	N.D.	Sens << Aff	CFA	mechanical hypersensitivity	wheel-running	mouse	Cobos *et al*., 2012
	COX-2 selective inhibitor	celecoxib	Sens < Aff	Sens < Aff			PEAP	rat	Boyce-Rustay *et al*., 2010
			N.D.				wheel-running	mouse	Cobos *et al*., 2012
Opioid	μ-opioid agonist	morphine	Sens >> Aff	Sens >> Aff	CARR	thermal hyperalgesia	LMA	rat	Zhu *et al*., 2012
			N.D.	Sens << Aff	CFA		CPA		Zhang *et al*., 2013
					CARR	mechanical hypersensitivity			van der Kam *et al*., 2008
			Sens = Aff	Sens < Aff	CCI	mechanical hyperalgesia	PEAP		Pedersen and Blackburn-Munro, 2006
			Sens > Aff	Sens > Aff	SPL	mechanical hypersensitivity			LaGraize *et al*., 2006
				Sens >> Aff	AA ip	writhing	LMA	mouse	Stevenson *et al*., 2009
			Only Sens				palatable solution		Miller *et al*., 2011
			Sens > Aff				wheel-running		
			N.D.	Sens << Aff	CFA	mechanical hypersensitivity			Cobos *et al*., 2012

Table 2. (*Continued*)

Drug type	Mechanism of action	Drug name	Efficacy	Potency	Pain model	Sensory endpoint	Affective endpoint	Animal specie	Reference
Anticonvulsionants *Benzod.*	GABA chloride channel agonist	etizolam	Only Aff	Sens << Aff	PSNL	thermal hyperalgesia	light-dark test; elevated plus-maze test	mouse	Narita *et al*., 2006
Anticonvulsionants	α2δ Ca^{2+} channel subunit ligand	gabapentin	Only Sens	Sens > Aff	CARR	thermal hyperalgesia	LMA	rat	Zhu *et al*., 2012
			Sens > Aff	Sens << Aff	CCI	mechanical hyperalgesia	PEAP		Pedersen and Blackburn-Munro, 2006
			Sens >> Aff			mechanical hypersensitivity			
Antidepressants	SNRI	duloxetine	Only Sens	Sens >> Aff	CARR	thermal hyperalgesia	LMA		Zhu *et al*., 2012
			Sens < Aff	Sens < Aff	CCI	mechanical hyperalgesia	PEAP		Pedersen and Blackburn-Munro, 2006
			Sens << Aff	Sens << Aff		mechanical hypersensitivity			
			Sens < Aff		CFA	mechanical hypersensitivity			Boyce-Rustay *et al*., 2010
	SSRI	fluoxetine	Only Sens	Sens > Aff					
	5-HT agonist	8-OH-DPAT	Sens < Aff	Sens < Aff	CCI	mechanical hyperalgesia			Pedersen and Blackburn-Munro, 2006
			Sens << Aff	Sens << Aff		mechanical hypersensitivity			
Others	dopamine D2 receptor antagonist	haloperidol	Only Sens	Sens >> Aff	AA ip	writhing	LMA	mouse	Stevenson *et al*., 2009
	NMDA receptor antagonist	phencyclidine			SPL	mechanical hyperalgesia	PEAP	rat	Boyette-Davis and Fuchs, 2011
		ketamine	Only Aff	Sens << Aff	SNI	mechanical hypersensitivity	sucrose preference		Wang *et al*., 2011
	muscarinic receptor antagonist	scopolamine	None	None	CFA		PEAP		Boyce-Rustay *et al*., 2010
	corticosteroid	prednisolone	N.D.	Sens << Aff			wheel-running	mouse	Cobos *et al*., 2012

Note that the "efficacy" might be related to the species and studied model.

CPA and PEAP tests to assess the level of unpleasantness evoked by painful stimuli; LMA test to assess spontaneous physical activity. Wheel running is a pleasure seeking activity for caged mice. The elevated plus maze test and light-dark test are widely used tests for measuring anxiety-like behavior. Decrease in sucrose preference is associated with depression-like behavior.

Aff: affective-motivational component of pain; Benzod: Benzodiazepine; CARR: carrageenan; CCI: chronic constriction injury; CFA: complete Freund's adjuvant; COX: ciclooxigenase; CPA: condicioned place avoidance; D2: dopamin receptor subtype 2; GABA: γ-aminobutyric acid; LMA: locomotor activity; N.D.: not determined; NSAIDs: nonsteroidal anti-inflammatory drugs; PSNL: partial sciatic nerve ligation; PEAP: place escape avoidance paradigm; SNRI: serotonin and norepinephrine reuptake inhibitor; Sens: sensory-discriminative component of pain; SNI: spared nerve injury; SPL: spinal nerve ligation; SSRI: selective serotonin reuptake inhibitors; 5-HT: serotonin receptor.

5. The sigma-1 receptor

Sigma receptor was first discovered in 1976 by William Martin and coworkers and was initially considered as a subtype of the opiate receptor because they bind certain synthetic opioids (Martin *et al.*, 1976). Subsequent studies confirmed that the sigma receptor is a non-opioid, non-G protein-coupled intracellular protein (Hanner *et al.*, 1996, Hayashi *et al.*, 2011). In the early 1990s, and based on the selectivity profile of some ligands, the molecular mass of the binding sites and the anatomical distribution, two distinct sigma receptor subtypes were identified: the sigma-1 (σ_1R) receptor and the sigma-2 (σ_2R) receptor (Bowen *et al.*, 1989; Hellewell and Bowen, 1990; Hellewell *et al.*, 1994). While σ_1R has been cloned (Hanner *et al.*, 1996; Kekuda *et al.*, 1996; Prasad *et al.*, 1998; Seth *et al.*, 1998; Mei and Pasternak, 2001), σ_2R has not yet been cloned. σ_1R consists of 223 amino acids (Hanner *et al.*, 1996) with two transmembrane domains (Aydar *et al.*, 2002). σ_1R has a molecular weight of ~25 kDa and although it has no homology to any other mammalian proteins (Hanner *et al.*, 1996), it shares a 30% identity with the yeast C8–C7 sterol isomerase (ERG2 protein), which is involved in sterol synthesis (Moebius *et al.*, 1997). σ_1R, however, does not contain sterol isomerase activity (Hanner *et al.*, 1996). The fact that σ_1R shares no homology with any mammalian protein made it difficult to predict the molecular function of σ_1R from the amino acid sequence. Nonetheless, a combination of subcellular localization studies, protein purification studies, coupling proteins identification studies and in vitro protein activity assays has begun to reveal the molecular function of σ_1R.

σ_1R has been identified as a ligand-responsive endoplasmic reticulum (ER) chaperone residing mainly at the ER-mitochondrion interface (MAM: the mitochondria-associated

ER membrane) (Hayashi and Su, 2007; Maurice and Su, 2009; Su *et al.*, 2010). In its dormant state, σ_1R forms a complex with another chaperone binding immunoglobulin protein (BiP), also known as 78 kDa glucose-regulated protein (GRP78), in the lumen of the ER. The presence of σ_1R agonists can trigger dissociation of the σ_1R from BiP, activating the σ_1R chaperone (Hayashi and Su, 2007; Su *et al.*, 2010). It was also demonstrated that σ_1R antagonists inhibit the action of agonists (Hayashi and Su, 2007). Therefore, synthetic drugs that can associate with σ_1R activate or inhibit their chaperone activity. The physical interaction that occurs between subcellular organelles provides an efficient mechanism for modulating cellular processes.

The MAM is a small section of the outer mitochondrial membrane tethered to the ER by lipid and protein filaments. MAM proteins, like σ_1R, contribute to multiple signaling pathways. σ_1R associating with BiP stabilizes inositol 1,4,5-triphosphate receptors type-3 at the MAM, leading to the regulation of Ca^{2+} influx into mitochondria and following ATP production, as an inter-organelle signaling modulator between the endoplasmic reticulum and mitochondria (Hayashi and Su, 2007; Su *et al.*, 2010). If stimulated by high concentrations of agonists or impacted by extreme ER stress, σ_1R is able to translocate from the MAM to the plasma membrane to directly bind to or indirectly regulate various ion channels (mainly sodium and potassium), kinases and receptors, including NMDA and some G-protein-coupled receptors, such as dopamine D1 receptors and μ-opioids receptors (Almansa and Vela, 2014). As a molecular chaperone, σ_1R and its ligands have no effect on ion channels and receptors under normal physiological conditions. However, in the presence of disease or dysfunction, the assistance of σ_1R chaperones may be demanded by changes in the activity of specific ion channels, receptors and other cellular signaling mediators (Su *et al.*, 2010).

σ_1R has been implicated in a myriad of cellular functions, biological processes and diseases. σ_1R interacts with endogenous neurosteroids, thus suggesting a link between the endocrine, nervous and immune systems (Su *et al.*, 1988). Additionally, σ_1R has been shown to be involved in the regulation of numerous neurotransmitter systems, such as cholinergic, dopaminergic and glutamatergic neurotransmission (Fu *et al.*, 2010; Cobos *et al.*, 2008). Although the signal transduction pathway after activation of σ_1R is not completely understood, there is increasing evidence suggesting that its represents a potential therapeutic target in many diseases.

σ_1R is expressed in nearly all mammalian cells. However, despite its ubiquity, no natural ligand for σ_1R has been clearly identified; thus, scientists have puzzled for years over the precise function of this receptor protein. Nevertheless, the σ_1R activity has been shown to be modulated by progesterone, sphingosine, and endogenous trace amines and their N-methyl and N,N-dimethyl derivatives (Saavedra and Axelrod, 1972). σ_1R is widely present in peripheral organs and tissues such as lung, liver, kidney and heart (Hellewell *et al.*, 1994; Alonso *et al.*, 2000; Guitart *et al.*, 2004). σ_1R is found in the immune, endocrine and nervous systems, with a characteristic distribution in the central nervous system. Interestingly, σ_1R has been described to be located in CNS regions involved in pain control (i.e., the superficial layers of the spinal cord dorsal horn, periaqueductal gray matter, locus coeruleus, and rostroventral medulla) (Alonso *et al.*, 2000).

Sigma-1 receptor and pain. Preclinical evidence has pointed out its potential as an adjuvant therapy to enhance opioid analgesia, without increasing the side effects associated with the use of opioids (Zamanillo *et al.*, 2013; Vidal-Torres, *et al.*, 2013). It was demonstrated that σ_1R is physically associated to μ-opioid receptors and that σ_1R

antagonists (but not agonists) potentiate opioid analgesia without influencing opioid receptor binding (Kim *et al.*, 2010). It is important to note that this increase in opioid potency appears to be limited to analgesia and not to side effects such as the inhibition of gastrointestinal transit (Chien and Pasternak, 1994; Vidal-Torres *et al.*, 2013). Therefore, σ_1R is a biochemically quite unique molecule that plays an important role in the modulation of opioid analgesia.

On the other hand, σ_1R ligands by themselves also play a role in modulating pain behaviour in the absence of opioids, particularly in sensitizing and chronic pain conditions. The site of action of this modulatory effect points to the spinal cord, where σ_1R is abundantly expressed in the superficial layers of the dorsal horn, and the underlying mechanism involved points to NMDA receptors and Ca^{2+}-dependent intracellular cascades. These aspects have been studied using σ_1R knock-out mice and selective σ_1R antagonists. Investigation using σ_1R knock-out mice has shown that mice lacking σ_1R are not able to completely develop the two phases of formalin-induced paw licking/biting behaviour. Both phases were reduced by >50% in comparison to wild-type animals, indicating that σ_1R is necessary for the full expression of formalin-induced pain (Cendan *et al.*, 2005b). The NMDA receptor plays an important role in this behavioural test. Mice lacking σ_1R are not able to develop mechanical hypersensitivity induced by intraplantar injection of capsaicin (Entrena *et al.*, 2009). Capsaicin administration induces secondary mechanical hypersensitivity through a central mechanism involving NMDA activation and extracellular signal-regulated kinase (pERK) phosphorylation (Kawasaki *et al.*, 2004). The behavioural phenotype of σ_1R knock-out mice sensitized by formalin or capsaicin was mimicked in wild-type

mice using systemically administered σ_1R antagonists which exerted antinociceptive (antiallodynic and antihyperalgesic) effects (Cendan *et al.*, 2005a, Entrena *et al.*, 2009).

Taken together, the above information highlights an important yet largely unexplored but promising area of research to examine the biological function and therapeutic potential of σ_1R. σ_1R ligands act as ideal therapeutic drugs effective only under pathological conditions, but inactive in normal, resting conditions (Hayashi and Su 2003; Tsai *et al.* 2009; Hayashi *et al.* 2010, 2011).

ESTEVE has been working in the field of σ_1R and has accumulated significant know-how and expertise. ESTEVE has developed its first-in-class new chemical entity, the pyrazole derivative E-52862 (S1RA), identified in a medicinal chemistry program as a highly active and selective σ_1R antagonist (Díaz *et al.*, 2012). E-52862 showed high affinity for σ_1R (Ki = 17 nM) and good σ_1/σ_2 selectivity ratio (>550) as well as antagonistic behaviour according to the phenytoin test. Moreover, it was highly selective over a 170 target panel and was shown to penetrate the blood-brain barrier and bind to the σ_1R in the CNS (Romero *et al.*, 2012). This σ_1R antagonist displays analgesic activity after systemic administration in different animal models of pain at doses devoid of side effects. Interestingly, a close correspondence (significant correlation) has been found between the extent of CNS receptor occupancy and the antinociceptive efficacy of E-52862 on different pain models (Romero *et al.*, 2012). When combined with opioids, E-52862 increases the opioid analgesia but not the undesirable side effects of opioids such as tolerance, hyperalgesia and other opioid-related side effects (Vidal-Torres *et al*., 2013).

E-52862 is a novel and highly selective σ_1R antagonist that provides a new approach to the management of pain. E-52862 is the only compound representing this new drug class (i.e., σ_1R antagonists for the treatment of pain) which has advanced to clinical development (Vela *et al.*, 2015). E-52862 has completed single- and multiple-dose phase I clinical studies and has shown a good safety, tolerability, pharmacodynamic and pharmacokinetic profile in healthy subjects (Abadias *et al.*, 2013). It is currently in phase II clinical development for multiple neuropathic pain indications.

II. Hypothesis

The sigma-1 receptor (σ_1R) is a unique ligand-regulated target class with chaperoning functions over different molecular targets. In its role as a molecular chaperone, σ_1R modulates downstream signalling pathways activated upon the stimulation of other systems (Su *et al.*, 2010). The function of σ_1R can be modulated by agonists and antagonists that differentially affect the ability of σ_1R to interact with different proteins. It has been postulated that σ_1R ligands have no effects by themselves but are able to modulate signalling pathways under pathological conditions. σ_1R is distributed in peripheral organs and in different areas of the central nervous system involved in memory, emotions, and sensory and motor functions (Wolfe *et al.*, 1989). In particular, it is expressed in key areas for pain control, such as the dorsal root ganglia, the superficial layers of the dorsal horn spinal cord, the periaqueductal grey matter, the locus coeruleus, and the rostroventral medulla (Zamanillo *et al.*, 2013). σ_1R was first linked to analgesia by Chien and Pasternak twenty years ago describing an endogenous anti-opioid system in acute pain models (Chien and Pasternak, 1994). Later studies using genetic (σ_1R knock-out mice) and pharmacological (selective σ_1R antagonists) approaches found a reduction in pain-like behaviours in the acute somatic pain induced by formalin and capsaicin (Cendan *et al.*, 2005a, b; Entrena *et al.*, 2009) suggesting a possible role of σ_1R in chronic pain.

Pain includes not only a sensory-discriminative but also a motivational-affective component (Fernandez and Turk, 1992; Auvray *et al.*, 2010). The sensory-discriminative component of pain provides information about the spatial and temporal localization of nociceptive inputs on the body and it is usually evaluated in the preclinical setting by reflex-based procedures (e.g., tail flick, licking, and guarding) induced by aversive stimulation (through the application of particular mechanical,

thermal, electrical, and chemical stimuli). Reflex-based procedures are important measures in preclinical pain studies, but are insufficient to capture the complexity of the pain experience which frequently triggers the onset of new disorders such as depression and/or anxiety (Cobos and Portillo-Salido, 2013).

The affective-motivational component of pain provides the input driving the negative emotional experience from the threatening long-term consequences of the nociceptive input, which underline several affective disorders such as depression (Simons *et al.*, 2011). Anhedonia, or markedly diminished interest or pleasure, is a hallmark symptom of depression and is usually evaluated in the preclinical setting by decreases on the expression of positively motivated behaviours (Der-Avakian and Markou, 2012), which are behaviours that have the ability to ensure a positive emotional state experienced as pleasure (Pfaffmann, 1960; Cytawa and Trojniar, 1978).

Considering that pain includes both a sensory-discriminative and an affective-motivational component and the role of σ_1R in areas controlling both sensory-discriminative function and emotions, **we hypothesized that modulation of σ_1R could attenuated not only the enhanced behavioural reflex in the sensory-discriminative component of acute and chronic pain but also the putative deficits on positively motivated behaviours of the affective-motivational component.**

III. Objectives

The global aim of this Thesis was to compare the role of σ_1R in both the sensory-discriminative and the affective-motivational components of visceral acute pain and chronic neuropathic pain.

To achieve this aim, the following specific objectives have been addressed:

1. To set-up the model of acetic acid (AA) as a model of acute visceral pain and the model of partial sciatic nerve ligation as a model of chronic neuropathic pain using reflex-based measurements, and consequently providing information about the sensory-discriminative component of pain.

2. To study the effect of the absence of σ_1R on the sensory-discriminative component of pain using reflex-based measurements in both acute and chronic pain models.

3. To study the effect of pharmacological blockade of σ_1R on the sensory-discriminative component of pain using reflex-based measurements in both acute and chronic pain models.

4. To set-up behavioural models based on hedonic-like behaviours providing information about the affective-motivational component of pain in the acute visceral and chronic neuropathic pain model.

5. To study the effect of the absence of σ_1R on the affective-motivational component of pain using changes of hedonic-like behaviours in both acute and chronic pain models.

6. To study the effect of pharmacological blockade of σ_1R on the affective-motivational component of pain using changes of hedonic-like behaviours in both acute and chronic pain models.

IV. Methods

Materials and methods used in the present Doctoral Thesis are widely described in its respective published article or manuscript. However, to provide an overview of the different experimental approaches, we have added the following tables where we present the summarized methodology.

Table 1. Summary of methods for the study of the sensory-discriminative component of pain.

Injury	Pain-related behaviours	Endpoint	Assessment methods	Drugs tested
AA i.p.	Writhing reflex	Writhes (n)	Manually record	E-52862, BD-1063, MOR, Ibu, DIC, DLX, CAF
PSNL	Hypersensitivity to mechanical stimuli	Hindpaw withdrawal latency (g)	von Frey filaments (up and down method)	E-52862
PSNL	Hypersensitivity to thermal stimuli	Hindpaw withdrawal latency (s)	Plantar test apparatus	N.T.
PSNL	Hypersensitivity to cold stimuli	Hindpaw elevations (n)	Hot/cold plate analgesiameter	N.T.

AA: acetic acid; PSNL: partial sciatic nerve ligation; MOR:morphine; Ibu: ibuprofen; DIC: diclofenac; DLX: duloxetine; CAF: caffeine; NT: no tested.

Table 2. Summary of methods for the study of affective-motivational component of pain.

Injury	Pain-related behaviours	Endpoint	Assessment methods	Drugs tested
AA i.p. / PSNL[(1)]	Decreased sweet preference	Solution intake (g)	Two-bottle choice, saccharin *versus* water	Ibu, CAF
AA i.p.	Decreased locomotor activity	Motion time (s)	Actimeter	Ibu, CAF
AA i.p. / PSNL[(2)]	Decreased approach behaviour, decreased consumption	Latency to eat (s), number of approaches (n), amount consumed (g), eating time (s)	Manually record	E-52862, BD-1063, MOR, Ibu, DIC, DLX, CAF

AA: acetic acid; PSNL: partial sciatic nerve ligation; LMA: Locomotor Activity; RSB: Reward Seeking Behavior; MOR :morphine; Ibu: ibuprofen; DIC: diclofenac; DLX: duloxetine; CAF: caffeine. [(1)]*No drug tested;* [(2)]*Only tested with E-52862.*

Behavioural studies were performed with CD-1 wild type (WT) mice (Charles River, L'Arbresle, France).

The effect of the absence of σ_1R in both components of pain was performed with **σ_1R knock-out ($\sigma_1R^{-/-}$) mice** generated by ESTEVE (Langa *et al.*, 2003) and backcrossed up to the 10th generation on CD-1 genetic background to reduce to less than 1% the genetic material remaining from the original background (Wong, 2002). The colony was maintained and distributed by Harlan Laboratories (Sant Feliu de Codines, Spain).

The effect of pharmacological blockade of σ_1R for the studying the role of this receptor in the sensory-discriminative and affective-motivational components of pain were performed with the highly selective σ_1R antagonist **E-52862** developed by ESTEVE and compared with other σ_1R antagonist or reference compounds.

We selected the intraperitoneal administration of diluted doses of **acetic acid (AA**) as a visceral noxious stimulus to model an acute pain state. These noxious stimuli not only lead nociceptive responses that trigger reflex responses but also could provide us with inputs from the affective consequences linked to visceral pain (Gebhart and Ness, 1991).

We selected the **partial sciatic nerve ligation (PSNL)** to model a chronic pain state. In this model, the damages in the peripheral nerve produce sensory-discriminative alterations as mechanical and thermal hypersensitivity but the negative affective consequences are less explored (Fig. 1).

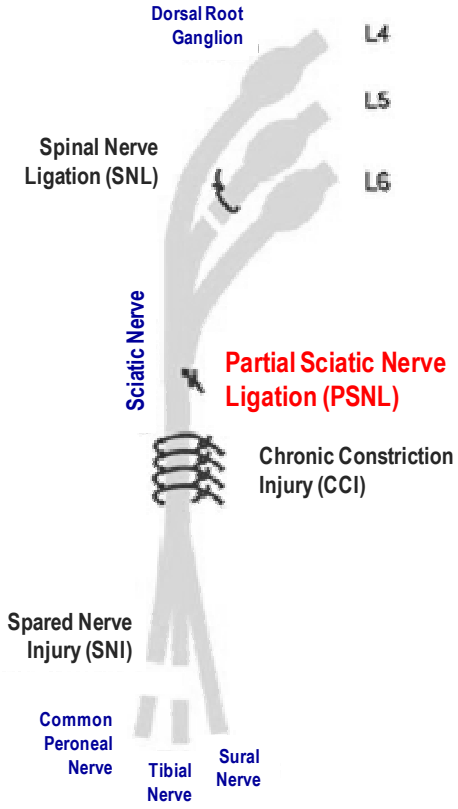


Fig. 1. Animals model of neuropathic pain. PSNL was the neuropathic pain model performed to study the sensory-discriminative and affective-motivational components of chronic pain. In the partial sciatic ligation (PSNL) model, a portion of the sciatic nerve is tightly ligated (Seltzer *et al.*, 1990). Other models depicted: SNL model (spinal nerve ligation); CCI model (chronic constriction injury, four loose chromic-gut ligatures on the sciatic nerve); SNI model (spared nerve injury) (Decosterd and Woolf, 2000). In all cases, only a portion of the afferents going to the foot are lesioned. The magnitude and duration of pain responses vary considerably depending on the suture material and strains (Yoon *et al.*, 1999). *From: Campbell JN and Meyer RA. Mechanisms of neuropathic pain. Neuron 2006;52:77-92.*

V. Results

1. Role of the sigma-1 receptor in the sensory-discriminative component of chronic neuropathic pain and acute visceral pain

1. Role of the sigma-1 receptor in the sensory-discriminative component of chronic neuropathic pain and acute visceral pain

In order to study the role of σ_1R in **the sensory-discriminative component of chronic neuropathic pain**, behavioural hypersensitivity to thermal and mechanical stimulation in the partial sciatic nerve ligation model in mice was evaluated and two experimental strategies were used. Firstly, $\sigma_1R^{-/-}$ mice were used to study the effect of the absence of σ_1R on the development of peripheral neuropathy. We accomplished the study using electrophysiological, immunohistochemical and immunoblot analysis. The results obtained from the experimental procedures conducted with $\sigma_1R^{-/-}$ mice are presented in **Article 1**. Secondly, E-52862 was used to selectively block σ_1R in the same model of peripheral neuropathy. The results obtained with this approach are presented in **Annex I**.

The abdominal writhing behaviour induced by intraperitoneal (i.p.) administration of acetic acid was selected to explore the role of σ_1R in the **sensory-discriminative component of acute visceral pain**. Similarly to the study of chronic neuropathic pain, a genetic and a pharmacological approach were used. Firstly, the writhing-reflex responses when the receptor was absent were assessed in $\sigma_1R^{-/-}$ mice. Secondly, selective σ_1R antagonists were used to block the receptor function. The results obtained with these two approaches are presented in **Annex II**.

1.1. ARTICLE 1

Sigma-1 receptors regulate activity-induced spinal sensitization and neuropathic pain after peripheral nerve injury

Beatriz de la Puente, Xavier Nadal, Enrique Portillo-Salido, Ricard Sánchez-Arroyos, Sergio Ovalle, Gabriel Palacios, Asunción Muro, Luz Romero, José Manuel Entrena, José Manuel Baeyens, José Antonio López-García, Rafael Maldonado, Daniel Zamanillo, José Miguel Vela

Department of Pharmacology, Drug Discovery and Preclinical Development, ESTEVE

Pain 2009; 145:294-303

Article Comment in: Central sensitization needs sigma receptors.
Drews E and Zimmer A. Pain 2009; 145:269-70.

PAIN® 145 (2009) 294–303

PAIN®

www.elsevier.com/locate/pain

Sigma-1 receptors regulate activity-induced spinal sensitization and neuropathic pain after peripheral nerve injury

Beatriz de la Puente [a], Xavier Nadal [b], Enrique Portillo-Salido [a], Ricard Sánchez-Arroyos [a], Sergio Ovalle [a], Gabriel Palacios [a], Asunción Muro [a], Luz Romero [a], José Manuel Entrena [c], José Manuel Baeyens [c], José Antonio López-García [d], Rafael Maldonado [b], Daniel Zamanillo [a], José Miguel Vela [a,*]

[a] *Department of Pharmacology, Laboratorios Esteve. Av. Mare de Déu de Montserrat, 221. 08041 Barcelona, Spain*
[b] *Laboratory of Neuropharmacology, Facultat de Ciències de la Salut i de la Vida, Universitat Pompeu Fabra. Doctor Aguader, 88. 08003 Barcelona, Spain*
[c] *Department of Pharmacology and Institute of Neuroscience, Faculty of Medicine, Universidad de Granada, Av. de Madrid 11. 18012 Granada, Spain*
[d] *Department of Physiology, Faculty of Medicine, Universidad de Alcalá de Henares. Alcalá de Henares, 28871 Madrid, Spain*

ARTICLE INFO

Article history:
Received 24 March 2009
Received in revised form 1 May 2009
Accepted 12 May 2009

Keywords:
Sigma-1 receptor
Neuropathic pain
Hyperalgesia
Allodynia
Spinal cord
ERK
Wind-up
Central sensitization

ABSTRACT

Sigma-1 receptor (σ_1R) is expressed in key CNS areas involved in nociceptive processing but only limited information is available about its functional role. In the present study we investigated the relevance of σ_1R in modulating nerve injury-evoked pain. For this purpose, wild-type mice and mice lacking the σ_1R gene were exposed to partial sciatic nerve ligation and neuropathic pain-related behaviors were investigated. To explore underlying mechanisms, spinal processing of repetitive nociceptive stimulation and expression of extracellular signal-regulated kinase (ERK) were also investigated. Sensitivity to noxious heat of homozygous σ_1R knockout mice did not differ from wild-type mice. Baseline values obtained in σ_1R knockout mice before nerve injury in the plantar, cold-plate and von Frey tests were also indistinguishable from those obtained in wild-type mice. However, cold and mechanical allodynia did not develop in σ_1R null mice exposed to partial sciatic nerve injury. Using isolated spinal cords we found that mice lacking σ_1R showed reduced wind-up responses respect to wild-type mice, as evidenced by a reduced number of action potentials induced by trains of C-fiber intensity stimuli. In addition, in contrast to wild-type mice, σ_1R knockout mice did not show increased phosphorylation of ERK in the spinal cord after sciatic nerve injury. Both wind-up and ERK activation have been related to mechanisms of spinal cord sensitization. Our findings identify σ_1R as a constituent of the mechanisms modulating activity-induced sensitization in pain pathways and point to σ_1R as a new potential target for drugs designed to alleviate neuropathic pain.

1. Introduction

Sigma receptors have been classified into two subtypes (σ_1R and σ_2R) [15,22]. Sigma-1 receptor (σ_1R) is the only subtype that has been cloned so far, and its sequence has no known homology with other mammalian protein. The gene encodes a protein of 223 amino acids with at least one putative transmembrane domain anchored to endoplasmic reticulum and plasma membranes [23,60,62].

From a functional point of view, the σ_1R has been proposed to be a modulator of a variety of receptors and ion channels, acting as amplifiers in signal transduction cascades [67]. At the endoplasmic reticulum the σ_1R acts as a chaperone regulating the flow of Ca^{2+} via inositol 1,4,5-trisphosphate (IP_3) receptors [26,27]. In addition, σ_1R regulates interorganellar Ca^{2+} signaling [28] and lipid transport from the endoplasmic reticulum to plasma membrane lipid rafts [29,30]. Other relevant functions are to regulate components of plasma membrane-bound signal transduction such as phospholipase C and protein kinase C activities [55] and, importantly, modulation of activity of neurotransmitter receptors and ion channels, including K^+ channels, Ca^{2+} channels, *N*-methyl-D-aspartate (NMDA), dopamine and γ-aminobutyric acid (GABA) receptors [2,44,51,56,61,66].

σ_1R has been proposed as a putative target in several therapeutic fields such as cognition and neurodegeneration, depression and anxiety, schizophrenia, and drug addiction [15,22,46,47,52]. Existing evidence also supports a role in pain. σ_1Rs are widely expressed in the nervous system [1,40,58,59,72] including areas important for pain control. During long time, data supporting a role for σ_1Rs in modulating pain were restricted to studies describing a tonic inhibitory control of σ_1R on opioid receptor-mediated antinociception: σ_1R agonists inhibit antinociception induced by morphine whereas σ_1R antagonists and σ_1R antisense

* Corresponding author. Tel.: +34 93 4466244; fax: +34 93 4466220.
E-mail address: jvela@esteve.es (J.M. Vela).

0304-3959/$36.00
doi:10.1016/j.pain.2009.05.013

oligodeoxynucleotides (ODNs) enhance the antinociceptive effect of morphine and other μ-, δ- and κ-opioid receptor agonists in acute thermal nociceptive tests [10,11,39,48,60]. The notion that σ_1R may also play a role in nociception in the absence of opioids came from recent studies showing that formalin-induced nociception was attenuated in σ_1R knockout mice [8] and by systemic administration of haloperidol and haloperidol metabolites I and II (with an order of potency which correlated with their affinity for σ_1Rs) [7]. Furthermore, intrathecal administration of σ_1R antagonists dose-dependently reduced formalin- and nerve injury-induced pain behaviors [37,63].

The selectivity and pharmacology of σ_1R ligands are unclear. Certain neurosteroids interact with σ_1Rs and have been proposed to be their endogenous ligands [47,52], but the precise nature of endogenous high-affinity σ_1R ligands is still under debate [20]. In this study, we took advantage of the genetic approach using σ_1R knockout mice to examine the role of σ_1Rs in mechanisms underlying neuropathic pain and spinal sensitization. We previously showed that deletion of this gene results in viable and fertile σ_1R-deficient mice with no detectable abnormalities [41]. Here we show that σ_1R modulates behavioral hypersensitivity and spinal hyperactivity secondary to nerve injury. Our findings identify σ_1R as a key constituent of the mechanisms modulating activity-induced sensitization in nociceptive pathways and point to σ_1R as a new potential target for the treatment of neuropathic pain.

2. Methods

2.1. Animals

Wild-type, heterozygous ($\sigma_1R^{+/-}$) and homozygous ($\sigma_1R^{-/-}$) male sigma receptor knockout mice backcrossed (N10 generation) to a CD1 albino genetic background (Harlan Ibérica, Barcelona, Spain) from 6 to 8 weeks old at the beginning of the experiments were used. Null mutant mice were generated by targeted removal of most of the coding region of the mSR1 gene, as described previously [41]. Mice had access to food and water *ad libitum* and were kept in controlled laboratory conditions with temperature at 21 ± 1 °C and a light–dark cycle of 12 h (lights on at 7:00 A.M.). Behavioral testing was conducted in a sound attenuated room and was done in blind respect to genotype and surgical procedure. Experimental procedures and animal husbandry were conducted according to ethical principles for the evaluation of pain in conscious animals [74] and to European guidelines regarding protection of animals used for experimental and other scientific purposes (Council Directive of 24 November, 1986, 86/609/ECC) and received approval by the local Ethical Committee.

2.2. Neuropathic pain model: partial sciatic nerve ligation

The partial sciatic nerve ligation model was used to induce neuropathic pain, according to the protocol previously described [43]. Briefly, mice were anesthetized with isoflurane (induction: 5%; surgery: 2%) and the common sciatic nerve was exposed at the level of the mid-thigh of the right hindpaw. At about 1 cm proximally to the nerve trifurcation, a tight ligation using 9–0 non-absorbable virgin silk suture (Alcon surgical, Texas, USA) was created enclosing the outer 33–50% of the diameter of the sciatic nerve, leaving the rest of the nerve "uninjured". Care was taken to ensure that the ligation was not too tight so as to occlude the perineural blood flow. The muscle was then stitched with 6–0 silk suture and the skin incision closed with wound clips. Control, sham-operated mice underwent the same surgical procedure and the sciatic nerve was exposed, but not ligated.

2.3. Nociceptive behavioral tests

2.3.1. Evaluation of thermal nociception (tail-flick and hot plate tests)

Acute heat nociceptive responses were assessed by the tail-flick and hot plate tests in uninjured independent groups of σ_1R knockout and wild-type mice (n = 12 per group). For the tail-flick test, animals were placed in a loose Plexiglas restrainer on a tail-flick apparatus (Panlab SL, Barcelona, Spain). A photobeam was focused on the tail about 4 cm from the tip, and the latency to tail-flick response was recorded automatically to the nearest 0.1 s. The intensity of the radiant heat source was adjusted to yield baseline latencies between 3 and 5 s in wild-type mice. A cut-off latency time of 10 s was imposed to avoid damage to tail tissues. Thermal nociceptive responses were also assessed by the hot plate test at 50 ± 0.5 °C. Briefly, mice were placed on the surface of the hot plate (Panlab SL, Barcelona, Spain) and the latency to the beginning of forepaw licking and jumping was recorded. Animals were removed from the hot plate after the jumping response was recorded. In order to avoid burns, the maximal time (cut-off) in the hot plate did not exceed 160 s.

2.3.2. Evaluation of neuropathic pain-related behaviors

Hyperalgesia to noxious thermal stimulus and allodynia to cold and mechanical stimuli were used as outcome measures of neuropathic pain in sham and nerve-injured mice (n = 12 per group) by using the plantar, cold-plate and von Frey test, respectively. Animals were first habituated for one hour to each different experimental test once daily for 4 days. After the habituation period, baseline responses were established during 2 consecutive days for each paradigm in the following sequence: von Frey, plantar (30 min later) and cold-plate test (15 min later). One day after baseline measurements, sciatic nerve injury was induced. σ_1R knockout and wild-type mice were tested in each paradigm on days 3, 6 and 10 after the surgical procedure using the same experimental sequence as for baseline responses.

Mechanical allodynia was quantified by measuring the hindpaw withdrawal response to von Frey filament stimulation [9]. Briefly, animals were placed into compartment enclosures in a test chamber with a framed metal mesh floor through which the von Frey monofilaments (bending force range from 0.008 to 2 g) (North Coast Medical, Inc., San Jose CA, USA) were applied and thresholds were measured using the up–down paradigm. The filament of 0.4 g was used at first. Then, the strength of the next filament was decreased when the animal responded or increased when the animal did not respond. This up–down procedure was stopped four measures after the first change in animal responding. The threshold of response was calculated by using the up–down Excel program generously provided by Basbaum's laboratory (UCSF, San Francisco, USA). Clear paw withdrawal, shaking or licking was considered as a nociceptive-like response. Both ipsilateral and contralateral hindpaws were tested.

Thermal hyperalgesia was assessed with a plantar test apparatus (Ugo Basile, Varese, Italy), as previously reported [24], by measuring hindpaw withdrawal latency in response to radiant heat. Briefly, mice were placed into compartment enclosures on a glass surface. The heat source was then positioned under the plantar surface of the hindpaw and activated with a light beam intensity chosen in preliminary studies to give baseline latencies from 8 to 9 s in control wild-type mice. The digital timer connected to the heat source automatically recorded the response latency for paw withdrawal to the nearest 0.1 s. A cut-off time of 20 s was imposed to prevent tissue damage in the absence of response. The mean withdrawal latencies for the ipsilateral and contralateral hindpaws were determined from the average of three separate trials, taken at 5-min intervals to prevent thermal sensitization and behavioral disturbances.

Cold allodynia was assessed by using a hot/cold-plate analgesia meter (Columbus, OH, USA) as previously described [4]. Briefly, mice were placed into compartment enclosures on the cold surface of the plate which is maintained at a temperature of 5 ± 0.5 °C. The number of elevations of each hindpaw was then recorded for 5 min. A score was calculated by subtracting the number of elevations of the right hindpaw (ipsilateral) from left hindpaw (contralateral). A positive difference score indicates development of cold allodynia.

2.4. ERK immunoblotting and immunohistochemistry

2.4.1. Western blotting

Immediately after deep anesthesia with sodium pentobarbital (100 mg/kg, i.p.), spinal cords were carefully removed from wild-type and $\sigma_1R^{-/-}$ mice 14 days ($n = 6$ per group) after surgery and ipsilateral spinal hemicord segments L4–S1 were dissected, frozen immediately in dry ice and stored at −80 °C. Spinal cord tissue was homogenized by sonication in 1% sodium dodecyl sulfate (SDS) containing a protease and phosphatase inhibitor cocktail purchased from Calbiochem (San Diego, CA, USA). The homogenate was centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was decanted from the pellet and used for Western blot analyses. The concentration of protein in the homogenate was measured using the DC Protein Assay from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Equal amounts of protein (50 μg) were fractionated by 8% (w/v) SDS–PAGE and transferred onto a polyvinylidene difluoride membrane, blocked with 5% non-fat dry milk in Tris–Tween 20-buffered Saline (T–TBS) overnight at 4 °C. Membranes were then incubated with rabbit primary polyclonal antibodies recognizing the mitogen-activated protein kinase (MAPK, total ERK 1/2) or mouse monoclonal antibodies recognizing the activated MAPK (diphosphorylated MAPK, p-ERK 1/2) obtained from Sigma–Aldrich Co. (Madrid, Spain) at a 1:20,000 and a 1:5000 dilution, respectively, in 5% non-fat dry milk in T–TBS. The blots were washed three times for 10 min with T–TBS and then incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG or goat anti-mouse IgG, purchased from Pierce Biotechnology Inc. (Rockford, IL, USA), diluted 1:5000 in 5% non-fat dry milk in T–TBS. The membranes were then washed with T–TBS three times for 10 min, one time with TBS for 10 min and the peroxidase reaction revealed by chemiluminescence (Immun-Star HRP Chemiluminescent Kit) from Bio-Rad. Chemiluminescence was detected with the ChemiDoc XRS System from Bio-Rad. The blots were then incubated for 15 min at room temperature in stripping buffer (Re-Blot Plus Strong Antibody Stripping Solution) from Chemicon International Inc. (Temecula, CA, USA) and re-probed for β-tubulin, using rabbit polyclonal antibodies diluted 1:1000 obtained from Abcam Inc. (Cambridge, MA, USA), as a loading control. The densitometric analysis of immunoreactive bands was done using the Quantity One software (Bio-Rad) and normalized respect to the intensity of the corresponding β-tubulin immunoreactive bands to obtain relative optical density ERK and p-ERK values. p-ERK was finally normalized respect to total ERK protein.

2.4.2. Immunohistochemistry

Wild-type and $\sigma_1R^{-/-}$ mice 14 days after surgery ($n = 12$ per group) were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused intracardially with 200 ml of cold saline solution followed by 500 ml of a cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). After perfusion, spinal cord segments L4–S1 were dissected out and postfixed for 4 h in 4% paraformaldehyde in PB at 4 °C. Then, spinal cord segments were washed in PB and serial coronal sections (40 μm-thick) were obtained with the aid of a vibratome (vibrocut FTB, Frankfurt, Germany) and collected in phosphate-buffered saline (PBS) to be processed immunohistochemically as free-floating sections. Sections were pre-incubated with 0.3% H_2O_2 in PBS for 30 min to block endogenous peroxidase activity and, after washing three times with PBS, with normal goat serum diluted 1:100 in PBS for 1 h at room temperature (RT) to prevent unspecific staining. Sections were then incubated for 48 h at 4 °C with the primary antibody (anti-p-ERK 1/2) diluted 1:500 in PBS with 1% bovine serum albumin and 0.4% Triton X-100. After washing three times for 10 min in PBS sections were incubated with goat anti-mouse biotinylated antibodies from Vector Laboratories Inc. (Burlingame, CA, USA) diluted 1:200 in PBS for 1 h at RT. After washing the sections three times in PBS, an avidin–biotin–peroxidase complex (Vector) was applied diluted 1:100 in PBS for 1 h at RT. The sections were washed again in PBS and placed in a chromogen solution containing 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2 in PBS for 5 min. The immunostained sections were placed on slides and coverslipped with glycergel mounting medium (DakoCytomation, Inc., Barcelona, Spain) for microscopic observation and photography.

Possible changes in p-ERK immunoreactivity in the dorsal horn were assessed by quantifying the density of immunostaining. Sections were simultaneously processed for immunohistochemistry in order to avoid methodological changes affecting the intensity of staining. Three appropriate fields per mice containing the dorsal horn were digitized by a video camera connected to a microscope and interfaced to a computer. The boundary of the dorsal horn (laminae I + II) was traced and the mean density of immunostaining was measured based on the inverse computer grayscale (from 0 = white to 255 = black) by using the National Institutes of Health (NIH) Image J software. Individual immunodensity values were corrected by subtracting the background (labeling in the white matter) for each section.

2.5. Electrophysiology

Spinal cords were obtained from newborn 5- to 10-day-old $\sigma_1R^{-/-}$ and wild-type mice after urethane (2 mg/kg, i.p.) anesthesia followed by dorsal laminectomy. In vitro AC recordings were performed as previously described [45] using a total of 13 spinal cords from wild-type and 10 cords from homozygous $\sigma_1R^{-/-}$ knockout mice. Briefly, the spinal cord was fixed to the Sylgard base of a recording chamber and continuously superfused (6–9 ml/min) with oxygenated (95% O_2; 5% CO_2) artificial cerebrospinal fluid (ACSF) at pH 7.4 and RT. The composition of the ACSF was (in mM): NaCl (128), KCl (1.9), KH_2PO_4 (1.2), $MgSO_4$ (1.3), $CaCl_2$ (2.4), $NaHCO_3$ (26) and glucose (10). A period of 60 min was allowed for the preparation to stabilize before testing spinal reflexes. The lumbar dorsal root (L4 or L5) and the corresponding ventral root were placed in tight-fitting glass suction electrodes. After setting up, the spinal cord was left untouched for one hour. Stimuli were then applied to the dorsal root in trains of shocks (20 stimuli at 1 Hz) and responses were recorded from the corresponding ventral root. Stimuli were adjusted to activate only thick and myelinated fibers (50 μs and 50 μA) or increased up to activate all fibers in the root including nociceptive C fibers (300 μs and 300 μA). The signal coming from the ventral root suction electrode was amplified to record fast compound spikes produced by the firing of action potentials by motor neurons using a Neurolog AC amplifier. Signals were digitized at 5 kHz and stored for offline computer-aided analysis using a CED 1401 interface and Spike 2 software from Cambridge Electronic Design Ltd. (Cambridge, UK). AC recordings from ventral roots were analyzed based on threshold criteria to count spikes to each stimulus of the train. Spike counts were performed for each stimulus of the train in a window between 20 and 950 ms from stimulus artifact.

2.6. Statistical analysis

Data obtained from thermal nociceptive tests were compared by one-way ANOVA and neuropathic pain-related behaviors were compared on each experimental day by using a two-way ANOVA repeated measures (paw and genotype as between factor of variation; day and surgery as within group levels) followed by corresponding one-way ANOVA and post hoc comparisons (Fisher's LSD test) when appropriate. Similarly, the difference between the groups for ERK immunoreaction in Western blots and immunohistochemistry was compared using ANOVA followed by post hoc comparison.

In electrophysiological studies, response profiles from the different groups of animals were compared by means of one-way ANOVA. Rise rate of spike counts was calculated as the maximal number of spikes minus the minimum number divided by the interval between them in seconds and non-paired Student's *t*-test was used for comparison.

In all cases, the criterion for statistical significance was established when p value was below 0.05.

3. Results

3.1. Nociceptive behavior of σ_1 receptor deficient mice: baseline control and neuropathic pain-related behaviors

3.1.1. Response of non-injured mice to thermal and mechanical stimuli

Sensitivity to noxious heat of homozygous σ_1R knockout mice ($\sigma_1R^{-/-}$), measured as the latency time of response to thermal stimulation in the hot plate (50 °C) and tail-flick tests, did not differ from wild-type mice (Fig. 1). Similarly, baseline values obtained in σ_1R knockout mice before nerve injury in the plantar, cold-plate and von Frey tests were indistinguishable from those obtained in wild-type mice (Fig. 2–4). Therefore, homozygous ($\sigma_1R^{-/-}$) knockout mice perceive and respond normally to acute thermal and mechanical nociceptive stimuli.

3.1.2. Thermal (heat) hyperalgesia (plantar test) secondary to nerve injury

No differences in baseline paw withdrawal latencies before surgery were found in the plantar test when compared to wild-type, σ_1R heterozygous ($\sigma_1R^{+/-}$) and homozygous ($\sigma_1R^{-/-}$) knockout mice (Fig. 2). Sham-operated wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice also showed similar paw withdrawal latencies 3, 6 and 10 days after surgery in both the ipsilateral and the contralateral paws.

As expected, a significant decrease in paw withdrawal latencies (thermal hyperalgesia) was observed in the ipsilateral, nerve-injured paw of wild-type mice 3, 6 and 10 days after surgery (Fig. 2). Thermal (heat) hyperalgesia also developed in heterozygous and homozygous σ_1R knockout mice exposed to sciatic nerve injury, as significantly reduced withdrawal latencies of their ipsilateral paws, indistinguishable from those in wild-type mice, were found 3, 6 and 10 days after surgery (Fig. 2). In the same way, no differences were found in the contralateral paw when compared paw withdrawal latencies of wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice exposed to sciatic nerve injury 3, 6 and 10 days after surgery (Fig. 2).

3.1.3. Cold allodynia (cold-plate test) secondary to nerve injury

Baseline values before surgery did not reveal significant differences between wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice. Likewise, sham-operated wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice showed similar cold-plate scores 3, 6 and 10 days after surgery (Fig. 3). The responses of the contralateral paw were similar in all the experimental groups at the different times.

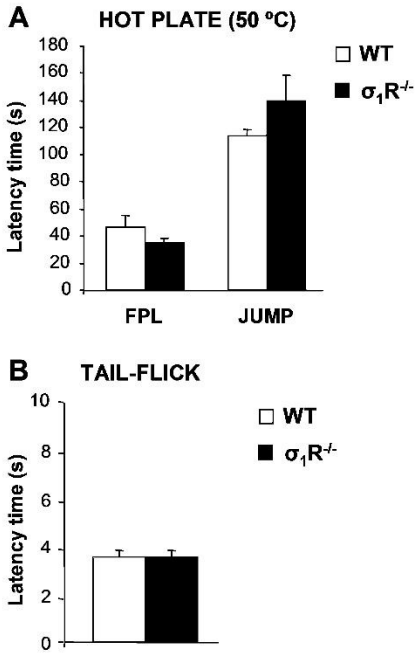


Fig. 1. Nociceptive response to noxious heat (hot plate and tail-flick tests) in control wild-type and homozygous $\sigma_1R^{-/-}$ mice. (A) The latency time for fore paw licking (FPL) and jumping (JUMP) responses on exposure to a hot plate kept at 50 °C did not differ in $\sigma_1R^{-/-}$ knockout mice respect to wild-type (WT) mice. (B) Similarly, the latency of the tail-flick response evoked by noxious heat stimulation using a light beam was in homozygous σ_1R knockout mice indistinguishable from wild-type mice. Each bar and vertical line represent the mean ± SEM (n = 12 per group). No statistically significant differences were found between wild-type and $\sigma_1R^{-/-}$ mice either in the hot plate or in the tail-flick test.

As expected, a significant increase of the cold-plate score (cold allodynia) was found in wild-type mice exposed to the sciatic nerve injury 3, 6 and 10 days after surgery when compared to sham-operated mice. Heterozygous $\sigma_1R^{+/-}$ mice exposed to the sciatic nerve injury also developed cold allodynia as significant increases of the cold-plate score 3, 6 and 10 days after surgery were found when compared to sham-operated mice (Fig. 3). However, cold-plate scores in homozygous $\sigma_1R^{-/-}$ exposed to nerve injury were not significantly different from values obtained in sham-operated mice at 3, 6 and 10 days after surgery (Fig. 3).

3.1.4. Mechanical allodynia (von Frey test) secondary to nerve injury

The pressure threshold eliciting paw withdrawal responses at baseline, before surgery, did not differ when wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice were compared (Fig. 4). In the same way, sham-operated wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice required similar pressure to elicit withdrawal of both the ipsilateral and the contralateral paw 3, 6 and 10 days after surgery. Wild-type, $\sigma_1R^{+/-}$ and $\sigma_1R^{-/-}$ mice exposed to sciatic nerve injury also required similar pressure to elicit withdrawal of the contralateral paw 3, 6 and 10 days after surgery.

Partial sciatic nerve ligation induced mechanical allodynia in wild-type mice, as evidenced by a significant reduction from day 3 post-injury of the pressure required to evoke withdrawal of the ipsilateral, nerve-injured paw compared to baseline pre-injury and contralateral paw values (Fig. 4). Heterozygous $\sigma_1R^{+/-}$ mice exposed to sciatic nerve injury also showed decreases in the pressure threshold eliciting withdrawal of the ipsilateral paw 3, 6 and 10 days after surgery. However, mechanical allodynia was significantly attenuated in $\sigma_1R^{+/-}$ mice, pressure thresholds being

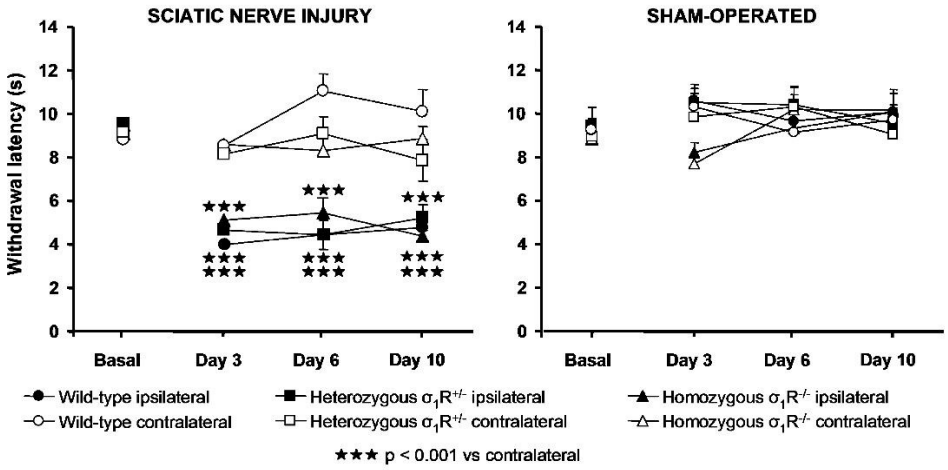


Fig. 2. Development of thermal (heat) hyperalgesia (plantar test) following nerve injury in control wild-type and σ_1R-deficient mice. Thermal hyperalgesia, evidenced as a significant reduction in the latency time for withdrawal of the ipsilateral, respect to the contralateral, hindpaw in response to radiant heat was found 3, 6 and 10 days after sciatic nerve injury in wild-type, σ_1R heterozygous and σ_1R homozygous knockout mice. The development of thermal hyperalgesia did not differ when the different genotypes were compared. Similarly, no effect of genotype was found when compared to responses in sham-operated mice. Each point and vertical line represent the mean ± SEM (n = 12 per group).

significantly modified respect to wild-type on days 6 and 10 after nerve injury. Interestingly, in the homozygous $\sigma_1R^{-/-}$, mechanical allodynia was strongly reduced 3 days after nerve injury and was not observed on days 6 and 10 post-injury (pressure thresholds eliciting withdrawal of the ipsilateral and contralateral paws were not significantly different). In fact, significant differences between $\sigma_1R^{-/-}$ knockout and wild-type mice were found in the withdrawal thresholds of the ipsilateral, nerve-injured paw on all evaluated days (3, 6 and 10 post-injury) (Fig. 4).

3.2. Activation of ERK in the spinal cord secondary to sciatic nerve injury

Activation (phosphorylation) of mitogen-activated protein kinases (MAPKs), particularly extracellular signal-regulated kinase (ERK 1/2; p44/42 MAPK), occurs in dorsal horn neurons and glial cells following nerve injury and contributes to the induction and maintenance of neuropathic pain [34,42,73]. We explored the possibility that differential activation of ERK in the spinal cord could underlie the differences in neuropathic pain-related behaviors (i.e., allodynia) between wild-type and $\sigma_1R^{-/-}$ knockout mice. ERK activation in spinal cords from sham-operated and nerve-injured mice was analyzed by Western immunoblotting and immunohistochemistry. Immunoblots using proteins from ipsilateral spinal hemicord segments L4–S1 14 days after surgery were done for total ERK and its phosphorylated form (p-ERK) (Fig. 5A). No significant changes in total ERK protein were found either in wild-type or in $\sigma_1R^{-/-}$ knockout mice when nerve-injured and sham-operated mice were compared. As expected, the phosphorylation of ERK was increased (7.8-fold; $p < 0.01$) in the ipsilateral spinal hemicord of wild-type mice exposed to sciatic nerve injury with respect to sham-operated wild-type mice. However, the significant

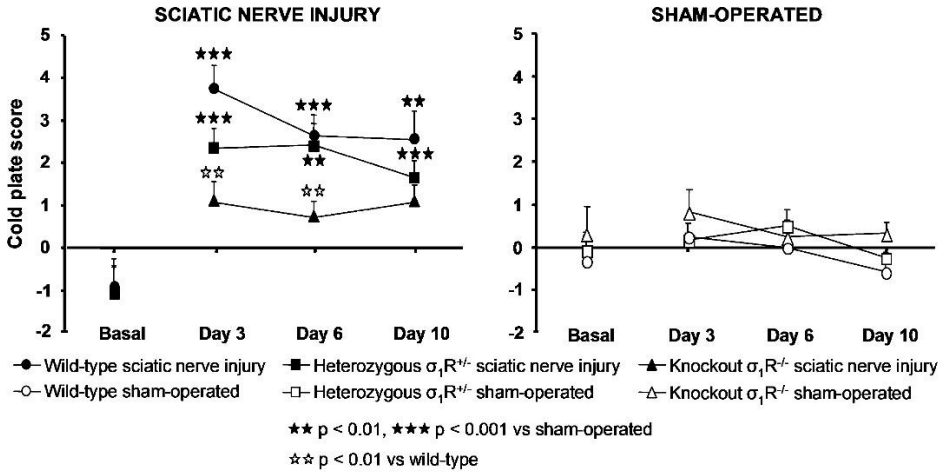


Fig. 3. Development of cold allodynia (cold-plate test) following nerve injury in control wild-type and σ_1R-deficient mice. Cold allodynia, evidenced as a significantly increased number of elevations of the right hindpaw (ipsilateral) respect to left hindpaw (contralateral) was evidenced in nerve-injured, respect to sham-operated mice, wild-type and σ_1R heterozygous mice, but not in σ_1R homozygous knockout mice. No effect of genotype was found when compared to responses in sham-operated mice. Each point and vertical line represent the mean ± SEM (n = 12 per group).

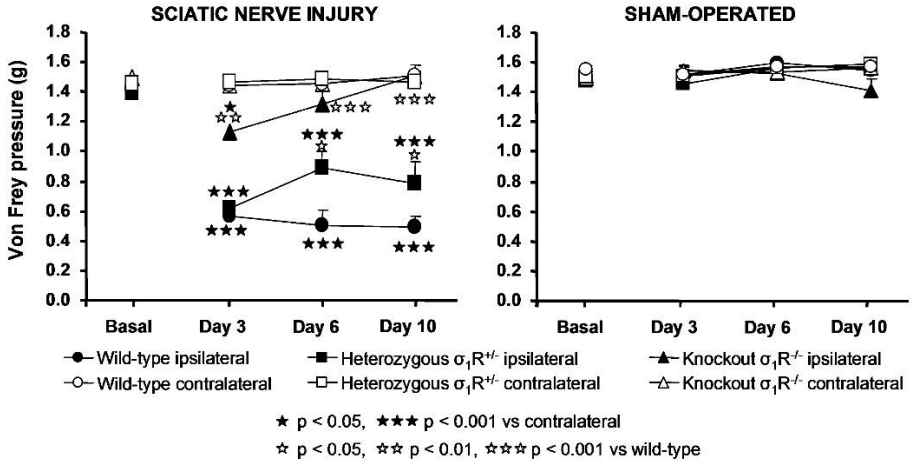


Fig. 4. Development of mechanical allodynia (von Frey test) following nerve injury in control wild-type and σ_1R-deficient mice. Mechanical allodynia, evidenced as a significant reduction in the pressure evoking withdrawal of the nerve-injured hindpaw was clearly evidenced in wild-type mice on all days of measurement. It was attenuated in heterozygous σ_1R mice on days 6 and 10 after nerve injury, and it was strongly reduced 3 days after nerve injury and was not observed on days 6 and 10 post-injury in homozygous σ_1R knockout mice. No effect of genotype was found when responses in sham-operated mice were compared. Each point and vertical line represent the mean ± SEM (n = 12 per group).

upregulation subsequent to nerve injury found in the wild-type did not occur in the knockout mice, as no significant change in the intensity of p-ERK immunoreactive bands was found when compared ipsilateral cords from $\sigma_1R^{-/-}$ mice exposed to nerve injury and sham operation (1.3-fold increase in nerve-injured mice with respect to sham-operated mice).

By immunohistochemistry, increased activation of ERK in the spinal cord (L4–S1 segments) was apparent in the ipsilateral dorsal horn of nerve-injured respect to sham-operated wild-type mice 14 days after surgery. This was evidenced by both the presence of increased numbers of p-ERK immunoreactive cells and the increased p-ERK immunostaining intensity showed by cells (Fig. 5B–H). Immunostaining for p-ERK was present in numerous cells in superficial laminae I and II, but also with smaller numbers in more ventral laminae, and was located in the soma as well as in the nucleus. In the $\sigma_1R^{-/-}$ knockout mice no differences in p-ERK immunostaining in the spinal cord were found when nerve-injured and sham-operated mice were compared. When p-ERK immunoreaction in the ipsilateral dorsal horn (laminae I–II) was quantified in the sections, the density was significantly increased after nerve injury (respect to sham operation) in wild-type but not in $\sigma_1R^{-/-}$ knockout mice (Fig. 5B).

3.3. Spinal processing of repetitive nociceptive stimulation

In order to provide an electrophysiological correlate to the nociceptive behavioral findings described above, in vitro AC recordings were performed in spinal cords from wild-type and homozygous $\sigma_1R^{-/-}$ knockout mice. Repetitive stimulation of the dorsal root at stimulus intensities sufficient to activate C-fibers produced a typical wind-up response in ventral root recordings, which manifested as a progressive increase in action potential firing (Fig. 6). Stimulus intensities insufficient to activate C-fibers (i.e., 50 μs and 50 μA) did not produce wind-up upon the arrival of the afferent volleys. Interestingly, spinal cords from wild-type animals showed stronger wind-up responses than those from homozygous $\sigma_1R^{-/-}$ animals (Fig. 6). This was evidenced as a reduction in the total number of action potentials induced by trains of C-fiber intensity stimuli in spinal cords from $\sigma_1R^{-/-}$ mice respect to wild-type mice. For 200 μs and 200 μA stimuli, wild-type cords produced the smaller mean spike count in response to the second stimulus of the train (22 ± 5 counts) and the greater in response to the last stimulus (223 ± 36 counts). The rise rate of the response was 8.7 ± 1.6 spikes per second in wild-type cords. For the same stimulus intensity, spinal cords from $\sigma_1R^{-/-}$ animals showed slightly larger spike counts than wild-types at the beginning of the train of stimuli (71 ± 26 counts) and much smaller spike counts towards the end of the train (141 ± 37 counts). This resulted in a significantly smaller rise rate for knockout animals (3.2 ± 0.9 spikes per second) compared to controls ($p < 0.05$; two-tailed unpaired t-test). Responses to repetitive A-fiber intensity stimulation did not produce spike wind-up and were similar in wild-type and $\sigma_1R^{-/-}$ knockout mice.

4. Discussion

In the present study we took advantage of a genetic approach using $\sigma_1R^{-/-}$ knockout mice to examine the role of σ_1R in nociception secondary to nerve injury. Our results indicated that increased sensitivity (allodynia) to mechanical and thermal (cold) stimuli found after nerve injury is markedly attenuated to absent in mice lacking σ_1Rs. Differential modulation of spinal excitability and ERK signaling could contribute to the reduced behavioral hypersensitivity seen in σ_1R knockout mice after nerve injury.

The sensitivity to noxious heat stimulation in the $\sigma_1R^{-/-}$ knockout mice did not differ from wild-type mice, as evidenced in the hot plate and tail-flick tests. In the same way, baseline (before surgery) responses of σ_1R-deficient mice to thermal (heat and cold) and mechanical stimuli were undistinguishable from wild-type ones. Responses of sham-operated mice also remained unchanged (respect to wild-type) in σ_1R-deficient mice. Thus, in the absence of nerve injury, no differences in the performance of the sensory/nociceptive pathway were seen when compared σ_1R-deficient mice to wild-type mice, suggesting that basic mechanisms for transduction, transmission and perception of sensory and nociceptive inputs are intact in mice lacking σ_1Rs. This is in agreement with previous pharmacological studies showing that σ_1 ligands did not influence nociceptive behaviors by themselves in normal conditions [7,11,18,38].

However, after sciatic nerve injury, when hypersensitivity in the ipsilateral paw would result in perception of pain from non-noxious stimuli (allodynia), the scenario is different. We found that

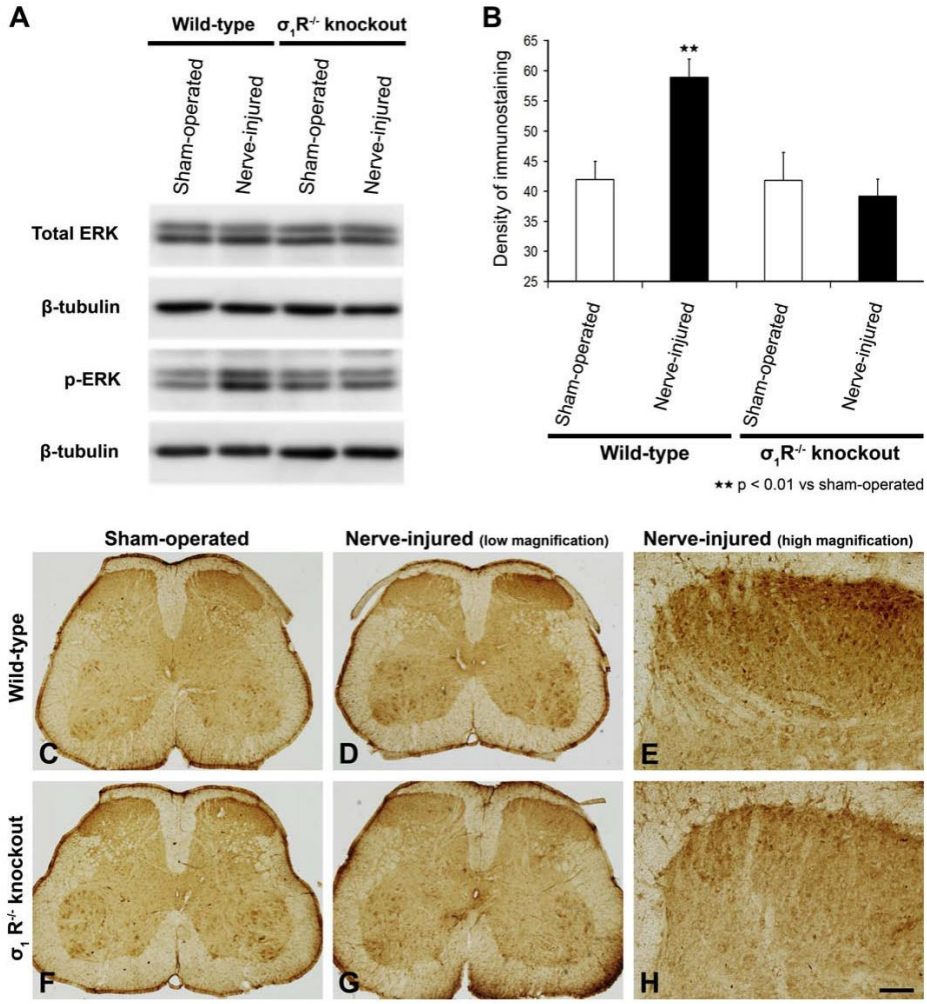


Fig. 5. Activation of ERK (immunoblotting and immunohistochemistry) following nerve injury in control wild-type and homozygous $\sigma_1R^{-/-}$ mice. (A) Western immunoblotting for total ERK and p-ERK in ipsilateral spinal hemicord segments L4–S1 14 days after surgery. β-tubulin was used as a loading control. (B) Quantification of immunostaining for p-ERK in the ipsilateral dorsal horn (laminae I and II) of spinal cord sections (L4–S1 segments) from sham-operated and nerve-injured wild-type and homozygous σ_1R knockout mice 14 days after surgery. Each bar and vertical line represent the mean ± SEM. (A and B) Note that p-ERK immunoreactivity was increased after nerve injury in wild-type but not in σ_1R null mice. (C–H) p-ERK immunostaining in spinal cord sections from sham-operated and nerve-injured wild-type and homozygous σ_1R knockout mice. The right side of photomicrographs C, D, F and G corresponds to the side of injury (ipsilateral side). Note that p-ERK immunoreactivity in superficial laminae of the ipsilateral dorsal horn after nerve injury was remarkably higher in wild-type (E) than in σ_1R null mice (H). Scale bar = 500 μm in C, D, F and G; and 100 μm in E and H.

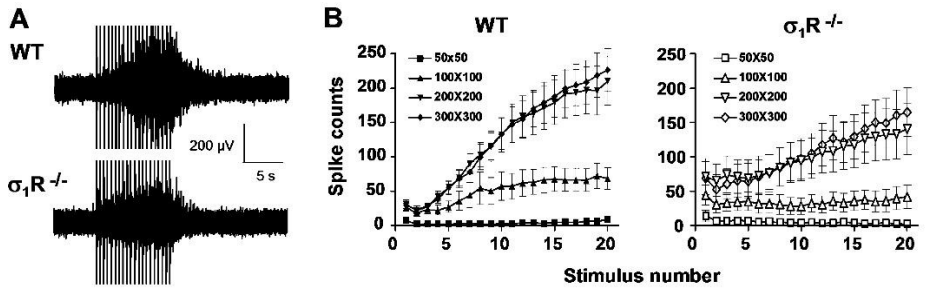


Fig. 6. Electrophysiological recordings of spinal cord responses upon application of repetitive electrical stimuli of constant intensity in control wild-type and homozygous $\sigma_1R^{-/-}$ mice. (A) Original AC recordings of responses to a train of 200 μs and 200 μA obtained from cords of different genotypes. Vertical lines at regular intervals correspond to stimuli artifact. Spikes are viewed as a progressive thickening of the baseline noise. (B) Spike counts obtained following stimulation at different intensities as stated (in μs × μA) obtained from cords of different genotypes. Note the large differences between phenotypes for high intensity (C-fiber intensity) stimuli (e.g., 200 μs × 200 μA). Significant differences ($p < 0.001$) were found between curves for high intensity stimuli (200 μs × 200 μA and 300 μs × 300 μA) in control and homozygous σ_1R knockout animals using two-way ANOVA.

mechanical allodynia was attenuated in heterozygous $\sigma_1R^{+/-}$ mice and was not observed in homozygous $\sigma_1R^{-/-}$ mice 6 and 10 days after nerve injury. Cold allodynia in homozygous (but not in heterozygous) σ_1R knockout mice exposed to nerve injury was also attenuated. In contrast, nerve injury-induced heat hyperalgesia was not modified in σ_1R knockout mice respect to wild-type mice. A differential modulation by σ_1Rs of sensory/nociceptive pathways depending on the stimulus quality and modality is thus suggested. This adds to previous findings that different receptor systems and mechanisms are involved in the diverse sensory abnormalities (i.e., mechanical versus thermal) associated with neuropathic pain [3,49,57,68].

The conception that σ_1Rs plays a role in modulating pain behaviors in sensitizing conditions is also supported in previous studies. Formalin-induced pain was reduced in σ_1R knockout mice [8] and haloperidol and its metabolites I and II, which behave as antagonists at this receptor [13,14], inhibited formalin-induced pain [7]. Similarly, intraplantar injection of capsaicin induced mechanical allodynia in wild-type but not in σ_1R knockout mice and σ_1R antagonists were able to inhibit this pain behavior [18]. Regarding the site of action, intrathecal pretreatment with the σ_1R antagonist BD-1047 has been shown to dose-dependently reduce pain behaviors in second phase of the formalin test [37]. The analgesic effect occurred concomitantly with a reduction in formalin-evoked Fos expression and phosphorylation of the NMDA receptor NR1 subunit (pNR1) at protein kinase C (PKC) and protein kinase A (PKA) dependent sites in spinal dorsal horn [37]. In separate recent studies, activation of spinal σ_1Rs has been found to have opposite effects: intrathecal administration of σ_1R agonists facilitated nociception involving Ca^{2+}-dependent second messenger cascades [64], and enhanced NMDA-induced pain in parallel to an increased pNR1 expression [38]. Interestingly, the effect of pharmacological blockage of spinal σ_1Rs in a neuropathic pain model, the chronic constriction injury of the sciatic nerve in rats, has recently been investigated [63]. The σ_1R antagonist BD1047 intrathecally administered on postoperative days 0–5 significantly attenuated the development of mechanical allodynia, but not thermal hyperalgesia, concurrent with an inhibition of nerve injury-induced NMDA receptor NR1 subunit expression and phosphorylation [63]. These results fully agree with the observation reported here that mechanical allodynia, but not thermal hyperalgesia, is suppressed in nerve-injured σ_1R knockout mice.

Collectively, results described above support a modulatory role of σ_1Rs in spinal sensitization and point to NMDA receptors and Ca^{2+}-dependent intracellular cascades as underlying mechanisms. This is not surprising as σ_1Rs are functionally coupled to NMDA receptors [5,25,44,51,53] and regulate intracellular Ca^{2+} concentration via phospholipase C (PLC) and IP_3 receptors [26,27,54,55,67]. The overall effect of activating σ_1Rs is to increase intracellular Ca^{2+} concentration by potentiating Ca^{2+} entry at the plasma membrane level (NMDA-induced Ca^{2+} influx) and Ca^{2+} mobilization from endoplasmic stores (IP_3-induced Ca^{2+} mobilization). Indeed, NMDA and IP_3 receptors, as well as downstream Ca^{2+}-dependent second messengers including PKC and other calcium/calmodulin-dependent kinases (e.g., CaMKII) are involved in central (spinal) sensitization and pain hypersensitivity [6,16,19,32,33,36,70]. Hence, according to our results, blocking/absence of σ_1Rs might be associated with reduced nerve injury-evoked activity of Ca^{2+}-permeable NMDA and IP_3 receptors (Fig. 7), thus causing reduction in central sensitization-related pain hypersensitivity.

The σ_1R is expressed abundantly in the spinal cord, mainly in the two superficial laminae, in dendritic processes and neuronal perikarya, both on the plasma and endoplasmic reticulum membranes [1]. Its expression in the spinal cord is upregulated early and transiently during the induction phase of neuropathic pain [63]. We focused on functional changes in excitability and synaptic plasticity in the spinal cord as an integrative correlate of the role played by σ_1Rs. Central sensitization is a complex synaptic plasticity phenomenon characterized by the rapid-onset, activity-dependent increase in the excitability of nociceptive spinal dorsal horn neurons. Several types of synaptic plasticity arise in response to noxious stimuli that modify nociceptive transmission in the spinal cord. These include immediate-onset phenomena such as wind-up and long-term potentiation (LTP). Wind-up is interpreted as an amplification in the spinal cord of the nociceptive message that arrives from peripheral nociceptors, whereby repetitive stimulation of dorsal horn neurons induces an increase in their evoked responses with each stimulus [17,31,50]. In our study, in spinal cords from wild-type mice, repetitive stimulation of primary afferent fibers in the dorsal root produced a typical wind-up response manifested as a progressive increase in action potential firing. Interestingly, spinal cords from mice lacking σ_1Rs showed reduced wind-up responses respect to wild-type ones, as evidenced by a smaller rise rate of the response and a subsequent reduction in the total number of action potentials induced by trains of stimuli. These data point to a modulatory role of σ_1R on spinal excitability after repetitive nociceptor stimulation, such as that expected to occur as a consequence of nerve injury. Similar to our findings in the spinal cord, σ_1Rs are known to modulate LTP in the hippocampus [44]. In this way, glutamatergic NMDA and substance P tachykinin receptors (i.e., NK1) have been identified as key mediators of wind-up and central sensitization [31,69], and σ_1Rs modulate elevations of cytosolic Ca^{2+} incurred not only upon activation of NMDA but also of NK1 receptors through the PLC-IP_3 pathway (Fig. 7).

Ca^{2+} entry into neurons via NMDA receptors or mobilized via IP_3 receptors can initiate the intracellular ERK signaling cascade. In fact, neurotransmitters released by primary afferent fibers, acting through a variety of receptors and ion channels, activate Ca^{2+}-dependent enzymes such as PKC, CaMKs, adenylate cyclase-PKA and tyrosine kinases, which lead to ERK activation (phosphorylation) in dorsal horn neurons [34–36] (Fig. 7). As a common effector of multiple signals, ERK activation is activity-dependent in dorsal horn neurons. Indeed, activation of ERK in dorsal horn neurons is induced by repetitive C-fiber electrical stimulation and blockage of spinal ERK suppresses wind-up [21]. Similarly, LTP of C-fiber-evoked field potentials induces ERK activation in spinal dorsal horn and inhibition of ERK blocks the induction and maintenance of LTP [71]. In this way, in addition to its kinase-dependent posttranslational regulation, the ERK pathway also regulates long-term pain hypersensitivity via transcriptional regulation of key gene products [36,65,71]. After nerve injury, ERK activation occurs early and it is long lasting. It is immediately induced in neurons of the superficial dorsal horn and this is followed by a widespread sequential induction in spinal microglia and astrocytes [42,73]. Activated glia produce inflammatory mediators that sensitize dorsal horn neurons. Activity of dorsal horn neurons, in turn, promotes activation of spinal glia. This neuron–glia interaction involves ERK signaling in the positive feedback enhancing and prolonging pain sensitization. Indeed, in several nerve injury models, intrathecal ERK inhibitors (i.e., MEK inhibitors or ERK antisense ODNs) reduce pain hypersensitivity, particularly mechanical allodynia, when administered in both the induction and maintenance phases of neuropathic pain [12,42,65,73]. In the present study, ERK activation was increased in spinal cords of wild-type mice exposed to sciatic nerve injury 14 days after surgery, but no significant changes respect to sham-operated mice were found in the spinal cords from nerve-injured $\sigma_1R^{-/-}$ knockout mice. This finding supports that σ_1R is involved in the regulation of intracellular cascades, probably Ca^{2+} dependent, that lead to phosphorylation of ERK (Fig. 7). The inability to upregulate ERK activation subsequent to nerve injury found in the spinal cords of σ_1R knockout mice could be interpreted along the lines of reduced sensitization and pain hypersensitivity

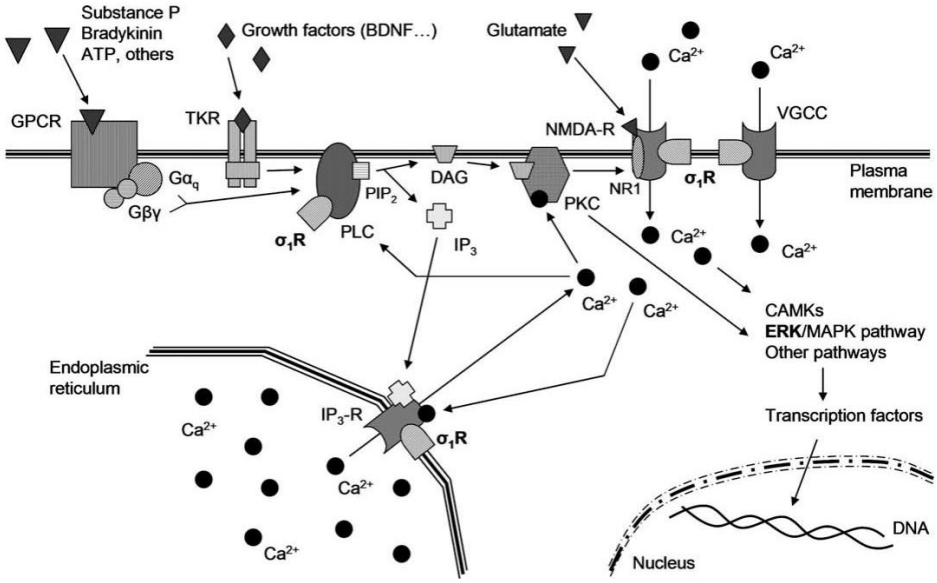


Fig. 7. Involvement of σ_1R in signal transduction pathways. Activation by nociceptive mediators released into the dorsal horn of G-protein-coupled receptors (GPCRs) involving $G\alpha_q$-coupling and other phospholipase C (PLC)-activating G proteins, and/or tyrosine kinase receptors (TKRs), stimulates PLC enzymes to hydrolyze phosphorylated phosphatidylinositols (e.g., PIP_2) to produce diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds then to IP_3 receptors (IP_3-R) in the endoplasmic reticulum to promote the efflux of Ca^{2+} to the cytoplasm. Raises of cytosolic Ca^{2+} are also produced by Ca^{2+} influx through ionotropic *N*-methyl-D-aspartate receptors (NMDA-Rs) and voltage-gated Ca^{2+} channels (VGCCs). In turn, some PLC isoforms are activated by Ca^{2+} and low increases in intracellular Ca^{2+} concentrations activate IP_3-R (Ca^{2+}-induced Ca^{2+} release) to promote more Ca^{2+} to be released from the endoplasmic reticulum. Interestingly, protein kinase C (PKC) as well as calcium/calmodulin-dependent kinases (CAMKs) is activated by Ca^{2+}. These enzymes phosphorylate diverse plasma membrane receptors and ion channels (e.g., NR1 subunit of NMDA-R), being responsible for their rapid sensitization, and activate different signaling pathways, including the ERK pathway, leading to both rapid kinase-dependent posttranslational regulation and long-term changes via transcriptional regulation in dorsal horn neurons. σ_1R plays a key role in the control of intracellular Ca^{2+} levels. Activation of σ_1Rs increases intracellular Ca^{2+} concentrations by potentiating both Ca^{2+} entry at the plasma membrane level (via NMDA-R and VGCC) and Ca^{2+} mobilization from endoplasmic stores (via PLC and IP_3-R), which is followed by increased kinase sensitization (rapid) and transcriptional activation (long-term) of key gene products underlying pain hypersensitivity. Absence/blocking of σ_1Rs would avoid upregulation of Ca^{2+}-dependent sensitizing intracellular cascades.

(allodynia) and it is consonant with the reduced wind-up responses found in spinal cords from mice lacking σ_1Rs.

Taken together, our findings propose the σ_1R as a new constituent of the mechanisms modulating activity-induced sensitization in nociceptive pathways and thus as a new potential target of action for drugs designed to alleviate neuropathic pain.

Conflict of interest

The authors state that there were no conflicts of interests in respect to the work reported in this paper.

Acknowledgements

We thank Mercè Olivet for her administrative assistance.

References

[1] Alonso G, Phan V, Guillemain I, Saunier M, Legrand A, Anoal M, Maurice T. Immunocytochemical localization of the sigma(1) receptor in the adult rat central nervous system. Neuroscience 2000;97:155–70.
[2] Aydar E, Palmer CP, Klyachko VA, Jackson MB. The sigma receptor as a ligand-regulated auxiliary potassium channel subunit. Neuron 2002;34:399–410.
[3] Baron R. Mechanisms of disease: neuropathic pain–a clinical perspective. Nat Clin Pract Neurol 2006;2:95–106.
[4] Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 1988;33:87–107.
[5] Bergeron R, de Montigny C, Debonnel G. Potentiation of neuronal NMDA response induced by dehydroepiandrosterone and its suppression by progesterone: effects mediated via sigma receptors. J Neurosci 1996;16:1193–202.
[6] Brenner GJ, Ji RR, Shaffer S, Woolf CJ. Peripheral noxious stimulation induces phosphorylation of the NMDA receptor NR1 subunit at the PKC-dependent site, serine-896, in spinal cord dorsal horn neurons. Eur J Neurosci 2004;20:375–84.
[7] Cendán CM, Pujalte JM, Portillo-Salido E, Baeyens JM. Antinociceptive effects of haloperidol and its metabolites in the formalin test in mice. Psychopharmacology (Berl) 2005;182:485–93.
[8] Cendán CM, Pujalte JM, Portillo-Salido E, Montoliu L, Baeyens JM. Formalin-induced pain is reduced in sigma(1) receptor knockout mice. Eur J Pharmacol 2005;511:73–4.
[9] Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 1994;53:55–63.
[10] Chien CC, Pasternak GW. Selective antagonism of opioid analgesia by a sigma system. J Pharmacol Exp Ther 1994;271:1583–90.
[11] Chien CC, Pasternak GW. Sigma antagonists potentiate opioid analgesia in rats. Neurosci Lett 1995;190:137–9.
[12] Ciruela A, Dixon AK, Bramwell S, Gonzalez MI, Pinnock RD, Lee K. Identification of MEK1 as a novel target for the treatment of neuropathic pain. Br J Pharmacol 2003;138:751–6.
[13] Cobos EJ, Baeyens JM, Del Pozo E. Phenytoin differentially modulates the affinity of agonist and antagonist ligands for sigma 1 receptors of guinea pig brain. Synapse 2005;55:192–5.
[14] Cobos EJ, del Pozo E, Baeyens JM. Irreversible blockade of sigma-1 receptors by haloperidol and its metabolites in guinea pig brain and SH-SY5Y human neuroblastoma cells. J Neurochem 2007;102:812–25.
[15] Cobos EJ, Entrena JM, Nieto FR, Cendán CM, Del Pozo E. Pharmacology and therapeutic potential of sigma1 receptor ligands. Curr Neuropharmacol 2008;6:344–66.
[16] Coderre TJ. The role of excitatory amino acid receptors and intracellular messengers in persistent nociception after tissue injury in rats. Mol Neurobiol 1993;7:229–46 [fall-winter].
[17] Dickenson AH, Sullivan AF. Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep rat dorsal horn nociceptive neurones following C fibre stimulation. Neuropharmacology 1987;26:1235–8.
[18] Entrena JM, Cobos EJ, Nieto FR, Cendán CM, Gris G, Del Pozo E, Zamanillo D, Baeyens JM. Sigma-1 receptors are essential for capsaicin-induced mechanical hypersensitivity: studies with selective sigma-1 ligands and sigma-1 knockout mice. Pain 2009;143:252–61.
[19] Fang L, Wu J, Lin Q, Willis WD. Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. J Neurosci 2002;22:4196–204.
[20] Fontanilla D, Johannessen M, Hajipour AR, Cozzi NV, Jackson MB, Ruoho AE. The hallucinogen *N,N*-dimethyltryptamine (DMT) is an endogenous sigma-1 receptor regulator. Science 2009;323:934–7.

[21] Fukui T, Dai Y, Iwata K, Kamo H, Yamanaka H, Obata K, Kobayashi K, Wang S, Cui X, Yoshiya S, Noguchi K. Frequency-dependent ERK phosphorylation in spinal neurons by electric stimulation of the sciatic nerve and the role in electrophysiological activity. Mol Pain 2007;16:3–18.
[22] Guitart X, Codony X, Monroy X. Sigma receptors: biology and therapeutic potential. Psychopharmacology (Berl) 2004;174:301–19.
[23] Hanner M, Moebius FF, Flandorfer A, Knaus HG, Striessnig J, Kempner E, Glossmann H. Purification, molecular cloning, and expression of the mammalian sigma1-binding site. Proc Natl Acad Sci USA 1996;93:8072–7.
[24] Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988;32:77–88.
[25] Hayashi T, Kagaya A, Takebayashi M, Shimizu M, Uchitomi Y, Motohashi N, Yamawaki S. Modulation by sigma ligands of intracellular free Ca^{++} mobilization by *N*-methyl-D-aspartate in primary culture of rat frontal cortical neurons. J Pharmacol Exp Ther 1995;275:207–14.
[26] Hayashi T, Maurice T, Su TP. Ca(2+) signaling via sigma(1)-receptors: novel regulatory mechanism affecting intracellular Ca(2+) concentration. J Pharmacol Exp Ther 2000;293:788–98.
[27] Hayashi T, Su TP. Regulating ankyrin dynamics: roles of sigma-1 receptors. Proc Natl Acad Sci USA 2001;98:491–6.
[28] Hayashi T, Su TP. Sigma-1 receptor chaperones at the ER–mitochondrion interface regulate Ca(2+) signaling and cell survival. Cell 2007;131:596–610.
[29] Hayashi T, Su TP. Sigma-1 receptors (sigma(1) binding sites) form raft-like microdomains and target lipid droplets on the endoplasmic reticulum: roles in endoplasmic reticulum lipid compartmentalization and export. J Pharmacol Exp Ther 2003;306:718–25.
[30] Hayashi T, Su TP. The potential role of sigma-1 receptors in lipid transport and lipid raft reconstitution in the brain: implication for drug abuse. Life Sci 2005;77:1612–24.
[31] Herrero JF, Laird JM, López-García JA. Wind-up of spinal cord neurones and pain sensation: much ado about something? Prog Neurobiol 2000;61:169–203.
[32] Hua XY, Chen P, Yaksh TL. Inhibition of spinal protein kinase C reduces nerve injury-induced tactile allodynia in neuropathic rats. Neurosci Lett 1999;276:99–102.
[33] Igwe OJ, Ning L. Regulation of the second-messenger systems in the rat spinal cord during prolonged peripheral inflammation. Pain 1994;58:63–75.
[34] Ji RR, Baba H, Brenner GJ, Woolf CJ. Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nat Neurosci 1999;2:1114–9.
[35] Ji RR, Woolf CJ. Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain. Neurobiol Dis 2001;8:1–10.
[36] Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van Der Meer C, Befort K, Woolf CJ, Ji RR. Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. J Neurosci 2004;24:8310–21.
[37] Kim HW, Kwon YB, Roh DH, Yoon SY, Han HJ, Kim KW, Beitz AJ, Lee JH. Intrathecal treatment with sigma1 receptor antagonists reduces formalin-induced phosphorylation of NMDA receptor subunit 1 and the second phase of formalin test in mice. Br J Pharmacol 2006;148:490–8.
[38] Kim HW, Roh DH, Yoon SY, Seo HS, Kwon YB, Han HJ, Kim KW, Beitz AJ, Lee JH. Activation of the spinal sigma-1 receptor enhances NMDA-induced pain via PKC- and PKA-dependent phosphorylation of the NR1 subunit in mice. Br J Pharmacol 2008;154:1125–34.
[39] King M, Pan YX, Mei J, Chang A, Xu J, Pasternak GW. Enhanced kappa-opioid receptor-mediated analgesia by antisense targeting the sigma1 receptor. Eur J Pharmacol 1997;331:R5–6.
[40] Kitaichi K, Chabot JG, Moebius FF, Flandorfer A, Glossmann H, Quirion R. Expression of the purported sigma(1) (sigma(1)) receptor in the mammalian brain and its possible relevance in deficits induced by antagonism of the NMDA receptor complex as revealed using an antisense strategy. J Chem Neuroanat 2000;20:375–87.
[41] Langa F, Codony X, Tovar V, Lavado A, Giménez E, Cozar P, Cantero M, Dordal A, Hernández E, Pérez R, Monroy X, Zamanillo D, Guitart X, Montoliu L. Generation and phenotypic analysis of sigma receptor type I (sigma 1) knockout mice. Eur J Neurosci 2003;18:2188–96.
[42] Ma W, Quirion R. The ERK/MAPK pathway, as a target for the treatment of neuropathic pain. Expert Opin Ther Targets 2005;9:699–713.
[43] Malmberg AB, Basbaum AI. Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. Pain 1998;76:215–22.
[44] Martina M, Turcotte ME, Halman S, Bergeron R. The sigma-1 receptor modulates NMDA receptor synaptic transmission and plasticity via SK channels in rat hippocampus. J Physiol 2007;578:143–57.
[45] Martinez-Gomez J, Lopez-Garcia JA. Simultaneous assessment of the effects of L-type current modulators on sensory and motor pathways of the mouse spinal cord in vitro. Neuropharmacology 2007;53:464–71.
[46] Martin-Fardon R, Maurice T, Aujla H, Bowen WD, Weiss F. Differential effects of sigma1 receptor blockade on self-administration and conditioned reinstatement motivated by cocaine vs natural reward. Neuropsychopharmacology 2007;32:1967–73.
[47] Maurice T. Neurosteroids and sigma1 receptors, biochemical and behavioral relevance. Pharmacopsychiatry 2004;37:S171–82.
[48] Mei J, Pasternak GW. Sigma1 receptor modulation of opioid analgesia in the mouse. J Pharmacol Exp Ther 2002;300:1070–4.
[49] Meller ST, Dykstra CL, Gebhart GF. Investigations of the possible role for carbon monoxide (CO) in thermal and mechanical hyperalgesia in the rat. Neuroreport 1994;5:2337–41.
[50] Mendell LM. Physiological properties of unmyelinated fiber projection to the spinal cord. Exp Neurol 1966;16:316–32.
[51] Monnet FP, Debonnel G, Junien JL, De Montigny C. *N*-methyl-D-aspartate-induced neuronal activation is selectively modulated by sigma receptors. Eur J Pharmacol 1990;179:441–5.
[52] Monnet FP, Maurice T. The sigma1 protein as a target for the non-genomic effects of neuro(active)steroids: molecular, physiological, and behavioral aspects. J Pharmacol Sci 2006;100:93–118.
[53] Monnet FP, Morin-Surun MP, Leger J, Combettes L. Protein kinase C-dependent potentiation of intracellular calcium influx by sigma1 receptor agonists in rat hippocampal neurons. J Pharmacol Exp Ther 2003;307:705–12.
[54] Monnet FP. Sigma-1 receptor as regulator of neuronal intracellular Ca^{2+}: clinical and therapeutic relevance. Biol Cell 2005;97:873–83.
[55] Morin-Surun MP, Collin T, Denavit-Saubié M, Baulieu EE, Monnet FP. Intracellular sigma1 receptor modulates phospholipase C and protein kinase C activities in the brainstem. Proc Natl Acad Sci USA 1999;96:8196–9.
[56] Mtchedlishvili Z, Kapur J. A presynaptic action of the neurosteroid pregnenolone sulfate on GABAergic synaptic transmission. Mol Pharmacol 2003;64:857–64.
[57] Nieto FR, Entrena JM, Cendán CM, Pozo ED, Vela JM, Baeyens JM. Tetrodotoxin inhibits the development and expression of neuropathic pain induced by paclitaxel in mice. Pain 2008;137:520–31.
[58] Palacios G, Muro A, Vela JM, Molina-Holgado E, Guitart X, Ovalle S, Zamanillo D. Immunohistochemical localization of the sigma1-receptor in oligodendrocytes in the rat central nervous system. Brain Res 2003;961:92–9.
[59] Palacios G, Muro A, Verdú E, Pumarola M, Vela JM. Immunohistochemical localization of the sigma1 receptor in Schwann cells of rat sciatic nerve. Brain Res 2004;1007:65–70.
[60] Pan YX, Mei J, Xu J, Wan BL, Zuckerman A, Pasternak GW. Cloning and characterization of a mouse sigma1 receptor. J Neurochem 1998;70:2279–85.
[61] Peeters M, Romieu P, Maurice T, Su TP, Maloteaux JM, Hermans E. Involvement of the sigma 1 receptor in the modulation of dopaminergic transmission by amantadine. Eur J Neurosci 2004;19:2212–20.
[62] Prasad PD, Li HW, Fei YJ, Ganapathy ME, Fujita T, Plumley LH, Yang-Feng TL, Leibach FH, Ganapathy V. Exon-intron structure, analysis of promoter region, and chromosomal localization of the human type 1 sigma receptor gene. J Neurochem 1998;70:443–51.
[63] Roh DH, Kim HW, Yoon SY, Seo HS, Kwon YB, Kim KW, Han HJ, Beitz AJ, Na HS, Lee JH. Intrathecal injection of the sigma(1) receptor antagonist BD1047 blocks both mechanical allodynia and increases in spinal NR1 expression during the induction phase of rodent neuropathic pain. Anesthesiology 2008;109:879–89.
[64] Roh DH, Kim HW, Yoon SY, Seo HS, Kwon YB, Kim KW, Han HJ, Betz AJ, Lee JH. Intrathecal administration of sigma-1 receptor agonists facilitates nociception: involvement of a protein kinase C-dependent pathway. J Neurosci Res 2008;86:3644–54.
[65] Song XS, Cao JL, Xu YB, He JH, Zhang LC, Zeng YM. Activation of ERK/CREB pathway in spinal cord contributes to chronic constrictive injury-induced neuropathic pain in rats. Acta Pharmacol Sin 2005;26:789–98.
[66] Soriani O, Le Foll F, Galas L, Roman F, Vaudry H, Cazin L. The sigma-ligand (+)-pentazocine depresses M current and enhances calcium conductances in frog melanotrophs. Am J Physiol 1999;277:E73–80.
[67] Su TP, Hayashi T. Understanding the molecular mechanism of sigma-1 receptors: towards a hypothesis that sigma-1 receptors are intracellular amplifiers for signal transduction. Curr Med Chem 2003;10:2073–80.
[68] Treede RD, Meyer RA, Raja SN, Campbell JN. Peripheral and central mechanisms of cutaneous hyperalgesia. Prog Neurobiol 1992;38:397–421.
[69] Willis WD. Role of neurotransmitters in sensitization of pain responses. Ann NY Acad Sci 2001;933:142–56.
[70] Woolf CJ, Thompson SW. The induction and maintenance of central sensitization is dependent on *N*-methyl-D-aspartic acid receptor activation; implications for the treatment of post-injury pain hypersensitivity states. Pain 1991;44:293–9.
[71] Xin WJ, Gong QJ, Xu JT, Yang HW, Zang Y, Zhang T, Li YY, Liu XG. Role of phosphorylation of ERK in induction and maintenance of LTP of the C-fiber evoked field potentials in spinal dorsal horn. J Neurosci Res 2006;84:934–43.
[72] Zamanillo D, Andreu F, Ovalle S, Pérez MP, Romero G, Farré AJ, Guitart X. Up-regulation of sigma(1) receptor mRNA in rat brain by a putative atypical antipsychotic and sigma receptor ligand. Neurosci Lett 2000;282:169–72.
[73] Zhuang ZY, Gerner P, Woolf CJ, Ji RR. ERK is sequentially activated in neurons, microglia, and astrocytes by spinal nerve ligation and contributes to mechanical allodynia in this neuropathic pain model. Pain 2005;114:149–59.
[74] Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16:109–10.

1.1.1. Annex I

Effect of pharmacological blockade of the sigma-1 receptor on the expression of hind paw withdrawal reflex after peripheral nerve injury

As previously shown, the withdrawal reflex to cold and mechanical stimulation did not develop normally in $\sigma_1R^{-/-}$ mice exposed to partial sciatic nerve injury. This result suggested that genetic σ_1R blockade modulates the behavioural manifestations of the sensory-discriminative component in chronic pain conditions (Article 1). In the present section, the effect of the pharmacological antagonism of σ_1R on the nociceptive reflex responses exhibited in wild-type mice exposed to partial sciatic nerve ligation (PSNL) was analysed to further evaluate the role of σ_1R in the sensory-discriminative component of chronic pain.

PSNL used to induce neuropathic pain was performed in female CD-1 mice according to the methodology previously explained in Article 1. The σ_1R selective antagonist E-52862 (S1RA) (supplied by Laboratorios Esteve) was used for pharmacological treatment in wild-type PSNL and sham-operated mice. Paw withdrawal was measured with calibrated von Frey filaments as explained in Article 1. Drug evaluation was conducted starting at 2 weeks after injury, when the maximum mechanical hypersensitivity was observed (see Article 4). The effects of single doses of E-52862 on the mechanical threshold were evaluated on days 15-17 post-surgery, according to a 3x3 Latin square design. All animals received the 3 doses of E-52862 (20, 40 and 80 mg/kg at a dose volume of 10 mL/kg) by intraperitoneal route. The hindpaw withdrawal threshold was evoked 30 min after drug administration. Finally, on day 18

post-surgery, the mechanical nociceptive threshold was evaluated after a 24-h drug washout period, and the responses were considered as an internal control to confirm that baseline thresholds were not influenced by previous treatments. In all cases both ipsilateral and contralateral paws were tested.

Behavioural data are expressed as mean ± SEM. The data obtained as baseline mechanical thresholds were analysed with the Student's two-independent-sample *t* test. The data obtained from the Latin square design were analysed with two-way repeated measures ANOVA (surgery and paw as main factors) followed by the Bonferroni's multiple comparison test as *post hoc* analysis. To calculate the ED_{50}, data were converted to percentage of antihypersensitivity effect based on the following formula: % antihypersensitivity effect = [(PW– PWi)/(PWc–PWi)] x 100, where PW is the paw withdrawal threshold (g) of the ipsilateral paw in drug treatment, PWi is the paw withdrawal threshold (g) of the ipsilateral paw at pre-treatment day, and PWc is the paw withdrawal threshold (g) of the contralateral paw at pre-treatment day. One way analysis of variance with Bonferroni *post hoc* comparison determined the differences between doses. A value of $P < 0.05$ was considered to be statistically significant.

Mechanical hypersensitivity, defined as a significant reduction in the pressure evoking withdrawal of the nerve-injured hindpaw, was clearly evidenced in PSNL mice ($P < 0.001$). The withdrawal threshold value for the right (ipsilateral) paw in PSNL and sham-operated mice was 0.18 ± 0.04 g and 1.43 ± 0.05 g, respectively (Fig. 1A, B). However, the responses to mechanical stimuli in sham-operated mice remained unchanged after surgery ($P > 0.05$). Withdrawal threshold values of 1.5 ± 0.03 g and 1.4 ± 0.04 g were obtained for the contralateral and ipsilateral paw, respectively.

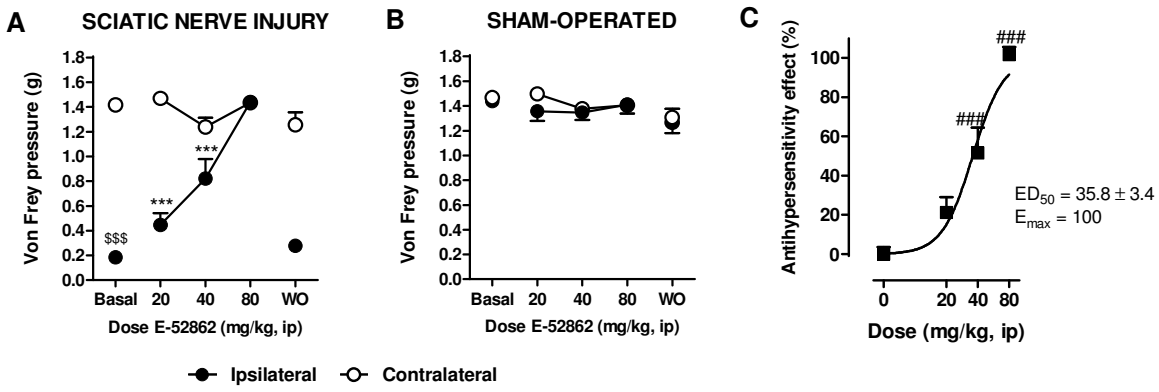


Fig. 1. Effects of the pharmacological blockade of σ_1R on the hind paw withdrawal reflex after mechanical stimulation in mice exposed to peripheral nerve injury. Threshold mechanical sensitivity was assessed with Von Frey filaments in PSNL (A) and sham-operated (B) mice in the ipsilateral and contralateral sides. PSNL surgery induced marked mechanical hypersensitivity in the ipsilateral hind paw 14 days after injury as compared to sham surgery (Basal in the graph). PSNL and sham-operated mice were administered σ_1R antagonist E-52862 (20, 40 and 80 mg/kg) daily as of days 15−17 post-surgery according to a Latin Square design, and paw withdrawal threshold measurements were performed 30 min after drug administration. After a 24-h drug washout period (WO in the graph), the hindpaw withdrawal threshold was measured at day 18 post-injury. (C) Dose-response curve of the antihypersensitivity effect of E-52862 (E_{max}=100; ED_{50} = 35.8 ± 3.4 mg/kg). Each point and vertical line represent the mean ± SEM. $^{\$\$\$}P < 0.001$, Student's two-sample t-test. $***P < 0.001$, two-way RM ANOVA, Bonferroni's *post hoc* test. $###P < 0.001$, one-way ANOVA, Bonferroni's *post hoc* test.

Pharmacological experiments showed that E-52862 had no effect on the responses of sham-operated mice after treatment [$F(4,64) = 2.27$, $P > 0.05$], thus suggesting that normal transmission and perception of sensory and nociceptive inputs remain intact following pharmacological antagonism of σ_1R. However, nerve injury-induced mechanical hypersensitivity was dose-dependently inhibited by the acute systemic administration of E-52862, with E_{max} = 100% and ED_{50} = 35.8 ± 3.4 mg/kg. A statistically significant dose effect was obtained in PSNL mice [$F(4,64) = 23,2$; $P < 0.001$]. Bonferroni's *post hoc* test revealed that E-52862 significantly increased the paw withdrawal threshold at 40 and 80 mg/kg ($P < 0.001$) as compared to the baseline

nerve-injured hindpaw value, while 20 mg/kg of E-52862 had no significant effect ($P > 0.05$) (Fig. 1C). The antinociceptive effect of E-52862 was consistent with the observation that $\sigma_1R^{-/-}$ mice failed to develop mechanical hypersensitivity following nerve injury in σ_1R, shown in Article 1.

The efficacy and potency of σ_1R antagonist E-52862 to reduce the mechanical hypersensitivity secondary to neuropathic pain was maximum and well within the range of doses where E-52862 was active in other pain models, including chemical sensitization with capsaicin (ED_{50} = 26.3 ± 5.5 mg/kg), formalin (ED_{50} = 40.0 ± 5.2 mg/kg), CFA (42.1 ± 7.1 mg/kg) or carrageenan (35.9 ± 9.8 mg/kg) (Gris *et al.*, 2014), and PSNL-induced mechanical allodynia (ED_{50} = 23.4 ± 0.9 mg/kg) (Romero *et al.*, 2012). Our results showed that the ED_{50} of E-52862 was higher than that obtained by Romero and co-workers in the same model of neuropathic pain in male mice, thus suggesting the possibility of gender influences. This study was performed in female mice for comparison with the affective-motivational studies (see Article 4) where females were used on the basis of studies of the literature, which indicate that females are more susceptible to psychological distress and severe pain than their male counterparts (Mogil and Chanda, 2005; Breivik *et al.*, 2006; Theis *et al.*, 2007; Fillingim *et al.*, 2009).

In summary, this pharmacological study showed that σ_1R blockade suppresses nociceptive reflex behaviours in a peripheral nerve injury model in mice, thus supporting the hypothesis that σ_1R plays a role in the sensory-discriminative component of chronic neuropathic pain.

1.1.2. Annex II

Effect of genetic and pharmacological blockade of the sigma-1 receptor on the expression of writhing reflex behaviour after AA-induced visceral pain

This study evaluated the role of σ_1R on the sensory-discriminative component of acute pain using the acetic acid (AA)-induced abdominal writhing. Intraperitoneal administration of a diluted dose of AA, which irritates the serous membranes, causes a very stereotyped behaviour in the mouse, characterized by abdominal contractions, movements of the body as a whole (particularly of the hind paws) and twisting of dorso-abdominal muscles (Le Bars *et al*., 2001). These behaviours are considered to be reflexes and thus evidence of the sensory-discriminative aspect of pain (Hammond, 1989; Le Bars *et al*., 2001). Similarly to chronic pain, writhing-reflex responses were assessed when the receptor was absent using $\sigma_1R^{-/-}$ mice and when the receptor function was blocked using the systemic administration of selective σ_1R antagonists E-52862 and BD-1063.

Experiments were performed in female wild-type mice (Charles River, Barcelona, Spain) and $\sigma_1R^{-/-}$ CD-1 mice (Laboratorios Esteve, Barcelona, Spain) weighing 25–30 g. $\sigma_1R^{-/-}$ mice were generated on a CD-1 background as previously described (Article 1). AA solutions were prepared by adding 0.015, 0.03, 0.06 and 0.09 mL of glacial acetic acid to double deionized water at a final volume of 10 mL. The injection volume of acetic acid was 10 mL/kg, i.p. E-52862 and BD-1063, purchased by Laboratories Esteve (Barcelona, Spain), were dissolved in HPMC 0.5% and administered intraperitoneally at a volume of 10 mL/kg.

The evaluation of visceral nociception was performed using writhing tests according to Koster and co-workers (Koster *et al*., 1959). In the time course and dose range study of AA-induced writhing, wild-type and $\sigma_1R^{-/-}$ mice received a single i.p. administration of AA at different doses (0.15, 0.3, 0.6 and 0.9%, w/v). After injection of AA each mouse was placed inside a Plexiglas observation cylinder (25 cm diameter; 30 cm high) and the number of writhes was recorded for 60 min. A writhe was defined as a wave of the contraction of the abdominal wall muscles followed by extension of the hind limbs. Based on the results of this experiment, the dose of 0.6% was selected to evaluate the effects of drugs injected 30 min before AA, and the number of abdominal contractions was counted for 10 min starting at 5 min after AA injection, where maximum contractions were obtained. In control mice, an appropriate amount of vehicle (HPMC 0.5%) was used.

Behavioural data are expressed as mean ± SEM. One-way repeated measures ANOVA was used to analyse writhing data measured for 60 min. The total number of writhing data was analysed with two-way ANOVA (genotype and AA doses as main factors) followed by Bonferroni's multiple comparison test as *post hoc* analysis. A value of $P < 0.05$ was considered to be statistically significant. Statistical analyses were carried out with the GraphPad Prism 5.00 program (GraphPad Software, San Diego, CA, USA). For ED_{50} calculation, data were converted to percentage of antinociception based on the following formula: Antinociception % = [(mean no. of writhes (test group) – mean no. of writhes (control group) / mean no. of writhes (control group)] × 100%. A dose-response curve was plotted by nonlinear regression analysis, and ED_{50} (dose of drug that produced half of its maximal response) and E_{max} (maximum effect) values were obtained. SEs were calculated on the basis of the best-fit values ± SEs of

regression with GraphPad Prism software, and data were analysed with one-way ANOVA followed by Bonferroni's *post hoc* test for individual inter-group comparisons.

Figure 1 shows the number of writhes induced by i.p. administration of different concentrations of diluted acetic acid. AA (0.15–0.9%, i.p.) evoked a dose-related abdominal writhing behaviour. The highest number of abdominal writhes was observed 5 min after AA administration for 10 min with both the 0.6 and 0.9% doses of AA. Then, a progressive decrease in this behaviour was observed. The effects of AA were no longer apparent after 60 min ($P < 0.005$, 0.9% AA, 0.6% AA, Fig.1A).

The time course of the writhing response evoked by i.p. administration of different doses of AA was indistinguishable between homozygous $\sigma_1R^{-/-}$ and wild-type mice (data not shown). Fig. 1B shows the total number of writhes counted for 60 min. Lower doses of AA (0.3% and 0.15%) induced a reduced number of writhes for both genotypes (0.15% AA: 17.0 ± 7.1 and 9.0 ± 2.9 writhes in wild-type and $\sigma_1R^{-/-}$ mice, respectively; 0.3% AA: 14.8 ± 7.9 and 23.3 ± 10.7 writhes in wild-type and $\sigma_1R^{-/-}$ mice, respectively). However, AA injected at doses of 0.6% and 0.9% dramatically increased abdominal contractions in both homozygous $\sigma_1R^{-/-}$ and wild-type mice. Consequently, no statistically significant differences were found between wild-type and $\sigma_1R^{-/-}$ mice in the total number of writhes measured for 60 min (0.6% AA: 107.0 ± 9.8 and 114.0 ± 18.7 in wild-type and $\sigma_1R^{-/-}$ mice, respectively; 0.9% AA: 145.9 ± 16.8 and 141.1 ± 14.0 writhes in wild-type and $\sigma_1R^{-/-}$ mice, respectively) (Fig. 1B). The fact that the absence of σ_1R failed to modify the reflex behaviour after AA-induced visceral pain suggested that σ_1R does not play a key role in this behaviour.

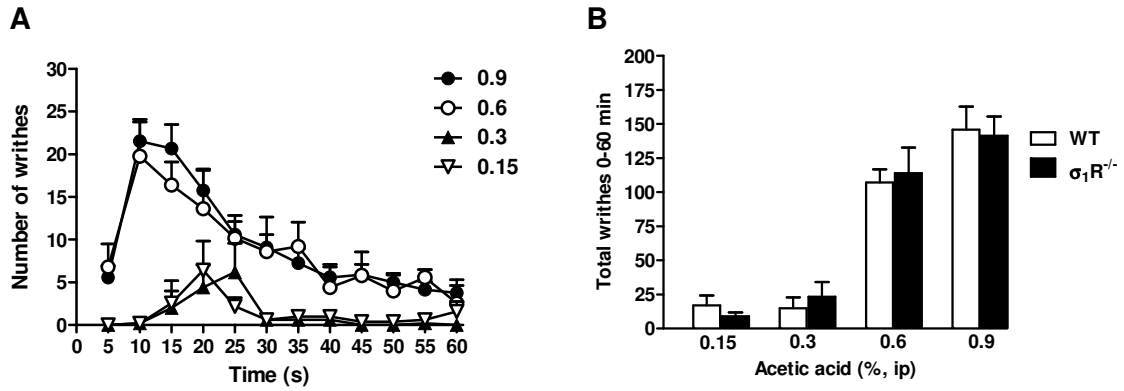


Fig. 1. **Writhing behaviour induced by i.p. administration of different concentrations of AA (0.15-0.9%) in mice.** The nociceptive reflex responses (writhes) were recorded for the first 60 min after administration of AA. (A) Time course of these reflex responses in wild-type mice. (B) Cumulative number of writhes during the 60 min period in wild-type (WT) and $\sigma_1R^{-/-}$ mice. Data are means ± SEM.

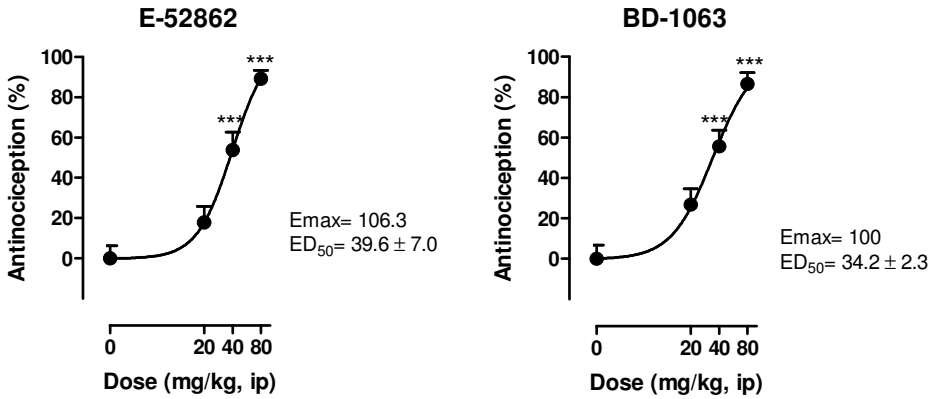


Fig. 2. Effects of σ_1R antagonists on the AA-induced writhing test in mice. A) σ_1R antagonists BD-1063 or E-52862 were tested in the AA-induced writhing test in mice. The number of writhes induced by 0.6% AA was completely blocked by 30 min pre-treatment with BD-1063 or E-52862. ***$P < 0.001$ vs. vehicle, one-way ANOVA, Bonferroni's *post hoc* test.

Also, the effect of σ_1R antagonists on the number of reflex behaviours evoked by AA after treatment with E-52862 or BD-1063 was evaluated. Both BD-1063 and E-52862 dose-dependently reduced the number of writhes (Fig. 2) with similar potency

(ED_{50} = 34.2 ± 2.3 and ED_{50} = 39.6 ± 7 mg/kg, respectively) and efficacy (E_{max} = 100% vs. 106.3%, respectively) (Fig. 2B).

As shown for chronic neuropathic pain described in Annex I, these ED_{50} values are well within the range of doses where E-52862 was active in other pain models (see Table 1). The fact that pharmacological blockade of σ_1R produced a dose-response decrease in the number of writhes induced by AA suggests that σ_1R plays a role in the behavioural expression of AA-induced reflex responses.

Table 1. ED_{50} values for E-52862 in different mouse models of pain.

Animal model	Pain etiology	Nociceptive response	E-52862 ED_{50} ± SE	$\sigma_1R^{-/-}$ vs WT[(1)]	References
CFA	Chronic inflammation	Mechanical hypersensitivity	42.1 ± 7.1	attenuated	Gris *et al*., 2014
Formalin test	Tonic nociception	Licking	40.0 ± 5.2	attenuated	Romero *et al*., 2012
Writhing assay	Acute visceral nociception	Writhing reflex	39.6 ± 7.0	indistinguishable	Annex II
Carregeenan	Acute inflammation	Mechanical hypersensitivity	35.9 ± 9.8	indistinguishable	Gris *et al*., 2014
PSNL	Chronic neuropathy	Mechanical hypersensitivity	35.8 ± 3.4	not developed	Annex I
Capsaicin test	Neurogenic nociception	Mechanical hypersensitivity	26.3 ± 5.5	not developed	Romero *et al*., 2012

[(1)]*Nociceptive responses obtained in $\sigma_1R^{-/-}$ mice as compared to wild-type (WT) mice after painful stimulation in the specified animal models. PSNL: Partial sciatic nerve ligation; CFA: complete Freund's adjuvant.*

These results have yielded apparently conflicting results. Indeed, while the absence of σ_1R failed to modify the nociceptive responses evoked by i.p. administration of AA, the pharmacological blockade of σ_1R produced a clear antinociceptive response. $\sigma_1R^{-/-}$ mice showed a "pain-resistant" phenotype in some experimental pain models such as the formalin, capsaicin or neuropathic pain models (i.e. nociceptive behaviours did not develop or were inhibited vs. wild-type) (Cendán *et al.*, 2005b; Entrena *et al.*, 2009; Nieto *et al.*, 2012) (Article 1). However, the genetic inactivation of σ_1R did not prevent the acquisition of CARR-induced and CFA-induced pain-related behaviours or PSNL-induced heat hypersensitivity (Article 1). As different injuries are known to recruit different pain pathways and mechanisms (Lee *et al.*, 1998; Dowdall *et al.*, 2005; Li *et al.*, 2013), the different phenotypes observed throughout the various models suggest a distinct involvement of the σ_1R system in the mechanisms underlying pain responses, depending on the pain model and the readout.

In summary, the main findings were that the pain-related behaviours (writhes) induced by i.p. administration of AA were similar in wild-type and $\sigma_1R^{-/-}$ mice, thus suggesting that σ_1R does not play a key role in the sensory-discriminative manifestation of acute pain. However, the pharmacological blockade of σ_1R inhibited the expression of the AA-induced reflex behaviour in wild-type mice, thus suggesting that the acute modulation of σ_1R by drugs can affect the sensory-discriminative component of acute pain.

2. Role of the sigma-1 receptor in the affective-motivational component of chronic neuropathic pain and acute visceral pain

2. Role of the sigma-1 receptor in the affective-motivational component of chronic neuropathic pain and acute visceral pain

Because depression is strongly linked to pain in humans (Simons *et al.*, 2014), we focused on depressive-like behaviours to study the role of σ_1R in the **affective-motivational component of acute and chronic pain**. To this end, the two different strategies previously used in the sensory-discriminative component of pain were followed. First, both AA-treated and PSNL mice and their respective controls were tested for anhedonia —a core symptom of depression defined as the inability to experience pleasure from activities typically found enjoyable— by means of the saccharin preference test. The results obtained from experimental procedures conducted in acute visceral pain model are reported in **Article 2**, and the results obtained from experimental procedures in chronic neuropathic pain model are reported in **Annex III**. The results obtained in these experimental approaches led us to devise a reward seeking behaviour (RSB) paradigm where not only consumption but also appetitive, goal-directed behaviours would be reliably disrupted by pain (**Article 3**). Finally, we compared the response to AA and PSNL of wild-type and $\sigma_1R^{-/-}$ mice on RSB and also characterized the dose response effect of E-52862 and known analgesics on pain-related depression of RSB. The results obtained from these experimental procedures conducted in the acute visceral pain model are reported in **Annex IV**. The results obtained in the chronic neuropathic pain model are reported in **Article 4.** This article also compares the effect of E-52862 with the sensory-discriminative component of pain (already shown in Annex I).

2.1. ARTICLE 2

Changes in saccharin preference behaviour as a primary outcome to evaluate pain and analgesia in acetic acid-induced visceral pain in mice

Beatriz de la Puente, Elizabeth Romero-Alejo, José Miguel Vela, Manuel Merlos, Daniel Zamanillo, Enrique Portillo-Salido

Department of Pharmacology, Drug Discovery and Preclinical Development, ESTEVE

Journal of Pain Research 2015; 8:663–673

Journal of Pain Research

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Changes in saccharin preference behavior as a primary outcome to evaluate pain and analgesia in acetic acid-induced visceral pain in mice

This article was published in the following Dove Press journal:
Journal of Pain Research
6 October 2015
Number of times this article has been viewed

Beatriz de la Puente
Elizabeth Romero-Alejo
José Miguel Vela
Manuel Merlos
Daniel Zamanillo
Enrique Portillo-Salido

Department of Pharmacology, Drug Discovery and Preclinical Development, ESTEVE, Barcelona, Spain

Abstract: Reflex-based procedures are important measures in preclinical pain studies that evaluate stimulated behaviors. These procedures, however, are insufficient to capture the complexity of the pain experience, which is often associated with the depression of several innate behaviors. While recent studies have made efforts to evidence the suppression of some positively motivated behaviors in certain pain models, they are still far from being routinely used as readouts for analgesic screening. Here, we characterized and compared the effect of the analgesic ibuprofen (Ibu) and the stimulant, caffeine, in assays of acute pain-stimulated and pain-depressed behavior. Intraperitoneal injection of acetic acid (AA) served as a noxious stimulus to stimulate a writhing response or depress saccharin preference and locomotor activity (LMA) in mice. AA injection caused the maximum number of writhes between 5 and 20 minutes after administration, and writhing almost disappeared 1 hour later. AA-treated mice showed signs of depression-like behaviors after writhing resolution, as evidenced by reduced locomotion and saccharin preference for at least 4 and 6 hours, respectively. Depression-like behaviors resolved within 24 hours after AA administration. A dose of Ibu (40 mg/kg) – inactive to reduce AA-induced abdominal writhing – administered before or after AA injection significantly reverted pain-induced saccharin preference deficit. The same dose of Ibu also significantly reverted the AA-depressed LMA, but only when it was administered after AA injection. Caffeine restored locomotion – but not saccharin preference – in AA-treated mice, thus suggesting that the reduction in saccharin preference – but not in locomotion – was specifically sensitive to analgesics. In conclusion, AA-induced acute pain attenuated saccharin preference and LMA beyond the resolution of writhing behavior, and the changes in the expression of hedonic behavior, such as sweet taste preference, can be used as a more sensitive and translational model to evaluate analgesics.

Keywords: saccharin preference, locomotor activity, pain, writhing, analgesia, ibuprofen, caffeine

Introduction

Most studies on pain and analgesia use reflex-based procedures (eg, tail flick, licking, and guarding) induced by aversive stimulation through the application of particular mechanical, thermal, electrical, and chemical stimuli to identify analgesics. This approach has been evaluated critically because it overfocuses on reflex behaviors and consequently neglects the key affective component of pain phenomena.[1–4] Consequently, the development of relevant new dependent variables to increase the validity of animal models of pain is increasingly pursued.[4–8] Among them, the evaluation of innate behaviors suppressed – instead of enhanced – by pain has been highlighted.[6,7,9–12] A claimed advantage of selecting these behaviors as endpoints is that those drugs

Correspondence: Enrique Portillo-Salido
Department of Pharmacology, Drug Discovery and Preclinical Development, ESTEVE, Carrer Baldiri Reixac, 4–8, Parc Científic de Barcelona, Barcelona 08028, Spain
Email eportillo@esteve.es

submit your manuscript | www.dovepress.com
Dovepress
http://dx.doi.org/10.2147/JPR.S91230

with analgesic properties will be associated with increased behavior rates, and, as a result, analgesic effects would be readily dissociable from motor impairment. In addition, the study of pain-suppressed behaviors should allow outlining the role of behavioral depression, which is normally associated with pain syndromes[13,14] and with others aspects related to the mechanisms and determinants of the affective component of pain.[15]

From this perspective, any behavior spontaneously performed by an animal can be selected as target behavior to evaluate whether pain is or is not able to depress it. However, hedonically oriented behaviors, which are behaviors that have the ability to ensure a positive emotional state experienced as pleasure,[16,17] are expected to be rapidly expressed and maintained by the animals at relatively high rates, which would help reduce methodological problems such as using food or water deprivation during the behavioral tasks. Furthermore, decreases in rate, frequency, duration, or intensity of highly preferred behaviors ("hedonic behaviors") caused by pain (or other insults) can be suggestive of a deterioration of the animal global welfare and/or quality of life, which makes hedonically oriented behaviors interesting in the testing of beneficial effects of analgesics – which should restore the normal hedonic behavior of the animals.

In this study, two positively motivated behaviors, such as the natural rodent preference for sweet taste and rodent locomotor activity (LMA) in a novel environment, were selected as the main dependent variables to measure the presence of pain or analgesia. Preference for sweet taste maintains a high rate in mice and requires an intact cognitive function as well as appetitive motivation.[18] A precise measurement of sweet taste preference is easy to conduct and can be determined in home cages without animal handling. This behavior has been shown to be sensitive to different pharmacological and environmental manipulations. It has been used to model anhedonia – the lack of interest or pleasure in response to hedonic stimuli or experiences – in the chronic mild stress animal model of depression.[19,20] LMA measures spontaneous, instinctive behaviors of rodents that are largely motivated by the exploration of a novel environment for means of escape. Decrease in locomotion as a consequence of pain has been consistently reported in both humans and rodents,[6,11,21–23] and psychomotor retardation – which includes motor impairment affecting gross locomotor skills – is also a central feature of depression.[24,25]

The classical preclinical pain test of acetic acid (AA)-induced abdominal constriction to induce pain was used. In this test, AA injection causes inflammation of the abdominal cavity wall and evokes sustained writhing behavior and reduced motor activity. The occurrence of this writhing behavior (abdominal cramps or stretching) per unit of time is commonly evaluated. These behaviors are considered to be reflexes and to be evidence of visceral pain,[26] but the frequency of writhing decreases spontaneously with time.

The goal of the present study was to compare the analgesic sensitivity of two pain-suppressed behaviors with the AA-induced standard reflexive outcome (writhing behavior). For that purpose, the time course of AA-induced behavior (writhing) and AA-depressed behavior (saccharin preference and LMA) was first studied. Secondly, the restorative effects on both LMA and saccharin preference behavior of a dose of ibuprofen (Ibu) devoid of efficacy on AA-induced writhing were evaluated. This was performed by administering the drug before (development protocol) and after (expression protocol) the induction of pain by AA. Finally, the effects of caffeine-induced behavioral activation to assess the specificity of the different tests were evaluated.

Methods

Animals

Female CD1 mice weighing 25–30 g were used in all experiments (Charles River, L'Arbresle, France). The study protocol was approved by the local Committee of Animal Use and Care of our institution (ESTEVE) and was in accordance with the guidelines for the Care and Use of Laboratory Animals of the European Community (European Directive 2010/63/EU) and with the International Association for the Study of Pain guidelines on ethical standards for investigation in animals.[27] Light/dark cycle (reverted 12/12 hours, lights on at 6 pm), temperature (22°C), and humidity (40%) were controlled. Animals had free access to food and water and were used after 14 days of acclimatization to housing conditions. All experiments were performed between 9 am and 6 pm.

Drugs

The drugs investigated were Ibu (40–320 mg/kg), supplied by Laboratorios Esteve (Barcelona, Spain), and caffeine (5–20 mg/kg), purchased from Sigma Chemical Co (Barcelona, Spain). Approximately 0.5% hydroxypropyl methylcellulose (HPMC) (Sigma Chemical Co) dissolved in saline was used as vehicle. The drugs (or the vehicle in the control group) were administered intraperitoneally (IP) at a volume of 10 mL/kg. The time of administration was chosen in order to evaluate the putative preventive or restorative effect of Ibu on target behaviors. To evaluate the preventive effect, the drug was administered 30 minutes

before AA challenge ("development protocol"). To evaluate a purely restorative effect, the drug was administered 120 or 150 minutes after AA challenge for saccharin preference and LMA, respectively ("expression protocol").

Assay of acetic acid-induced writhing

For the time course study, mice were injected 10 mL/kg of AA (0.6%) or vehicle (distilled water) by IP route. Each mouse was then placed in an individual, clear plastic observation chamber and the total number of writhes was counted for 1 hour after administration.

Based on the results of this protocol, the interval ranging between 5 and 15 minutes after AA injection was selected to evaluate the effects of Ibu and caffeine on the number of writhes. Separate groups of mice were administered vehicle (HPMC 0.5%), Ibu, or caffeine, IP, 30 minutes before 0.6% AA injection.

For scoring purposes, a "writhe" was defined as a contraction of the abdominal muscles accompanied by body elongation and hind limb extension. Data are expressed as the mean number of writhes over the 10-minute observation period.

Saccharin preference test

Mice were habituated to saccharin (0.1%, Sigma Aldrich Co, St Louis, MO, USA) consumption by means of saccharin solution diluted in tap water as sole drinking fluid for 48 hours. After habituation, the baseline saccharin preference was measured for 6 hours 1 day before the test. During the saccharin preference test, fluid consumption was measured for 24 hours with a two-bottle protocol, whereby mice were exposed to a bottle each of tap water and 0.1% saccharin solution. Water and saccharin solution intake was estimated simultaneously in control and experimental groups by weighing the bottles at 2, 4, 6, 8, and 24 hours. The animals were not previously deprived of water and food, but had no access to food during the first 6-hour preference tests. For each mouse on each day, the ratio of solution preference was calculated according to the formula below:

$$\text{Ratio (\%)} = \text{Saccharin solution intake}/(\text{Saccharin solution intake} + \text{Water intake}) \times 100$$

Novelty induced LMA evaluation

LMA was scored automatically in independent experiments. Eight standard actimeters (Linton Instrumentation Inc., Norfolk, UK) equipped with infrared beam motion detectors were used. On the day of the experiment, mice were evaluated in a dark environment. Mice were marked and weighed at the beginning of each experimental session. After administering AA, the compounds, or their vehicles, the animals were returned to their home cages and then placed in the LMA cages at the scheduled time. In the time course experiment, LMA was evaluated in separate groups of mice exposed to the chamber only once at the scheduled post-AA time (1, 2, 3, 4 or 5 hours post-AA). Moving time (seconds) was measured for 60 minutes in each separate group, with readings performed every 5 minutes.

Data analysis

Data are expressed as mean ± standard error of mean. For studies of LMA and saccharin preference, data were analyzed with two-way repeated measures analysis of variance (ANOVA), with pain and treatment drug as factors. One-way ANOVA was used for area under the curve (AUC, from 0 to 24 hours) comparison. One-way repeated measures ANOVA was used to analyze writhing test data, and one-way ANOVA with Bonferroni's multiple comparison test as post hoc analysis was used to analyze drug treatment data. $P<0.05$ was considered statistically significant. Statistical analyses were carried out with the GraphPad Prism 5.00 program (GraphPad Software, San Diego, CA, USA).

Results

Acetic acid-induced stimulation of writhing and depressed sweet preference behavior

IP injection of 0.6% AA robustly induced the appearance of abdominal constrictions (writhing) in mice (Figure 1, left axis). The number of writhes peaked 5–20 minutes after AA administration ($P<0.001$). Then, a progressive decrease in this behavior was observed, and the effects of AA were no longer apparent after 60 minutes ($P>0.05$). The right axis of Figure 1 shows the preference for a saccharin solution (0.1%) in animals pretreated with AA (pain group) or its vehicle (control group). The baseline values for saccharin preference measured 1 day earlier did not vary significantly between the pain and the control groups (80.1±2.1 and 77.3±3.3%, respectively). Mice treated with the vehicle of AA demonstrated a preference for saccharin solution over water of 65%–75% at different times. Variations between baseline and test day values were observed across all experiments. These variations were attributed to the different baseline recording times – 6 hours on a continuous basis – and to mice handling on the test day, which included IP injection. Pretreatment with 0.6% but not 0.3% (data not shown) AA significantly decreased the expression of saccharin preference behavior as compared to control mice.

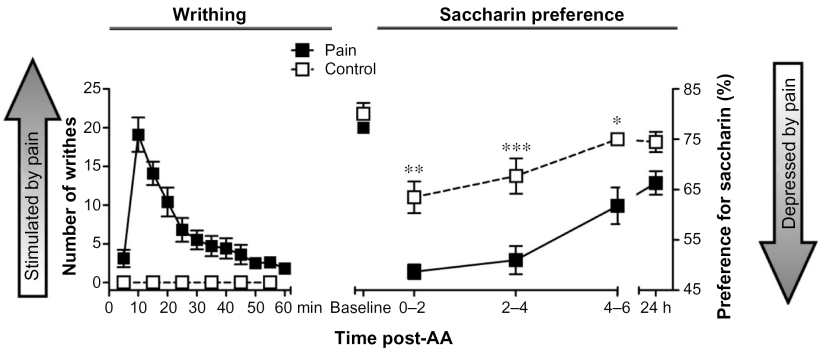


Figure 1 Time course of AA-induced writhing and AA-depressed saccharin preference measured over a period of 24 hours post-AA.
Notes: The administration of AA (0.6%) (pain group, black squares) induced the appearance of writhing behavior (left axis) that almost disappeared after 1 hour. In contrast, depressed sweet preference behavior (right axis) remained for at least 6 hours as compared to control mice (white squares). Data are mean ± SEM. *$P<0.05$ (4-6), **$P<0.01$ (0-2), ***$P<0.001$ (2-4) vs pain.
Abbreviations: AA, acetic acid; SEM, standard error of mean; h, hours; min, minutes.

Two-way repeated measures ANOVA (time × pain) showed a significant effect of time [$F(4,48) = 28.24$, $P<0.001$] and pain [$F(1,12) = 15.46$, $P<0.01$] and a significant interaction between these two factors [$F(4,48) = 2.76$, $P<0.05$]. AA-induced deficit in the expression of this hedonic behavior was observed for at least 6 hours, and normal preference behavior was restored 24 hours after AA administration (Figure 1, right axis). Post hoc testing showed significantly reduced saccharin preference rates in AA-induced pain in mice at 2, 4, and 6 hours ($P<0.01$, $P<0.001$, and $P<0.05$, respectively), but not at 24 hours.

Acetic acid-induced decrease of LMA

LMA as a function of pretreatment interval at the same concentration of AA tested in the saccharin preference experiment is shown in Figure 2. Control mice showed peak activity during the first 5 minutes. After that, mice became habituated to the environment and their locomotion behavior progressively declined. The administration of 0.3% AA did not change LMA as compared to vehicle-treated mice (data not shown). However, mice treated with 0.6% of AA showed a strongly shortened motion time as compared to control mice (vehicle). A repeated measures two-way ANOVA showed significant effects of pain at 60 minutes [$F(1,28) = 20.69$, $P<0.001$], 120 minutes [$F(1,28) = 16.40$, $P<0.001$], 180 minutes [$F(1,28) = 9.23$, $P<0.01$], and 240 minutes [$F(1,28) = 18.22$, $P<0.001$], but not at 300 minutes [$F(1,28) = 0.17$, $P>0.05$]. A significant interaction between pain and time was detected at 60 minutes [$F(11,308) = 6.35$, $P<0.001$], 120 minutes [$F(11,308) = 7.23$, $P<0.001$], and 180 minutes [$F(11,308) = 2.14$, $P<0.05$], but not at 240 minutes [$F(11,308) = 0.92$, $P>0.05$] or 300 minutes [$F(11,308) = 0.62$, $P>0.05$].

Effects of ibuprofen and caffeine on acetic acid-induced writhing

Ibu administration 30 minutes before AA led to a significant, dose-related inhibition of AA-induced writhing in mice. Doses of 160

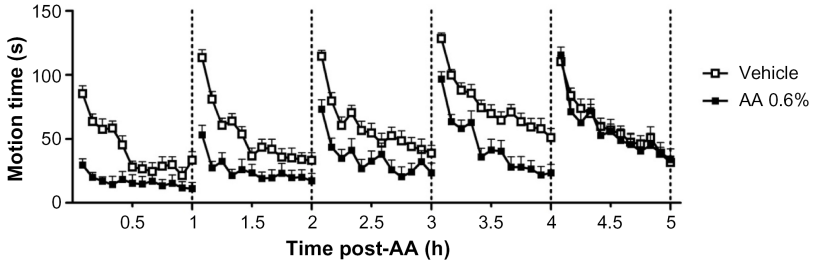


Figure 2 Time-related effect after AA injection on AA-depressed exploratory behavior.
Notes: Separate groups of animals were injected with AA 0.6% (black squares) or its vehicle (white squares) at 0, 1, 2, 3, and 4 hours before each LMA evaluation. Each group of mice was exposed to the chamber only once on the indicated post-AA time. Motion time (seconds) was measured between 0 and 60 minutes, every 5 minutes. Note that LMA depression in mice injected with AA 0.6% remained for at least 4 hours as compared to vehicle-treated mice. Data are mean ± SEM.
Abbreviations: AA, acetic acid; LMA, locomotor activity; SEM, standard error of mean; h, hours; s, seconds.

and 320, but not 40 or 80 mg/kg, significantly inhibited AA-induced writhing behavior ($P<0.01$, Figure 3A). Caffeine administration, however, failed to significantly inhibit AA-induced writhing in mice at the doses of 5, 10, and 20 mg/kg IP (Figure 3B, NS).

Effects of ibuprofen on AA-induced deficit in saccharin preference behavior

Next, we aimed at determining whether an analgesic was able to revert AA-induced deficit in the saccharin preference behavior of mice in two different administration protocols, the "development" and the "expression" protocols.

In the development protocol, 40 mg/kg of Ibu – a dose that failed to produce any analgesic effect evaluated by AA-induced writhing – or vehicle were administered 30 minutes before AA challenge. Mice receiving vehicle (vehicle + AA group) before AA injection showed a significantly depressed saccharin preference behavior as compared to control mice (vehicle + vehicle group). Repeated measures two-way ANOVA (time × pain) showed a significant effect of time [$F(5,80)=27.17$, $P<0.001$] and pain [$F(1,80)=4.81$, $P<0.05$], and a significant time × pain interaction between these two factors [$F(5,80)=2.45$, $P<0.05$]. Ibu did not affect the normal saccharin preference of vehicle-injected mice (Ibu + vehicle group) and did not prevent decreased saccharin preference in AA-treated mice (Ibu + AA group) before 2 hours, but it was able to revert the AA-induced deficit in the preference for saccharin from 2 to 6 hours (Figure 4A). Repeated measures two-way ANOVA (time × treatment) showed a significant effect of time [$F(5,80)=39.29$, $P<0.001$], treatment [$F(1,80)=11.91$, $P<0.05$], and interaction between these two factors [$F(5,80)=3.49$, $P<0.01$]. One-way ANOVA followed by Bonferroni's post hoc test of the AUC (from 0 to 24 hours) globally suggested total restoration of saccharin preference behavior in AA-treated mice (Figure 4B; $P<0.01$).

We took advantage of the long-term duration of the AA-induced decrease in saccharin preference to evaluate whether Ibu was able to revert the deficit once established ("expression protocol"). Thus, Ibu or vehicle was administered 2 hours after AA or vehicle challenge (arrow in Figure 4C). AA-injected mice treated with vehicle (vehicle + AA group) showed a significant decrease in saccharin preference behavior as compared to those injected with vehicle (vehicle + vehicle group). Two-way repeated measures ANOVA (time × pain) showed a significant effect of time [$F(5,70)=31.34$, $P<0.001$], pain [$F(1,70)=9.13$, $P<0.01$], and interaction between these two factors [$F(5,70)=3.58$, $P<0.01$]. Similar to the development protocol, Ibu did not change the preference for saccharin of the vehicle-injected mice, but was able to revert the deficit of the AA-injected mice (Figure 4C). Repeated measures ANOVA (time × treatment) showed a significant effect of time [$F(5,70)=34.52$, $P<0.001$], treatment [$F(1,70)=5.09$, $P<0.05$], and interaction between these two factors [$F(5,70)=4.63$, $P<0.001$]. Similarly, one-way ANOVA followed by Bonferroni's post hoc test of the AUC was also suggestive of a restored saccharin preference behavior in AA-treated mice (Figure 4D, $P<0.01$).

Effects of ibuprofen on the AA-induced deficit in LMA

We next aimed to determinate whether Ibu was able to revert the AA-induced deficit in the exploratory behavior of mice, also using the two administration protocols ("development" and "expression" protocols).

In order to prevent deficit, Ibu was again administered at 40 mg/kg (inactive dose evaluated by writhing behaviors) 30 minutes before AA challenge ("development protocol").

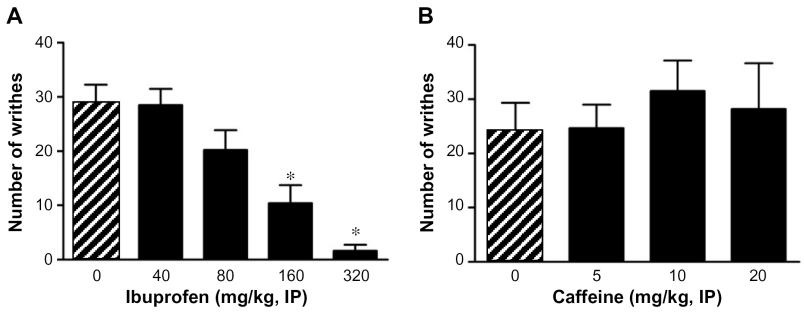


Figure 3 Effect of Ibu (**A**) and caffeine (**B**) on AA-induced writhing.
Notes: Ibu (40, 80, 160, and 320 mg/kg) and caffeine (5, 10, and 20 mg/kg) were administered IP 30 minutes prior to writhing test. Note that 40 mg/kg of Ibu did not significantly reduce the number of writhes. *$P<0.001$ vs vehicle.
Abbreviations: AA, acetic acid; Ibu, ibuprofen; IP, intraperitoneal.

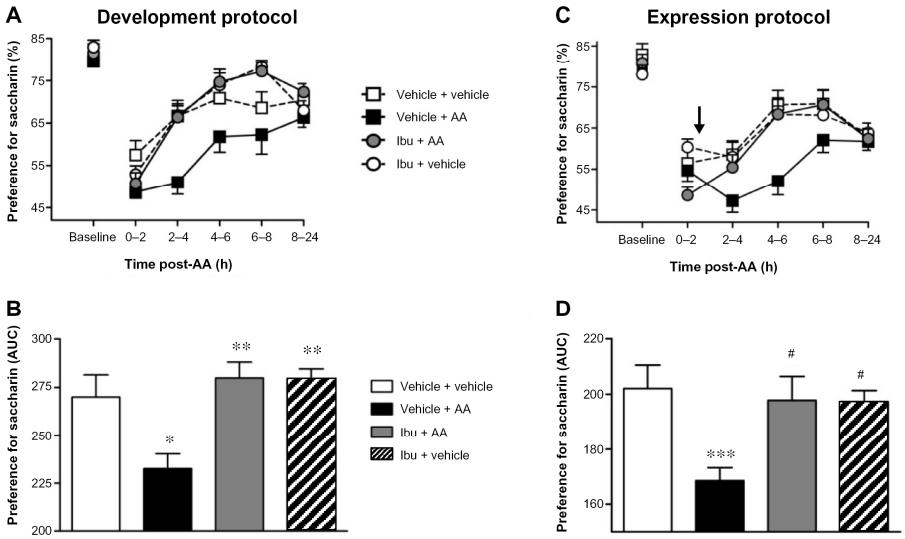


Figure 4 Effect of Ibu on AA-depressed saccharin preference.

Notes: Administration of 40 mg/kg of Ibu 30 minutes before (development protocol) and 2 hours after (expression protocol) AA injection reverted (Ibu + AA group; gray circle in **A** and **C**, and gray solid bar in **B** and **D**) saccharin preference behavior deficit in AA-treated mice (vehicle + AA group; black square in **A** and **C**, and black bar in **B** and **D**). The vehicle + vehicle group (white square in **A** and **C**, or white bar in **B** and **D**) includes control mice injected with vehicle instead of AA and treated with the vehicle of Ibu. The Ibu + vehicle group (white circle in **A** and **C**, or lined bar in **B** and **D**) includes control mice injected with vehicle instead of AA and treated with Ibu. Bar graphs (**B** and **D**) show the AUC values calculated for each experimental group in both protocols. Data are mean ± SEM. $^{*}P<0.05$ vs vehicle group; $^{**}P<0.01$ vs AA-induced pain group; $^{***}P<0.01$ vs vehicle group; $^{\#}P<0.05$ vs AA-induced pain group.

Abbreviations: AA, acetic acid; AUC, area under the curve; Ibu, ibuprofen; SEM, standard error of mean; h, hours.

Vehicle-treated mice injected with AA (vehicle + AA group) showed a significantly depressed LMA behavior as compared to control mice (vehicle + vehicle group). Repeated measures two-way ANOVA (time × pain) showed a significant effect of time [$F(11,154)=9.81$, $P<0.001$], pain [$F(1,154)=20.10$, $P<0.001$], and no interaction between these two factors [$F(11,154)=1.68$, $P>0.05$]. While Ibu administration failed to significantly affect the expression of the LMA behavior in the animals injected with AA vehicle (Ibu + vehicle group), it did slightly increase the LMA of AA-injected mice (Ibu + AA group), particularly over the first 10 minutes (Figure 5A). Repeated measures ANOVA (time × treatment) showed a significant effect of time [$F(11,154)=31.02$, $P<0.0001$], treatment [$F(1,154)=10.72$, $P<0.001$], and interaction between factors [$F(11,154)=2.43$, $P<0.01$]. While AUC values calculated for each experimental group indicated a partially restored saccharin preference behavior caused by Ibu in AA-injected mice (Figure 5B), one-way ANOVA followed by Bonferroni's post hoc test of the AUC showed no statistically significant differences.

The effects of Ibu 2 hours after AA challenge ("expression protocol") are shown in Figure 5C. Vehicle-treated mice after AA injection (vehicle + AA group) showed a significant decrease in exploratory behavior as compared to control mice (vehicle + vehicle group). Repeated measures two-way ANOVA (time × pain) showed a significant effect of time [$F(11,198)=15.85$, $P<0.001$], pain [$F(1,198)=33.35$, $P<0.001$], and interaction between these two factors [$F(11,198)=1.05$, $P<0.05$]. Ibu did not change the LMA of the mice injected with AA vehicle (Ibu + vehicle group). However, Ibu was able to fully revert LMA decrease in the AA-injected mice (Ibu + AA group). Repeated measures ANOVA (time × treatment) showed a significant effect of time [$F(11,198)=30.98$, $P<0.001$] and treatment [$F(1,198)=25.14$, $P<0.001$], but no interaction between these two factors [$F(11,198)=0.27$, $P>0.05$]. One-way ANOVA followed by Bonferroni's post hoc test of the AUC was also suggestive of fully restored LMA behavior in AA-treated mice (Figure 5D, $P<0.01$).

Effects of caffeine on the AA-induced depression in saccharin preference and LMA behavior

In order to study the specificity of the endpoints, we tested the effects of caffeine, a nonanalgesic stimulant producing behavioral increases, on AA-induced depression in both LMA and saccharin preference behaviors using the development protocol. The effects on LMA are shown

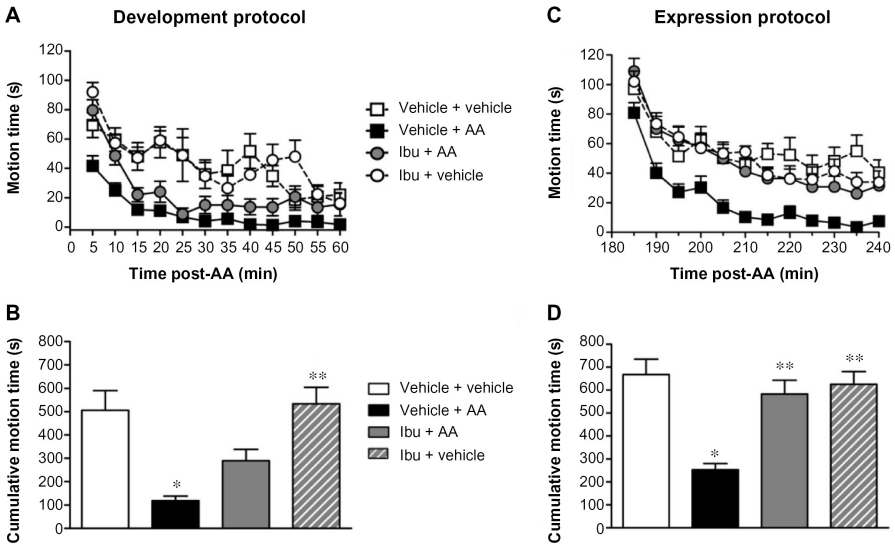


Figure 5 Effect of Ibu on AA-decreased exploratory behavior.

Notes: Administration of 40 mg/kg 30 minutes before AA (development protocol) partially prevented, but not significantly reverted (Ibu + AA group; gray circle in **A**, and gray solid bar in **B**), locomotor activity deficit in AA-treated mice (vehicle + AA group; black square in **A**, and black bar in **B**). Ibu administered 150 minutes after AA (expression protocol) completely restored (Ibu + AA group; gray circle in **C**, and gray solid bar in **D**) locomotor activity in AA-treated mice (vehicle + AA group; black square in **C**, and black bar in **D**). The vehicle + vehicle group (white square in **A** and **C**, or white bar in **B** and **D**) includes control mice injected with vehicle instead of AA and treated with the vehicle of Ibu. The Ibu + vehicle group (white circle in **A** and **C**, or lined bar in **B** and **D**) includes control mice injected with vehicle instead of AA and treated with Ibu. Bar graphs (**B** and **D**) show the AUC values calculated for each experimental group in both protocols. Data are mean ± SEM. *$P<0.001$ compared to the vehicle-treated group; **$P<0.001$ vs AA-induced pain group.

Abbreviations: AA, acetic acid; AUC, area under the curve; Ibu, ibuprofen; SEM, standard error of mean; min, minutes; s, seconds.

in Figure 6A. Caffeine was administered at 10 mg/kg (IP) 30 minutes before AA challenge. As expected, AA-injected mice treated with vehicle (vehicle + AA group) showed a significantly depressed LMA behavior as compared to control mice (vehicle + vehicle group). Repeated measures two-way ANOVA (time × pain) showed a significant effect of time [$F(11,275)=16.59$, $P<0.001$], pain [$F(1,275)=46.21$, $P<0.001$], and interaction between these two factors [$F(11,275)=1.50$, $P>0.05$]. Caffeine administration caused the expected increase in vehicle-injected mice (caffeine + vehicle group), but also increased the LMA of AA-injected mice (caffeine + AA group) as compared to control mice (vehicle + vehicle group) (Figure 6A). Repeated measures two-way (time × treatment) ANOVA showed a significant effect of time [$F(11,187)=59.63$, $P<0.001$], treatment [$F(1,187)=22.32$, $P<0.001$], and interaction between these two factors [$F(11,187)=10.55$, $P<0.001$]. One-way ANOVA followed by Bonferroni's post hoc test of the AUC confirmed the caffeine-induced increase in LMA of both AA-treated and vehicle-treated mice (Figure 6B).

The effects on saccharin preference behavior are shown in Figure 6C. Caffeine was administered at the same dose of 10 mg/kg (IP) 30 minutes before AA challenge. AA-injected mice treated with vehicle (AA + vehicle group) showed a significant decrease in saccharin preference behavior as compared to control mice (vehicle + vehicle group). Two-way repeated measures ANOVA (time × pain) showed a significant effect of time [$F(4,64)=21.36$, $P<0.001$], pain [$F(1,64)=21.33$, $P<0.001$], and interaction between these two factors [$F(4,64)=6.18$, $P<0,001$]. Caffeine administration did not significantly affect the expression of the saccharin preference behavior of the animals injected with the vehicle of AA (caffeine + vehicle group) as compared to control mice (vehicle + vehicle group). Interestingly, caffeine administration to AA-injected mice (caffeine + AA group) did not revert the AA-induced decrease in saccharin preference. Two-way repeated measures ANOVA (time × treatment) showed a significant effect of time [$F(4,68)=59.75$, $P<0.001$], but not treatment [$F(1,68)=0.12$, $P>0.05$] or interaction between these two factors [$F(4,68)=0.72$, $P>0.05$]. AUC calculation clearly indicates the absence of caffeine effect on saccharin preference behavior deficit in AA-treated mice (Figure 6D).

Discussion

Efforts have recently been made to investigate pain and analgesia using novel paradigms that do not rely

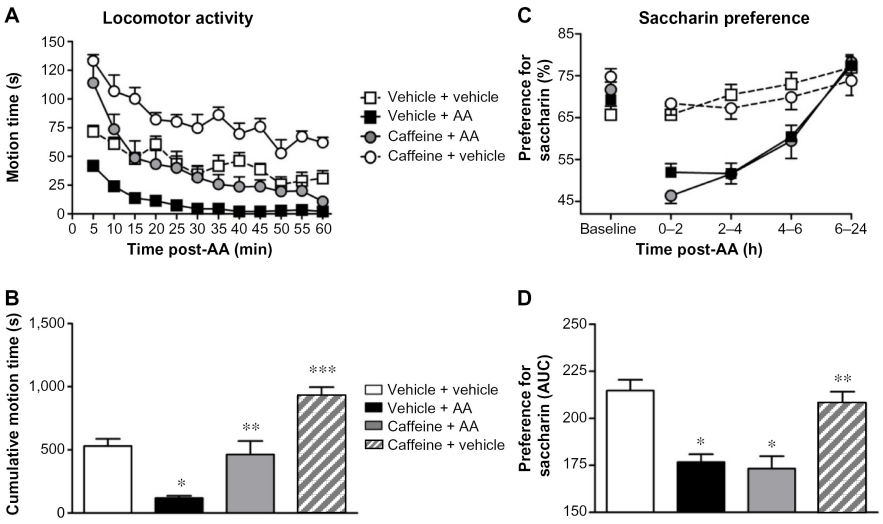


Figure 6 Effect of caffeine on AA-decreased sweet preference (**C** and **D**) and exploratory (**A** and **B**) behaviors.
Notes: Administration of 10 mg/kg of caffeine 30 minutes before AA (caffeine + AA group; gray circle in **A** and **C**, and gray solid bar in **B** and **D**) reverted locomotor activity deficit, but not saccharin preference depression in AA-treated mice (vehicle + AA group; black square in **A** and **C**, and black bar in **B** and **D**). The vehicle + vehicle group (white square in **A** and **C**, or white bar in **B** and **D**) includes control mice injected with vehicle instead of AA and treated with the vehicle of caffeine. The caffeine + vehicle group (white circle in **A** and **C**, or lined bar in **B** and **D**) includes control mice injected with vehicle instead of AA and treated with caffeine. Note that this control group increases the cumulative motion time as compared to the vehicle + vehicle group in the locomotor activity experiments (**A** and **B**), but not in the saccharin preference endpoint (**C** and **D**). Bar graphs (**B** and **D**) show the AUC values calculated for each experimental group in both protocols. *$P<0.001$ compared to the vehicle-treated group; **$P<0.01$, ***$P<0.001$ vs AA-induced pain group.
Abbreviations: AA, acetic acid; AUC, area under the curve; Ibu, ibuprofen; h, hours; min, minutes; s, seconds.

solely on reflex-based withdrawal responses.[4] Decreases in burrowing,[28] nesting,[29] feeding,[10] intracranial self-stimulation,[30] wheel running,[31,32] and food-maintained operant responding[7] to evaluate the presence of pain and analgesia have also been reported. Also, decreased LMA as a consequence of pain has been consistently reported in both humans and rodents.[6,11,21–23] The present study provides evidence that the hedonic behavior of sweet taste preference using saccharin in mice was strongly depressed by AA and that it can be used to detect the analgesic effects of drugs. The characteristic pain writhing behavior induced by AA, which lasted less than 1 hour, was followed by a substantially longer "behavioral depression" manifested by a strongly decreased expression of both saccharin preference and LMA for at least 4 hours. Pain-suppressed behaviors long after AA-induced writhing behavior is consistent with the results of a previous study showing a similarly decreased LMA for 5 hours after treatment with 0.56% AA in male ICR mice.[11] However, to our knowledge, this is the first time that such a sustained depression (for at least 6 hours) of sweet taste preference after AA administration is described. Previously, the time of feeding suppression using a Liquid Ensure™ protein drink was determined 1 hour after 0.56% AA administration.[10]

In the present study, a visceral noxious stimulus was selected to induce pain. Visceral pain presents with important differences as compared to cutaneous somatic pain. Somatic and visceral pain are mediated, at least in part, through different neural pathways at spinal and supraspinal sites, and evoke different emotional responses.[33–38] Cutaneous somatic pain is escapable, can be controlled, and characteristically evokes active emotional coping responses such as agitation, hyperactivity, fight–flight, and hypertension. In contrast, visceral pain is inescapable, cannot be controlled by the subjects themselves, and usually evokes passive coping or "conservation-withdrawal" strategies, characterized by "disengagement from" the environment, ie, behavioral quiescence and immobility, decreased reactivity to the environment, hypotension, and bradycardia.[36,38] The behavioral inhibition observed after the visceral noxious stimulation in our study is consistent with this view. Recent data from our laboratory – where formalin administration to the paw, a somatic pain model, was unable to alter saccharin preference behavior in mice – further support this view (unpublished data).

Ibu started to produce significant effect in the attenuation of the number of writhes at the dose of 160 mg/kg, but the pharmacological effect of 40 mg/kg of Ibu in the

saccharin preference paradigm was already consistent with analgesia, considering the whole 0–24 hours measurement period. This dose, however, was ineffective to prevent AA-depressed saccharin preference in the first 2 hours of the saccharin preference test (development protocol). A logical explanation for this is that the saccharin preference behavior reductions observed during the first 2 hours may be caused by AA-induced writhing behavior, and are not inhibited by Ibu at 40 mg/kg. These two behaviors (writhing and saccharin preference) seem incompatible because mice cannot drink and writhe at the same time. Interestingly, Ibu clearly prevented sweet preference behavior deficit after 2 hours.

The fact that 40 mg/kg of Ibu – ineffective to block pain-induced writhing – was actually effective on the pain-depressed behaviors of saccharin preference raises the possibility that the analgesic effects of drugs can be better observed with a pain-depressed endpoint than with a pain-stimulated endpoint. This conclusion agrees with those of several previous studies showing that some analgesics such as Ibu, morphine, pregabalin, or acetaminophen attenuate the affective component of pain more potently than its sensory component.[8,39–41] In the present study, where decreased saccharin preference behavior reflects the affective component and increased writhing behavior reflects the sensory component of AA-induced pain, Ibu was better against the affective component than against the sensory component of pain. Furthermore, this could be indicating that a drug can have analgesic properties without inhibiting the writhing behavior. This may be of particular importance in a drug discovery context because possible analgesics may be currently being discarded based on a lack of efficacy on sensory-based pain screening experimental models.

The "expression protocol" allowed us to test the effect of the drug using a within-subject design in animals where the AA-induced deficit had already been established and once the AA-induced writhing behavior had disappeared. Before Ibu administration, AA-treated mice showed the expected depression in saccharin preference shown by the decrease observed during the first 2 hours as compared to control animals. When these animals were treated with Ibu, the preference for saccharin returned to that of vehicle-treated animals. This approach allowed avoiding the potential effect of AA-stimulated behaviors likely to compete with the target depressed behavior, as we have previously hypothesized to explain the lack of effect in the development protocol of the saccharin intake in the first 2 hours, ie, when writhing is occurring at a relatively high rate, mice cannot perform the intake of liquid.

In the present study, Ibu administration 30 minutes before AA (development protocol) was able to only partially prevent AA-induced decrease in LMA. The results obtained during the entire hour period suggest that the dose of 40 mg/kg was not sufficient to completely restore pain-depressed LMA behavior. The partial efficacy of Ibu on AA-induced deficit in LMA is consistent with the lack of efficacy observed in the AA-induced writhing test and during the first 2 hours of the saccharin preference test.

The administration of Ibu after AA (expression protocol) restored LMA, with the activity of AA-injected animals returning to that of vehicle-injected animals. In this protocol, mice received Ibu or its vehicle 150 minutes after AA injection (30 minutes before the behavioral test). Animals pretreated with Ibu – but not animals treated with the vehicle – showed LMA restoration, which is consistent with the results observed in the expression protocol of the saccharin test.

Finally, caffeine was used as a nonanalgesic stimulant to evaluate the specificity in relation with pain of the two target behaviors. Caffeine prevented LMA decrease in AA-injected mice, but also induced a strong LMA increase in vehicle-injected animals. In contrast, caffeine was unable to change the depressed saccharin preference behavior in AA-treated mice. Therefore, despite the fact that caffeine induced LMA normalization in AA-injected mice to the level of control animals, the deficit in saccharin preference behavior was not sensitive to this behavioral arousal induced by caffeine. In a previous study, Stevenson et al[11] did not find such effect of caffeine on AA-depressed LMA. The reasons for this discrepancy are not clear. Similar to this study, Stevenson et al[11] found that caffeine significantly increased LMA in nondepressed mice. However, they only found a nonsignificant tendency of caffeine to revert acid-depressed LMA. Discrepancy may be sex related because we used female mice and the Stevenson et al[11] study used male mice. However, no sex-related differences in caffeine-induced LMA increase have been found.[42] Discrepancy might also arise from the different light/dark cycles in which the two behavioral experiments were performed. In order to favor the higher levels of LMA associated with the dark (active) phase of the animal's activity cycle, our experiments were conducted under dark conditions, while the Stevenson et al's[11] study was conducted under light conditions. While the effects of caffeine on LMA did not seem to be altered by ambient lighting,[43,44] circadian fluctuations in visceral sensory functions have been reported.[45] Finally, despite standardization, systematic differences in behavior across laboratories have been well documented.[46]

In summary, saccharin preference and LMA behaviors were altered by a visceral noxious stimulus. AA-treated mice showed signs of depression-like behaviors after writhing resolution, as evidenced by reduced saccharin preference and locomotion for at least 6 and 4 hours, respectively. The decrease observed after AA administration in sweet taste preference was probably due to ongoing pain because it was specifically reverted by an analgesic drug such as Ibu but not by the stimulant drug caffeine. The decrease observed in novelty induced locomotion after AA injection was probably also due to ongoing pain because it was reverted by Ibu. However, the AA-depressed LMA was also reverted by the stimulant caffeine, thus suggesting that this behavioral endpoint is not robust enough to evaluate analgesic drugs and should be complemented with another pain-depressed behavior endpoint. The affective and sensory components of pain were selectively affected by Ibu because the same dose of Ibu was ineffective to block writhing behavior but effective to improve pain-depressed behaviors (saccharin preference).

Consequently, hedonic behaviors are more sensitive, and translational readouts to evaluate analgesics and changes in the expression of hedonic behavior – such as sweet taste preference described in this study – can be used as a primary outcome measure to evaluate pain in mice and may complement the more traditional procedures used to assess candidate analgesics.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Mogil JS, Crager SE. What should we be measuring in behavioral studies of chronic pain in animals? *Pain*. 2004;112(1/2):12–15.
2. Vierck CJ, Hansson PT, Yezierski RP. Clinical and pre-clinical pain assessment: are we measuring the same thing? *Pain*. 2008;135(1/2):7–10.
3. Mogil JS. Animal models of pain: progress and challenges. *Nat Rev Neurosci*. 2009;10(4):283–294.
4. Cobos EJ, Portillo-Salido, E. "Bedside-to-bench" behavioral outcomes in animal models of pain: beyond the evaluation of reflexes. *Curr Neuropharmacol*. 2013;11(6):560–591.
5. Colpaert FC, Tarayre JP, Alliaga M, Bruins Slot LA, Attal N, Koek W. Opiate self-administration as a measure of chronic nociceptive pain in arthritic rats. *Pain*. 2001;91(1/2):33–45.
6. Martin TJ, Buechler NL, Kahn W, Crews JC, Eisenach JC. Effects of laparotomy on spontaneous exploratory activity and conditioned operant responding in the rat: a model for postoperative pain. *Anesthesiology*. 2004;101(1):191–203.
7. Martin TJ, Kahn WR, Eisenach JC. Abdominal surgery decreases food-reinforced operant responding in rats: relevance of incisional pain. *Anesthesiology*. 2005;103(3):629–637.
8. Langford DJ, Bailey AL, Chanda ML, et al. Coding of facial expressions of pain in the laboratory mouse. *Nat Methods*. 2010;7(6):447–449.
9. Negus SS, Vanderah TW, Brandt MR, Bilsky EJ, Becerra L, Borsook D. Preclinical assessment of candidate analgesic drugs: recent advances and future challenges. *J Pharmacol Exp Ther*. 2006;319(2):507–514.
10. Stevenson GW, Bilsky EJ, Negus SS. Targeting pain-suppressed behaviors in preclinical assays of pain and analgesia: effects of morphine on acetic acid-suppressed feeding in C57BL/6J mice. *J Pain*. 2006;7(6):408–416.
11. Stevenson GW, Cormier J, Mercer H, et al. Targeting pain-depressed behaviors in preclinical assays of pain and analgesia: drug effects on acetic acid-depressed locomotor activity in ICR mice. *Life Sci*. 2009;85(7/8):309–315.
12. Morgan D, Carter CS, DuPree JP, Yezierski RP, Vierck CJ Jr. Evaluation of prescription opioids using operant-based pain measures in rats. *Exp Clin Psychopharmacol*. 2008;16(5):367–375.
13. Kroenke K, Wu J, Bair MJ, Krebs EE, Damush TM, Tu W. Reciprocal relationship between pain and depression: a 12-month longitudinal analysis in primary care. *J Pain*. 2011;12(9):964–973.
14. Campbell LC, Clauw DJ, Keefe FJ. Persistent pain and depression: a biopsychosocial perspective. *Biol Psychiatry*. 2003;54(3):399–409.
15. Fernandez E, Turk DC. Sensory and affective components of pain: separation and synthesis. *Psychol Bull*. 1992;112(2):205–217.
16. Pfaffmann C. The pleasures of sensation. *Psychol Rev*. 1960;67:253–268.
17. Cytawa J, Trojniar W. Hedonesthesia: the nervous process determining motivated ingestive behavior. *Acta Neurobiol Exp (Wars)*. 1978;38(2/3):139–151.
18. Valenstein ES, Kakolewski JW, Cox VC. Sex differences in taste preference for glucose and saccharin solutions. *Science*. 1967;156(3777):942–943.
19. Willner P, Muscat R, Papp M. Chronic mild stress-induced anhedonia: a realistic animal model of depression. *Neurosci Biobehav Rev*. 1992;16(4):525–534.
20. Harkin A, Houlihan DD, Kelly JP. Reduction in preference for saccharin by repeated unpredictable stress in mice and its prevention by imipramine. *J Psychopharmacol*. 2002;16(2):115–123.
21. Lazarus H, Neumann C. Assessing undertreatment of pain: the patients' perspectives. *J Pharm Care Pain Symptom Control*. 2001;9(4):5–34.
22. Zhang L, Zhang X, Westlund KN. Restoration of spontaneous exploratory behaviors with an intrathecal NMDA receptor antagonist or a PKC inhibitor in rats with acute pancreatitis. *Pharmacol Biochem Behav*. 2004;77(1):145–153.
23. Matson DJ, Broom DC, Cortright DN. Locomotor activity in a novel environment as a test of inflammatory pain in rats. *Methods Mol Biol*. 2010;617:67–78.
24. Volkers AC, Tulen JH, van den Broek WW, Bruijn JA, Passchier J, Pepplinkhuizen L. Motor activity and autonomic cardiac functioning in major depressive disorder. *J Affect Disord*. 2003;76(1–3):23–30.
25. Bennabi D, Vandel P, Papaxanthis C, Pozzo T, Haffen E. Psychomotor retardation in depression: a systematic review of diagnostic, pathophysiologic, and therapeutic implications. *Biomed Res Int*. 2013;2013:158746.
26. Le Bars D, Gozariu M, Cadden SW. Animal models of nociception. *Pharmacol Rev*. 2001;53(4):597–652.
27. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain*. 1983;16(2):109–110.
28. Andrews N, Legg E, Lisak D, et al. Spontaneous burrowing behaviour in the rat is reduced by peripheral nerve injury or inflammation associated pain. *Eur J Pain*. 2012;16(4):485–495.
29. Negus SS, Neddenriep B, Altarifi AA, Carroll FI, Leitl MD, Miller LL. Effects of ketoprofen, morphine, and kappa opioids on pain-related depression of nesting in mice. *Pain*. 2015;156(6):1153–1160.
30. Do Carmo GP, Stevenson GW, Carlezon WA, Negus SS. Effects of pain- and analgesia-related manipulations on intracranial self-stimulation in rats: further studies on pain-depressed behavior. *Pain*. 2009;144(1/2):170–177.

31. Stevenson GW, Mercer H, Cormier J, et al. Monosodium iodoacetate-induced osteoarthritis produces pain-depressed wheel running in rats: implications for preclinical behavioral assessment of chronic pain. *Pharmacol Biochem Behav*. 2011;98(1):35–42.
32. Cobos EJ, Ghasemlou N, Araldi D, Segal D, Duong K, Woolf CJ. Woolf inflammation-induced decrease in voluntary locomotion in mice: an operant test for evaluating inflammatory pain and analgesia. *Pain*. 2012;153(4):876–884.
33. Tanimoto S, Nakagawa T, Yamauchi Y, Minami M, Satoh M. Differential contributions of the basolateral and central nuclei of the amygdala in the negative affective component of chemical somatic and visceral pains in rats. *Eur J Neurosci*. 2003;18(8):2343–2350.
34. Menétrey D, Gannon A, Levine JD, Basbaum AI. Expression of c-fos protein in interneurons and projection neurons of the rat spinal cord in response to noxious somatic, articular, and visceral stimulation. *J Comp Neurol*. 1989;285(2):177–195.
35. Burstein AH, Reiss WG, Kantor E, Anderson GD. Cytochrome P450 3A4 activity in premenopausal and postmenopausal women, based on 6-beta-hydroxycortisol: cortisol ratios. *Pharmacotherapy*. 1998;18(6):1271–1276.
36. Bandler R, Shipley MT. Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? *Trends Neurosci*. 1994;17(9):379–389.
37. Clement CI, Keay KA, Podzebenko K, Gordon BD, Bandler R. Spinal sources of noxious visceral and noxious deep somatic afferent drive onto the ventrolateral periaqueductal gray of the rat. *J Comp Neurol*. 2000;425(3):323–344.
38. Nakagawa T, Katsuya A, Tanimoto S, et al. Differential patterns of c-fos mRNA expression in the amygdaloid nuclei induced by chemical somatic and visceral noxious stimuli in rats. *Neurosci Lett*. 2003;344(3):197–200.
39. Jensen TS. Opioids in the brain: supraspinal mechanisms in pain control. *Acta Anaesthesiol Scand*. 1997;41(1 Pt 2):123–132.
40. Hummel M, Lu P, Cummons TA, Whiteside GT. The persistence of a long-term negative affective state following the induction of either acute or chronic pain. *Pain*. 2008;140(3):436–445.
41. Rutten K, De Vry J, Robens A, Tzschentke TM, van der Kam EL. Dissociation of rewarding, anti-aversive and anti-nociceptive effects of different classes of anti-nociceptives in the rat. *Eur J Pain*. 2011;15(3):299–305.
42. Uzbay T, Kose A, Kayir H, Ulusoy G, Celik T. Sex-related effects of agmatine on caffeine-induced locomotor activity in Swiss Webster mice. *Eur J Pharmacol*. 2010;630(1–3):69–73.
43. Kallman WM, Isaac W. The effects of age and illumination on the dose-response curves for three stimulants. *Psychopharmacologia*. 1975;40(4):313–318.
44. Garcia AM, Cardenas FP, Morato S. The effects of pentylenetetrazol, chlordiazepoxide and caffeine in rats tested in the elevated plus-maze depend on the experimental illumination. *Behav Brain Res*. 2011;217(1):171–177.
45. Gschossmann JM, Buenger L, Adam B, et al. Diurnal variation of abdominal motor responses to colorectal distension and plasma cortisol levels in rats. *Neurogastroenterol Motil*. 2001;13(6):585–589.
46. Crabbe JC, Wahlsten D, Dudek BC. Genetics of mouse behavior: interactions with laboratory environment. *Science*. 1999;284(5420):1670–1672.

2.1.1. Annex III

Peripheral nerve injury failed to significantly alter the saccharine preference behaviour in mice

Our previous studies using the AA model in mice have shown that the behavioural manifestations of the sensory-discriminative aspect of acute pain were of short duration (less than 1 hour). However, the affective consequences were substantially longer as evidenced by a general "behavioural depression" (a strongly decreased expression of both saccharine preference and LMA for at least 6 hours) (see Article 2). We have now used the same saccharine preference test as a readout to unmask the possible affective consequences of chronic neuropathic pain in mice.

To this end, the effect of 11-week exposure to sciatic nerve injury on the preference for the 0.1% saccharin solution presented over 6-h periods of exposure were analysed. Female wild-type CD-1 mice (Charles River, Barcelona, Spain) weighing 25–30 g underwent partial sciatic nerve ligation to induce neuropathic pain, and sham-operated mice were used as controls as previously described in Article 1. The assessment of the anhedonic-like behaviour using the saccharin preference test was performed weekly before and after injury and for up to 11 weeks thereafter, according to the methodology explained in Article 2. Data were analysed with two-way repeated measures ANOVA with genotype and time (weeks) as main factors. A value of $P < 0.05$ was considered to be statistically significant. Statistical analyses were carried out with the GraphPad Prism 5.00 program (GraphPad Software, San Diego, CA, USA).

The results showed that sciatic nerve injury failed to significantly alter the saccharine preference behaviour (Fig. 1). Statistical analyses showed no significant differences between both experimental groups at any time point. This result suggests that this behavioural endpoint/design was not adequate to detect the affective consequences of neuropathic pain in PSNL mice.

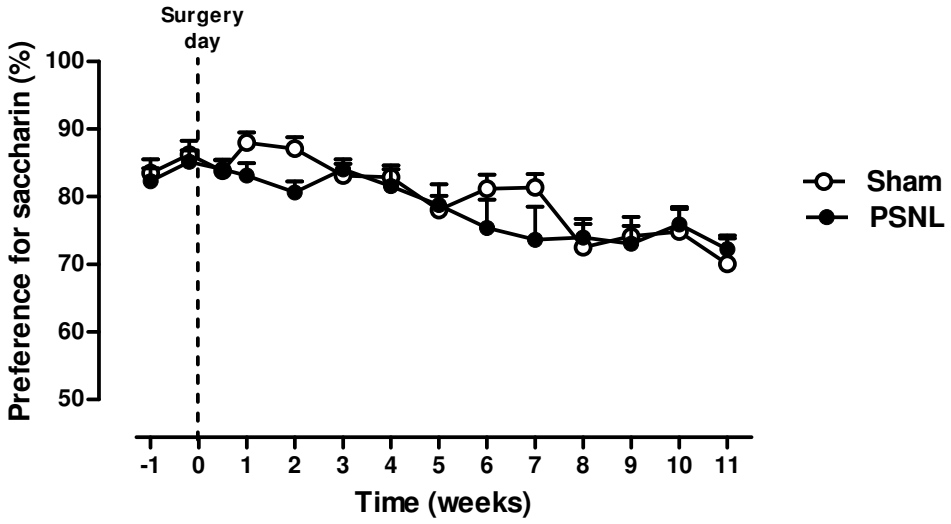


Fig. 1. Effect of 11-week exposure to PSNL on the preference for the 0.1% saccharin solution presented over 6-h periods of exposure. The affective-motivational component of chronic neuropathic pain was not evidenced by this sweet preference test. Preference for saccharin solutions was similar in PSNL and sham-operated mice. Values are mean ± SEM. ($P > 0.05$, two-way RM ANOVA).

A previous report found that PSNL reduced sucrose preference compared to sham-operated mice from 99% to 96% (Bura *et al*., 2013). In our study we found a similar reduction in saccharin preference two weeks after the nerve injury (from 87% to 81%) but this decrease was not maintained in subsequent weeks. Animal studies investigating the relationships between neuropathic pain and mood disorders are limited

and contradictory (Cobos and Portillo-Salido, 2013). Some research groups have observed depression-related phenotypes in rodents with chronic pain using the sucrose preference test, (Wang *et al.*, 2011; Kim *et al.*, 2012; Goffer *et al.*, 2013) or the exploratory activity test (Gonçalves *et al.*, 2008; Hu *et al.*, 2009). Other studies, however, have failed to show any association between neuropathic pain and depression-related behaviours using the sweet preference test (Kontinen *et al.*, 1999; Urban *et al.*, 2011; Schwartz *et al.*, 2014).

The sweet preference test is normally used to evaluate anhedonia, which has traditionally been viewed as a deficit in the experience of pleasure. A reduction in sucrose and saccharine consumption following experimental manipulations are considered to be related to consummatory deficits. However, recent evidence suggests that anhedonia should be refined attending to the distinction between deficits in the hedonic response to rewards ("consummatory anhedonia") and a diminished motivation to pursue them ("motivational anhedonia") (Treadway and Zald, 2011). Therefore, the negative results found in our study could be due to the lack of analysis of "motivational anhedonia", which cannot be obtained from the saccharine preference test used in the present study.

2.2. ARTICLE 3

Pharmacological characterization of pain-related depression of the reward seeking behaviour in mice

Beatriz de la Puente, Daniel Zamanillo, Luz Romero, José Miguel Vela, Manuel Merlos, Enrique Portillo-Salido

Department of Pharmacology, Drug Discovery and Preclinical Development, ESTEVE

Manuscript to be submitted

Pharmacological characterization of pain-related depression of the reward seeking behaviour in mice

Abstract

Disruption and restoration of reward seeking behaviour (RSB) by pain and analgesics respectively can help to model the affective dimension of pain. The expression of RSB comprises two main phases triggered by an incentive: the appetitive phase and the consummatory phase. In the present study we evaluated the effects of intraperitoneal acetic acid (AA) administration and its pharmacological modulation on a RSB task where both the appetitive and consummatory components of motivated behaviours were assessed. Female CD-1 mice were offered a highly palatable food (white chocolate). Pain was induced by i.p. administration of AA (0.6-1.2%, 10 mL/kg). The latency and the number of approaches to eat were evaluated as appetitive behaviours on the RSB. The duration of eating and the amount consumed were assessed as consummatory behaviours**.** The effects of i.p. administration of ibuprofen, diclofenac, morphine, duloxetine and caffeine on AA-induced writhing and the changes in the four parameters of RSB were compared. AA produced a concentration- and time-dependent increase in the latency to eat and a decrease in the other three parameters. Ibuprofen and diclofenac inhibited AA-induced writhing (ED_{50}=123 and 60 mg/kg, respectively). These, however, were much more potent in reversing AA-induced changes in RSB: latency to eat (ED_{50}=1 and 0.004 mg/kg) and amount consumed (ED_{50}=7 and 0.06 mg/kg). Morphine and duloxetine inhibited the writhing response (ED_{50}=0.7 and 6 mg/kg, respectively) but failed to modify AA-induced changes in RSB. Caffeine was ineffective in both AA-induced writhing and RSB changes. Changes in the expression of RSB and can be used as a primary outcome measure to evaluate pain in mice and may complement the more traditional procedures used to assess candidate analgesics..

Introduction

The presence of pain has many negative consequences, including changes in the affective state and the activities of daily living (Gatchel *et al.*, 2007). Current analgesics are not fully effective and the development of new ones is probably impeded by the possible lack of translation from the preclinical to the clinical setting (Cobos and Portillo-Salido, 2013). The majority of preclinical pain research uses reflex-based procedures (e.g., tail flick, licking, and guarding) induced by aversive stimulation (through the application of particular mechanical, thermal, electrical, and chemical stimuli) to identify analgesics. Clinical pain research, however, uses scales and verbal reports where not only pain but also physical and emotional functioning are evaluated (Turk *et al.*, 2003). The analgesic-induced recovery of normal physical and emotional functioning seems to be more important than the analgesic-induced interruption of the reflex behaviour induced by injuries. The reflex response observed usually involves tissue injury and sometimes plays a protective role (withdrawal reflex to protect the injured area). Hypersensitivity is also expressed after a short physical stimulation and is easily avoided by the body, at least in the experimental setting. Furthermore, hypersensitivity does not seem to be the most significant clinical problem in humans with chronic pain (Backonja and Stacey, 2004; Maier *et al.*, 2010, Bennett, 2012; Mogil, 2012). Instead, ongoing pain —i.e. sensing pain in the absence of external stimuli and likely caused by ectopic impulse generation within the nociceptive pathways— seems to be present at a much higher frequency and intensity than the hypersensitivity-related symptoms commonly studied in the laboratory, such as increased pain caused by touch or heat (Backonja and Stacey, 2004).

While the study of ongoing pain in preclinical models is complex, some innovative approaches different from those based on behavioural hypersensitivity measures have been tested (Cobos and Portillo-Salido, 2013). One approach is to investigate positively motivated behaviours in animals with experimental pain (Low and Fitzgerald, 2012; Farmer *et al.*, 2014; Schwartz *et al.*, 2014). These motivation-related approaches make sense in view of the interruptive consequences of both acute and chronic pain on ongoing behaviour (Eccleston and Crombez, 1999; Van Damme *et al.*, 2010). From this perspective, while pain is fundamentally threatening, interruptive and aversive, it also interferes in the activities of daily living and its persistence could cause the associated stresses of depression (Van Damme *et al.*, 2008). The study of motivational deficits is interesting because these underline hopelessness and depression, where a retarded initiation of voluntary responses (motivational symptom) has been delimited (Abramson *et al.*, 1989). Consequently, changes in motivated behaviours can help model depressive symptoms commonly associated with pain in both human and non-human subjects (Li, 2015; Chopra and Arora, 2014). In general, motivated behaviour is thought to comprise two main phases triggered by an incentive (any stimulus that activates an approach behaviour): the appetitive phase and the consummatory phase. Appetitive behaviours (also known as anticipatory, appetitive, preparatory, approach, or seeking behaviour) are flexible, non-stereotyped responses that bring the experimental animal subject in physical proximity with the goal object (e.g. the reward or reinforcer), whereas consummatory behaviours are the final reflexive, stereotyped responses after decisions and efforts have been made to reach the motivational stimulus or goal object (Keen-Rhinehart *et al.*, 2013). These different aspects of motivated behaviours are experimentally dissociable (Barbano and Cador, 2006; Huang *et al.*, 2010).

The purpose of this study was to evaluate the effects of an experimental prototype noxious stimulus (intraperitoneal acetic acid, AA), several analgesics (morphine, duloxetine, ibuprofen and diclofenac) and a non analgesic (caffeine) on a reward seeking behaviour (RSB) task where both appetitive and consummatory behaviours to a strongly rewarding stimulus such as white chocolate were assessed**.** The AA-induced visceral pain model has been previously used to depress innate behaviour (i.e. feeding, locomotion, wheel running, nesting, saccharine preference) (Stevenson *et al.*, 2006; Stevenson *et al.*, 2009; Miller *et al.*, 2011; Negus *et al.*, 2015; de la Puente *et al.*, 2015) and also to refine preclinical animal pain methodologies in the laboratory mouse by evaluating its effects on complex behaviours such as empathy, social behaviour, and pain facial expressions (Langford *et al.*, 2006; Langford *et al.*, 2010). In order to obtain a more detailed profile of AA-induced alterations on RSB, a total of four dependent measures were evaluated: latency to eat and number of approaches to eat as appetitive motivation, and duration of eating and amount of chocolate consumed as consummatory dimension. The influence of neophobia, pretreatment time and dose of AA on RSB were analysed. Furthermore, in order to compare drug sensitivity between nonreflexive and reflexive outcomes, dose-response curves for known analgesics with different mechanisms of action were compared for both abdominal contractions (writhing, reflexive-like behaviour) and RSB (nonreflexive-like behaviour). We hypothesized that AA would change the appetitive and consummatory responses, and that analgesics would prevent AA-induced changes in RSB.

Material and methods

Animals

Females CD1 mice aged 4-5 weeks were purchased from Charles River (France) and housed in groups of five in transparent polypropylene cages with suitable beddings (wood shavings) and food and water *ad libitum*. Animals were left undisturbed for two weeks to acclimatize to laboratory conditions in a testing room with light and temperature maintained at 20-22 ºC with a 12 h light/12 h dark cycle (lights on at 06:00 and off at 18:00). All trials were performed between 8.00 am and 13.00 pm. All animal research was conducted in accordance with protocols approved by the local Committee of Animal Use and Care of our Institution, with the Care and Use of Laboratory Animals Guidelines of the European Community (European Directive 2010/63/EU), and with the International Association for the Study of Pain Guidelines on ethical standards for investigation in animals (Zimmermann, 1983).

Drugs and compounds

Ibuprofen and duloxetine were supplied by Laboratorios Esteve. Caffeine and diclofenac were purchased from Sigma Chemical Co. (Barcelona, Spain). Morphine hydrochloride was obtained from the Spanish Drug Agency (Agencia Española de Medicamentos y Productos Sanitarios, Area Estupefacientes, Madrid, Spain). All compounds were dissolved in 0.5% HPMC and administered intraperitoneally at a volume of 10 mL/kg. Drugs were administered 30 min before the test. AA solutions were prepared by adding 0.06, 0.09 or 0.12 mL of glacial acetic acid to double deionized water at a final volume of 10 mL. The injection volume of AA was 10 mL/kg, i.p.

Assay of reward seeking behaviour (RSB) based on the approach and consumption of white chocolate.

The basic test procedure was adapted from Merali *et al.* (2003), who showed that offering highly palatable, familiar snacks in the home cage resulted in a rapid approach and consumption of food. In this study, among the different food sources offered to nondeprived mice, white chocolate (Milkybar®, Nestlé, S.A.) was chosen based on preliminary experiments indicating a stronger consumption as compared to other rewarding stimuli, such as dark chocolate (Supreme Mini-Treats™, Bio-Serv Inc). Each mouse was placed in one corner of the home cage (floor size 25 x 50 cm) on the opposite side from a Petri dish (4-cm-diameter, 1-cm-deep) containing a piece of white chocolate (~2g). The animal was tested for 10 min and, in order to obtain a more detailed profile of pain-induced alterations of the feeding behaviour, a total of four dependent measures were used: latency to eat, duration of eating behaviour, number of approaches to eat, and amount of chocolate consumed. Latency to eat was defined as the time between the placement of the animal in the home cage containing the chocolate and the beginning of eating. The number of times and the time taken to eat chocolate were chosen because they have been shown to be affected by several experimental manipulations (Whishaw *et al.*, 1992; Krebs *et al.*, 1997). The amount consumed was assessed by weighing the piece of chocolate before and after testing.

We first studied the stability of RSB and the influence of neophobia (a reduction in consumption of a novel tastant because of fear to the unknown post-ingestive consequences; Barnett, 1958). After the acclimatization period to the experimental room, mice were divided into two groups: Group 1, mice habituated to white chocolate for three consecutive days (non-neophobic), and Group 2, mice not habituated

(neophobic). Then, the RSB of mice habituated or not habituated to white chocolate was tested, as described above, for three consecutive days. From these initial experiments we observed that, under non-neophobic conditions, the feeding behaviour was maintained high and stable in all parameters as of day two. Based on this, a protocol was selected where, testing was initiated at day 2 after the baseline determination (day 1).

Second, the concentration (0-1.2 %) and the pretreatment time (0-24h) of AA were systematically manipulated in order to identify conditions under which AA reliably depressed the reward-seeking behaviour (RSB). RSB was evaluated in different groups of mice at 15, 60, 120 and 180 min. A group of mice tested at 120 min was again tested at 24h. On the basis of these studies, an AA concentration of 0.9% and 120 min (tonic "affective-related phase" where the writhing behaviour was no longer apparent) were used for the remainder of the study.

Third, the effects of a range of doses of intraperitoneal (i.p.) morphine (0.03-5 mg/kg), ibuprofen (0.3-320 mg/kg), diclofenac (0.001-160 mg/kg), duloxetine (1.25-40 mg/kg) and caffeine (5-40 mg/kg) were examined on AA-depressed and non-depressed RSB (vehicle treated condition) in the "affective-related phase".

Assay of AA-Induced Writhing

In the time course study, mice were injected i.p. with 10 mL/kg of AA (0.9 %). Each mouse was then placed in an individual clear plastic observation chamber and the total number of writhes was counted for 1 h after administration. Based on the results obtained, a 5-min pretreatment time was used for the remainder of the writhing experiment. To evaluate the effects of drugs, separate groups of mice were administered

vehicle or a dose of the corresponding drug by i.p. route followed by an intraperitoneal injection of 0.9% AA 30 min later. The number of writhes was counted from 5 to 15 min after AA administration (35-45 min after vehicle or drug). For scoring purposes, a "writhe" was defined as a contraction of the abdominal muscles accompanied by elongation of the body and extension of the hindlimbs. Data are expressed as the mean number of writhes during the 10-min observation period.

Statistical analysis

Data are expressed as mean ± SEM. For the purpose of pretreatment time of AA, data were analysed with a two-way ANOVA; white chocolate habituation studies were analysed with a two-way repeated measures ANOVA. Writhing data were analysed using a one-way repeated measures ANOVA. AA concentration data and the drug treatment study were analysed with one-way ANOVA. Bonferroni's *post hoc* analyses were performed in all cases to assess specific group comparisons. $P < 0.05$ was considered to be statistically significant.

A dose–response curve was plotted using nonlinear regression analysis, and ED_{50} (dose of drug producing half of its maximal response) and E_{max} (maximum effect) values were obtained. SEs were calculated on the basis of the best-fit values ± SEs of regression with GraphPad Prism software (version 5.0; GraphPad Software Inc., La Jolla, California, USA)

To calculate the ED_{50}, data were converted to the percentage of analgesia based on the following formula for the writhing test: [mean no. of writhes (control group) – (mean no. of writhes (test group)] / [mean no. of writhes (control group)] x 100 and the

following calculation for behavioural parameters: ([mean value test group – mean value pain-AA group] / [mean value control group – mean value pain-AA group]) × 100%.

Results

Influence of neophobia on RSB.

In trial 1 the latency to eat of mice exposed to chocolate for the first time (non-habituated) was much higher that the latency to eat of mice previously habituated to chocolate (Fig. 1A). In trials 2 and 3 a progressive decrease in the latency to eat was observed in non-habituated mice, without reaching the latency of habituated mice. A two-way RM ANOVA test showed a significant effect of habituation condition [$F(1,51) = 202.6$; $P < 0.001$], trial number [$F(2,51) = 31.0$; $P < 0.001$] and a significant effect between factors [$F(2,51) = 17.1$; $P < 0.001$]. Interestingly, no differences in the number of approaches to consume were observed between habituated and non-habituated mice [$F(1,51) = 3.1$; $P > 0.05$] but there was a significant effect in the number of trials [$F(2,51) = 4.4$, $P < 0.05$] or interaction between approaches to consume and the number of trials [$F(2,51) = 11.0$, $P < 0.001$] (Fig. 1B). The amount consumed was significantly higher in mice previously exposed to white chocolate as compared to the non-habituated group (Fig. 1C). In trial 1 the amount consumed by non-habituated mice was almost inexistent. In trials 2 and 3 an increased consumption was observed in these mice, without reaching the consumption of habituated mice. There was a significant effect of habituation [$F(1,51) = 362.6$; $P < 0.001$] and number of trials [$F(2,51) = 12.3$; $P < 0.001$], and the interaction between factors was also significant [$F(2,51) = 6.3$; $P < 0.01$].

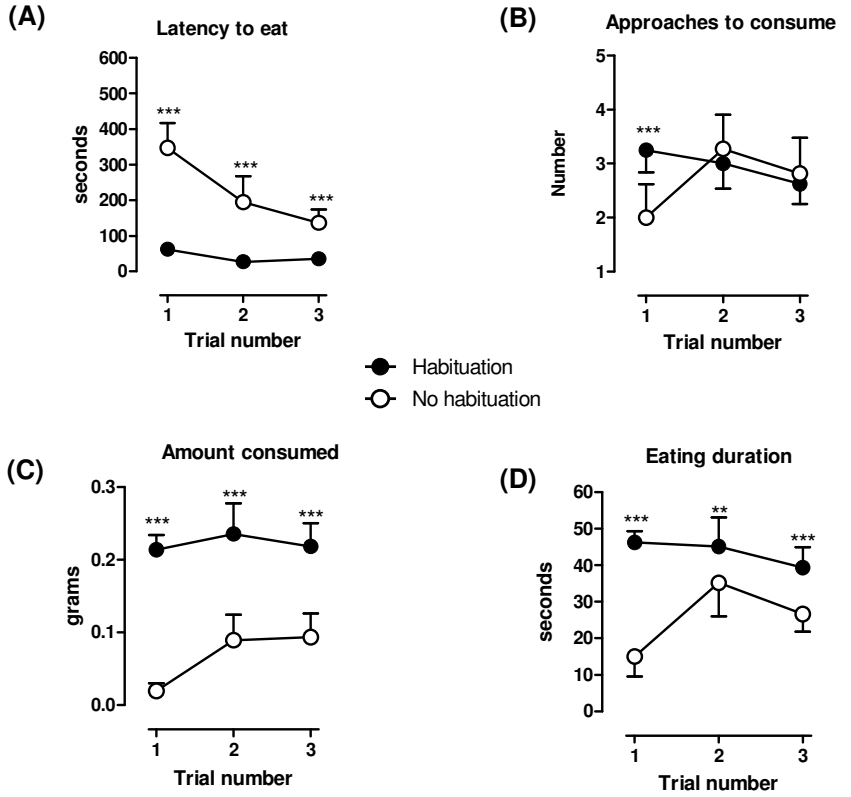
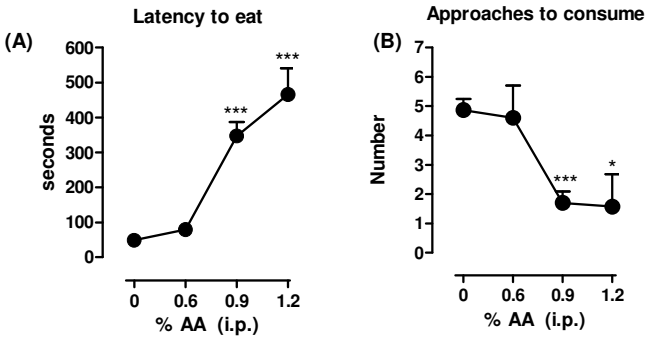


Fig. 1. Influence of neophobia on RSB. A neophobic response to white chocolate was detected by three of the four dependent measures. Habituated female mice showed a much higher latency to eat (A) as compared to non-habituated mice. Amount consumed (C) and eating duration (D) were higher in habituated than in non-habituated mice. Approaches to consume were unaffected by habituation although statistical *post hoc* analysis displayed significant differences in the first trial between non-habituated and habituated mice. **$P < 0.01$, ***$P < 0.001$, two-way RM ANOVA, Bonferroni's *post hoc* test.

Finally, both groups (habituated and non-habituated to white chocolate) showed a significant effect of habituation on the eating duration [$F(1,51) = 108.9$; $P < 0.001$], trial number [$F(2,51) = 11.1$; $P < 0.001$] , or interaction between factors [$F(2,51) = 15.1$; $P < 0.001$] (Fig. 1 D). A neophobic response to white chocolate was detected by three of the four dependent measures. Therefore, further studies were conducted using mice habituated to chocolate.

Effects of concentration on AA-depressed RSB.

The RSB after i.p. injection of a range of AA concentrations in mice is shown in Fig. 2. AA administration produced changes in a concentration-dependent manner in the four endpoints analysed. Under control conditions (mice injected with HPMC and saline; the solvents of drugs and AA, respectively), the latency to eat the piece of white chocolate was 48.9 ± 9.1 seconds. 0.6% AA failed to produce significant changes (79 ± 13.8; $P < 0.05$) on the latency to eat the palatable food, but 0.9% and 1.2% AA induced a strong increase (346.9 ± 39.6; $P < 0.001$; 465.3 ± 75.1 seconds; $P < 0.001$, respectively, Fig. 2A). Similarly, the number of approaches to consume was not affected by 0.6% AA (from 4.9 ± 0.4 to 4.6 ± 1.1; $P > 0.05$) but was strongly decreased by both 0.9% (1.7 ± 0.4; $P < 0.001$) and 1.2% (1.6 ± 1.1; $P < 0.05$, Fig. 2B) doses of AA. However, the amount of white chocolate consumed following 0.6% AA was significantly decreased (from 0.19 ± 0.03 to 0.10 ± 0.02 g; $P < 0.05$) and almost suppressed by 0.9% and 1.2% AA as compared to the control values of consumption (0.03 ± 0.009 g and 0.02 ± 0.02 g consumed, respectively; $P < 0.001$, Fig. 1C). Finally, doses of 0.9% and 1.2% AA, but not 0.6% AA, robustly decreased eating duration (from 54.1 ± 9.3 to 9.1 ± 2.5 and 4.7 ± 3.6 seconds, respectively; $P < 0.001$ and $P < 0.01$ respectly, Fig. 2D). On account of these results, further experiments were conducted using 0.9% AA.



(E)

	Parameter measured	ED_{50} (% AA)
Appetitive behaviour	Latency to eat (s)	0.9 ± 0.02
	Approaches to consume (n)	0.9 ± 0.06
Consummatory behaviour	Amount consumed (g)	0.7 ± 0.05
	Eating duration (s)	0.7 ± 0.08

Fig. 2. Effects of concentration on AA-depressed RSB. The administration of AA produced changes in a concentration-dependent manner in the four endpoints analysed. Doses of 0.9% and 1.2% AA produced significant changes in latency to eat (A), approaches to consume (B), amount consumed (C), and eating duration (D). (E) Values of ED_{50} for the behavioural parameters evaluated. Appetitive behaviours (latency to eat and approaches to consume) showed similar ED_{50} values (ED_{50} = 0.9%) and were higher than those obtained in consummatory behaviours (ED_{50} = 0.7%), thus suggesting that consummatory behaviours are more sensitive to changes in AA-induced pain than appetitive behaviours. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, one-way ANOVA, Bonferroni's *post hoc* test.

Time-course of AA-induced writhing behaviour and AA-depressed RSB.

The time course of writhing behaviour and RSB following the injection of AA is shown in Fig. 3. As expected, intraperitoneal administration of 0.9% AA robustly induced the appearance of abdominal constrictions (writhing) in mice (Fig. 3A, right axis). The number of writhes was maximal 5 to 15 minutes after AA (*P* < 0.001). Then, a

progressive decrease in this behaviour was observed, and the effects of AA were not significant after 25 min ($P > 0.05$) and no longer apparent after 60 min.

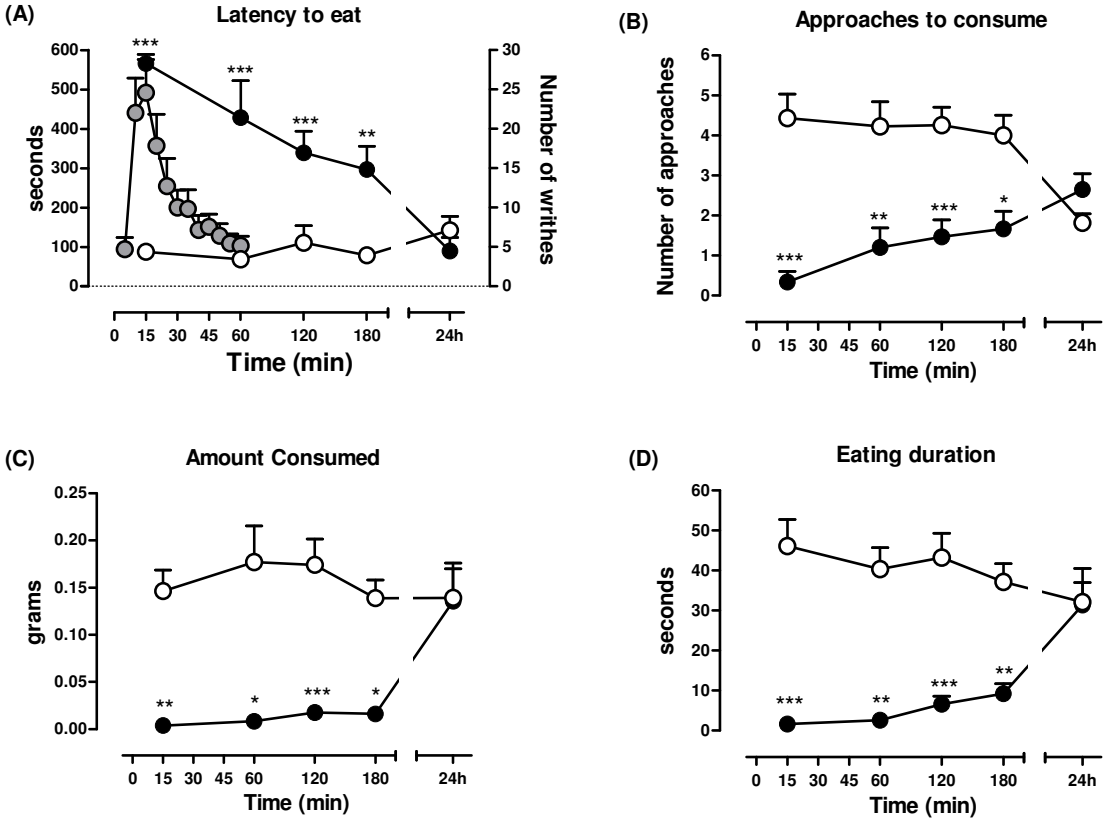


Fig. 3. Time course of AA-induced writhing behaviour and AA-depressed RSB. AA-treated mice showed RSB depression for at least 3 h. (A) Latency to eat, (B) approaches to consume, (C) amount consumed, (D) eating duration. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, two-way ANOVA, Bonferroni's *post hoc* test.

AA administration produced profound changes for at least 180 min in the four RSB-related endpoints analysed. The left axis in Fig. 3A shows the influence of the pretreatment time of AA or its vehicle on the latency to eat white chocolate. A two-way ANOVA revealed significant effects of AA [$F(1,165) = 1387.2$; $P < 0.001$] over time

after AA injection [F(4,165) = 122.3; $P < 0.001$] and interaction between factors [F(4,165) = 178.0; $P < 0.001$] as compared to control mice treated with the AA vehicle . 15 min after AA administration, the latency to eat was near the cut-off point (575.4 ± 17 seconds) and the latency-to-eat values in the AA-treated mice were decreased progressively as of the first hour (427.8 ± 94.7 seconds; $P < 0.001$) and restored to control values 24 h later ($P > 0.05$).

The number of approaches to consume of AA-treated mice is shown in Fig. 3B. There were virtually no approaches 15 min after AA-induced pain (0.3 ± 0.2 times). As of the first hour a slight increase in the number of approaches (1.2 ± 0.5 times; $P < 0.01$) was observed, and the number of approaches was similar in both AA-treated and control mice after 24 h ($P > 0.05$). While two-way ANOVA failed to indicate significant effects over time [F(4,169) = 0.6; $P > 0,05$], there were significant effects of AA [F(1,139) = 46.4; $P < 0.001$] and interaction between factors [F(4,139) = 6.2, $P < 0.001$].

As shown in Fig. 3C, the amount consumed was highly depressed by AA administration. Two-way ANOVA indicated significant effects of AA [F(1,139) = 37.5; $P < 0.001$]. However, no differences in the amount consumed over time [F(4,139) = 1.3; $P > 0.05$] and no interaction between the amount consumed and the time after AA injection [F(4,139) = 2.4; $P > 0.05$] were found. Virtually there was no palatable food intake between 15 (0.004 ± 0.003 g) and 180 (0.016 ± 0.005 g) min. The amount consumed was restored to control values by 24 hours after AA-induced pain ($P > 0.05$). Similarly, there were significant effects of AA on eating duration [F(1,139) = 62.7; $P < 0.001$] (Fig. 3D), but not over time [F(4,139) = 0.9; $P > 0.05$]. Control mice treated with the AA vehicle showed a similar eating duration at all time points analysed

($P > 0.05$ compared with 15 min). The eating duration was strongly reduced by AA at 15, 60, 120 and 180 min as compared to control values. The analysis of variance indicated that duration varied significantly between both groups [$F(1,139) = 62.7$; $P < 0.001$]. The eating duration was restored to control values by 24 hours after AA-induced pain AA ($P > 0.05$). On account of these results, subsequent RSB experiments were conducted using a 120-min pretreatment with 0.9% AA.

Effects of morphine, ibuprofen, diclofenac, duloxetine and caffeine on AA-induced changes in the reward seeking behaviour.

The effects of morphine (0.08-5 mg/kg), ibuprofen (0.3-40 mg/kg), diclofenac (0.001 - 5 mg/kg), duloxetine (1.25-5 mg/kg) and caffeine (5-40 mg/kg) on the changes in RSB observed in mice with visceral pain induced by AA are compared in Fig. 4. Treatment with the nonsteroidal anti-inflammatory drugs (NSAIDs) diclofenac and ibuprofen fully restored to control values, in a dose-dependent manner, the latency to eat (Fig. 4A), the number of approaches to consume (Fig. 4B), the amount consumed (Fig. 4C), and the eating duration (Fig. 4D) of AA-treated mice. The statistical analysis showed significant effects at the doses of 2.5-40 mg/kg and 0.08-5 mg/kg for ibuprofen and diclofenac, respectively. However, morphine, duloxetine and caffeine were ineffective at any dose tested in mice pretreated with AA in any parameter assessed in RSB.

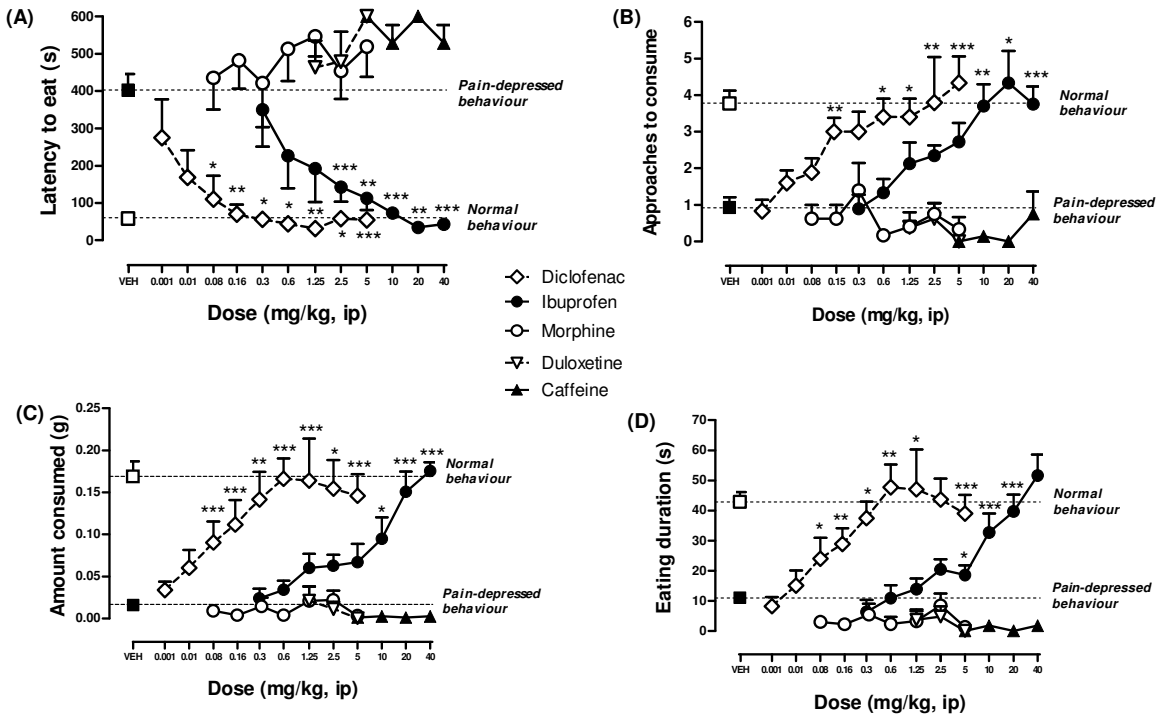


Fig. 4. Dose response effects of diclofenac, ibuprofen, morphine, duloxetine and caffeine on the changes in RSB observed in mice with AA-induced visceral pain. NSAIDs drugs ibuprofen and diclofenac showed significant effects to reverse depressive-like behaviours. (A) Latency to eat, (B) approaches to consume, (C) amount consumed and (D) eating duration. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one way-ANOVA, Bonferroni's *post hoc* test.

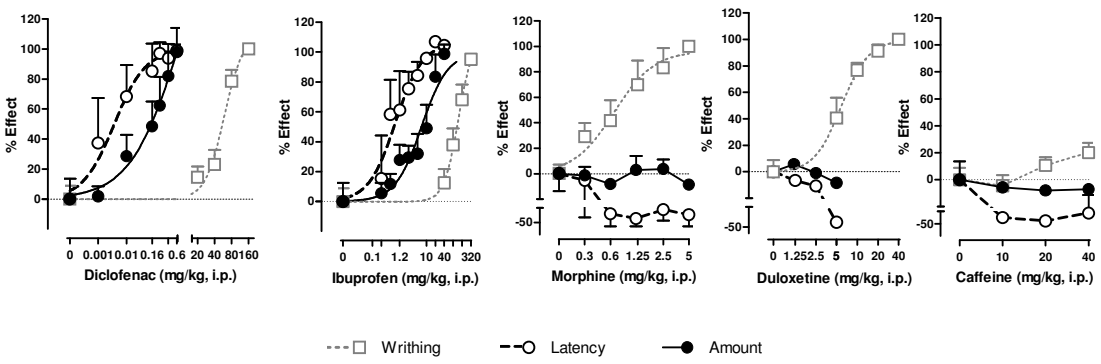
To explore whether any of the drugs tested had effects on RSB likely to interfere in the interpretation of results, the doses of each drug tested in AA-treated mice were administered to non AA-treated mice. Table 1 shows latency to eat, duration of eating behaviour, number of approaches to eat and amount of chocolate consumed, expressed as means ± SEM for each drug and tested dose.

	Dose (mg/kg)	Latency to eat (s)	Approaches to consume	Amount consumed (g)	Eating duration (s)
Diclofenac	**0**	75.9 ± 28.1	4.8 ± 0.9	0.1965 ± 0.04	49.4 ± 8.2
	2.5	84.3 ± 27.7	2.6 ± 0.7	0.1950 ± 0.04	33.1 ± 6.7
	5	73.4 ± 22.3	3.8 ± 0.7	0.1716 ± 0.03	37.0 ± 8.3
Ibuprofen	**0**	70.3 ± 13.7	4.3 ± 0.3	0.1833 ± 0.02	48.2 ± 5.2
	2.5	66.9 ± 20.1	4.6 ± 0.6	0.2043 ± 0.05	47.2 ± 11.4
	5	75.0 ± 19.4	4.1 ± 0.6	0.1690 ± 0.04	42.6 ± 8.0
	10	65.5 ± 14.1	4.6 ± 0.7	0.2010 ± 0.03	45.8 ± 5.7
	20	76.0 ± 17.5	4.3 ± 0.5	0.1906 ± 0.05	47.3 ± 7.9
	40	25.8 ± 3.2	4.1 ± 0.8	0.3387 ± 0.06	75.9 ± 13.8
Morphine	**0**	65.6 ± 18.5	4.5 ± 0.5	0.1688 ± 0.02	57.2 ± 10.1
	0.3	91.9 ± 33.3	3.7 ± 0.5	0.2651 ± 0.07	72.7 ± 22.3
	0.6	159.0 ± 59.0	2.8 ± 0.5	0.1226 ± 0.04	37.2 ± 11.4
	1.25	105.6 ± 51.3	3.7 ± 0.7	0.1449 ± 0.02	42.5 ± 7.4
	2.5	145.1 ± 58.8	4.6 ± 1.0	0.1667 ± 0.04	40.1 ± 11.3
	5	278.4 ± 85.2	2.6 ± 0.7	0.0942 ± 0.05	21.6 ± 10.0
Duloxetine	**0**	54.5 ± 43.5	2.5 ± 0.5	0.1680 ± 0.03	30.0 ± 1.0
	1.25	209.4 ± 88.0	3.1 ± 0.7	0.0814 ± 0.04	30.8 ± 11.1
	2.5	293.4 ± 84.8	1.8 ± 0.5	0.0973 ± 0.06	29.6 ± 16.0
	5	479.0 ± 69.6	0.6 ± 0.3	0.0135 ± 0.01	6.3 ± 3.8
Caffeine	**0**	77.8 ± 29.7	2.8 ± 0.3	0.1386 ± 0.04	30.5 ± 8.2
	10	103.5 ± 21.0	2.5 ± 0.4	0.1600 ± 0.03	32.1 ± 4.8
	20	42.0 ± 21.5	5.7 ± 1.1	0.2532 ± 0.06	62.7 ± 12.8
	40	104.0 ± 44.2	5.3 ± 0.9	0.2068 ± 0.06	82.3 ± 18.1

Table 1. Effects of morphine, ibuprofen, diclofenac, duloxetine and caffeine on RSB in non AA-treated mice. Latency to eat (s), approaches to consume (number), amount of chocolate consumed (g) and duration of eating behaviour (s). Data are expressed as means ± SEM for each drug and tested dose.

Effects of morphine, ibuprofen, diclofenac, duloxetine and caffeine on AA-induced changes in the reward seeking behaviour expressed as percent control.

(A)



(B)

	Writhing	*Consumatory*		*Appetitive*	
	Number of writhes (n)	Amount consumed (g)		Latency to consume (s)	
	ED 50	*ED 50*		*ED 50*	
Diclofenac	59,6 ± 24,8	0.06 ± 0.03	114,9	0.004 ± 0.003	107,3
Ibuprofen	122,6 ± 80,3	6.8 ± 1.3	123,1	0.9 ± 0.5	102,8
Morphine	0,7 ± 0,3	N.D.	N.D.	N.D.	N.D.
Duloxetine	5,9 ± 1,9	N.D.	N.D.	N.D.	N.D.
Caffeine	N.D.	N.D.	N.D.	N.D.	N.D.

Fig. 5. (A) Dose response curves expressed as % of effect of diclofenac, ibuprofen, morphine, duloxetine and caffeine on two parameters of RSB (latency to eat and amount consumed) and on AA-induced writhing in mice. Ibuprofen and diclofenac displayed more analgesic efficacy in depressive-like responses than in stimulation-like responses to AA-induced pain, showing in turn that they were more potent in restoring hedonic behaviour in latency to eat as compared to amount consumed. Morphine and duloxetine totally eliminated writhing but failed to show any effect on restored depressive-like behaviours. Caffeine, a stimulant non-analgesic drug, lacked activity in any of the behavioural responses evaluated. (B) ED_{50} values obtained in the pain-depressed responses (consumatory and appetitive components of hedonic response) and pain-induced responses (writhing) for all drugs evaluated. N.D.: not determined.

Here, only the latency to eat and the amount consumed are shown as measures of the appetitive-approach and consummatory dimensions of motivated behaviour,

respectively. The dose response effects of morphine, ibuprofen, diclofenac, duloxetine and caffeine on these two parameters of RSB and on AA-induced writhing are shown in Fig. 5A. ED_{50} and E_{max} are shown in Fig. 5B. Both diclofenac and ibuprofen were much more potent in inhibiting AA-induced changes in latency to eat (ED_{50} = 0.004 ± 0.003 for diclofenac and ED_{50} = 0.9 ± 0.5 for ibuprofen) and amount consumed (ED_{50} = 0.06 ± 0.03 for diclofenac and ED_{50} = 6.8 ± 1.3 for ibuprofen) than in inhibiting AA-induced writhing (ED_{50} = 59.6 ± 24.8 for diclofenac and ED_{50} = 122.6 ± 80.3 for ibuprofen). Diclofenac and ibuprofen were more potent in restoring the latency to eat (ED_{50} = 0.004 ± 0.003 for diclofenac and ED_{50} = 0.9 ± 0.5 for ibuprofen) than in restoring the amount consumed (ED_{50} = 0.06 ± 0.03 for diclofenac and ED_{50} = 6.8 ± 1.3 for ibuprofen). Finally, morphine and duloxetine inhibited AA-induced writhing in a dose-dependent manner without affecting AA-induced changes in latency to eat or amount consumed, whereas caffeine failed to shown effects on any AA-induced change.

Discussion

Recent research has demonstrated the utility of preclinical pain assays that measure pain-suppressed behaviour in addition to traditional assays that measure pain-elicited behaviour (Martin *et al.*, 2004; Stevenson *et al.*, 2006, 2009; Matson *et al.*, 2007; Pereira Do Carmo *et al.*, 2009; Negus *et al.*, 2010; Miller, 2011; de la Puente *et al.*, 2015). In line with these, the purpose of the present research was to devise a reproducible reward seeking behaviour (RSB) paradigm where not only consumption but also appetitive, goal-directed behaviours would be reliably disrupted by pain and sensitive to known analgesics. To this end, the approach (appetitive) and consummatory

behaviours triggered by a highly rewarding food such as white chocolate were characterized to analyse the effects of acute visceral pain induced by AA and the effects of morphine, ibuprofen, diclofenac, duloxetine and caffeine on AA-treated mice. The main findings of this study were: (i) a commonly used noxious stimulus (intraperitoneal AA injection) produced a long-lasting (beyond the resolution of writhing behaviour) and concentration-dependent suppression of both the appetitive and consummatory components of motivated behaviour in mice; (ii) this pain-related suppression of behaviour was dose-dependently prevented by treatment with ibuprofen and diclofenac but not by morphine, duloxetine or caffeine; and (iii) ibuprofen and diclofenac were much more potent in reversing AA-induced changes in appetitive and consummatory components than AA-induced writhing.

Methodological issues of sex, type of palatable food and neophobia were addressed in the study design. We found that male mice preferred white chocolate over dark chocolate. We also found that male mice ate less white chocolate than female mice (data not shown). This sexually dimorphic behaviour is a well known phenomenon in rodents (Valenstein *et al.*, 1967; Barron *et al.*, 1995). A similar result where white chocolate was preferred over milk chocolate in a conditioned place preference test has been previously reported (Ventura *et al.*, 2008). Although neophobia to new taste is a well described phenomenon in rodents (Kronenberger and Médioni, 1985), we performed experiments where one group of female mice was given an opportunity to eat white chocolate together with the standard food, while only standard laboratory food was made available to a second group. As expected, we found that those receiving chocolate ate much more than those receiving standard laboratory food only. All these data indicated that the expression of sweet feeding behaviour critically depends on factors

such as sex, type and previous knowledge of the food stimulus. From these experiments we adopted a strategy to optimize the expression of feeding behaviour by using non-neophobic female mice and white chocolate.

Our behavioural analysis included the recording of four related outcomes which intend to study the distinct components of motivational behaviour. Appetitive motivation for the rewarding stimulus was inferred from changes in the latency to start the ingestion of the palatable food (free approach) and the number of approaches to eat, whereas the consumption behaviours were evaluated by amount consumed and time of consumption. Similar approaches to appetitive motivation have been used by other investigators to study motivated behaviours for natural rewards (Ghiglieri *et al.*, 1997; Barbano and Cador, 2006; Bai *et al.*, 2014). For instance, the time required for the mouse to traverse the alley in the "runaway" model has proven to be a reliable index of the animal's motivation to seek the incentive that is made available to it upon goal box entry. In this paradigm, the changes in the subject's motivation to seek an incentive are considered to produce predictable and reliable shifts in the run times required to get the incentive (Ettenberg, 2009). Increased run time indicates a decreased motivation to reach the food reward and the degree of wanting is determined by analysing the latency to begin to consume reward during the behavioural task. In our study, the latency to eat was short and stable, thus suggesting that mice were highly motivated for the incentive. AA administration caused a dose-dependent increase in this parameter, thus suggesting that mice appetitive motivation was affected by pain. Interestingly, our AA dose response study found that while the latency to eat the reward was not significantly affected by the dose of 0.6% AA, the amount of reward consumed was reduced by approximately 50%. This result suggests that the approach-related parameters of motivational behaviour

were more resistant to acute visceral pain. This could make sense from an evolutionary perspective since strong impediments in approach motivation — which occur before the consummatory-related behaviours— could seriously affect body survival.

Our results showed that 0.9% AA induced a transient behavioural depression characterized by a strong reduction in the reward-seeking behaviour which lasted at least 3 hours and reverted to baseline levels 24 hours after injection. This offset in the recovery time was similar to a previous result obtained in the saccharine preference test, where the preference for this sweet solution was depressed for 6 hours (de la Puente *et al.*, 2015).

Another study found that the consumption of palatable food was decreased upon exposure to the same noxious stimuli (Stevenson *et al.,* 2006). However, the present study incorporated measures of the motivational dimension of behaviour by recording the latency to eat and the number of approaches to consume. Furthermore, our study took advantage of the long term behavioural depression observed to evaluate the pharmacological modulation two hours later, when the writhing behaviour was absent.

We found that NSAIDs, ibuprofen and diclofenac dose-dependently restored the RSB deficit induced by AA to control values. Ibuprofen fully restored both appetitive and consummatory aspects of RSB. Interestingly, the maximum effect of ibuprofen on the latency to eat was observed at the dose of 10 mg/kg (with an ED_{50} = 1 mg/kg), while in the amount parameter it was observed at 40 mg/kg (ED_{50} = 7 mg/kg)—the dose failing to show significant effects on writhing. In fact, the ED_{50} for the ibuprofen-induced decrease in writhing behaviour was 123 mg/kg. Therefore, as far as the decrease in RSB reflects the affective component and the increase in writhing behaviour reflects the

sensory component of AA-induced pain, ibuprofen was able to reduce the affective component of pain at doses that did not affect the sensory component of pain. This conclusion agrees with those of several previous clinical and preclinical studies showing that some analgesics such as ibuprofen, morphine, pregabalin or paracetamol attenuate the affective component of pain more potently than its sensory component (Jensen, 1997; Roberts *et al*., 2006; Hummel *et al*., 2008; Langford *et al*., 2010; Rutten *et al*., 2011; Gilron *et al*., 2013; de la Puente *et al*., 2015). Similarly, diclofenac, another NSAID, dose-dependently restored all parameters evaluated over the dose range of 0.001-0.6 mg/kg, and was at least 2.3-fold more potent than ibuprofen. The maximum effect on the latency to eat was obtained at the dose of 0.16 mg/kg (ED_{50}=0.004 mg/kg), while the dose of 0.6 mg/kg showed the maximum effect in the amount consumed (ED_{50}=0.06 mg/kg). Surprisingly, the dose of 20 mg/kg failed to show any effect on the writhing behaviour (ED_{50} observed to inhibit writhing behaviour was 60 mg/kg). Clearly, diclofenac was effective to restore the affective component of pain at much lower doses than those required to restore the sensory component of pain. In addition, both drugs were more potent in restoring the motivational aspect of the hedonic response than in restoring the consummatory aspect of it, thus suggesting that approach motivation was more sensitive to analgesic drugs than consummatory pleasure. Moreover, ibuprofen and diclofenac at doses producing antianhedonic responses in mice with AA-induced pain had no effect on control mice administered with AA vehicle. This suggests that the present results are not due to non-specific increases in the hedonic response as a result of NSAIDs administration.

Morphine dose-dependently decreased the number of writhes resulting from intraperitoneal injection of AA at a dose range of 0.3-5 mg/kg i.p. The observation that

morphine reversed AA-induced writhing in the present study is consistent with previous studies that have shown similar results with morphine and other opioids in assays of acid-stimulated writhing in rats and mice (Cowan *et al.*, 1977; Fürst, 1991; Stevenson *et al.*, 2006; Meymandi and Sepehri, 2008; Pereira Do Carmo *et al.*, 2009; Miranda *et al.*, 2008, 2013). Morphine, however, failed to restore any parameter evaluated in the RSB task at any dose tested, including those that produced a restored effect in the assay of pain-elicited behaviour. The well-known central rewarding properties of morphine may interfere, making the natural reward in our experimental conditions less attractive. Our findings are consistent with the previous results obtained in mice (Miller *et al.*, 2011), where morphine was ineffective in attenuating the suppression of feeding caused by i.p. injections of AA, but not with other studies performed in mice (Stevenson *et al.*, 2006), where morphine was effective in restoring it. The reasons for this discrepancy are still unclear. There are many differences in the experimental parameters of the two studies (e.g. solution or solid food, time and amount of exposure to noxius stimuli, hedonic test duration) that might contribute to different findings.

Duloxetine showed significant analgesic effects in the writhing assay (5-40 mg/kg). The effects of duloxetine in this assay have been previously reported by others (Jones *et al.*, 2005). The administration of duloxetine (1.25-5 mg/kg, i.p.) in the RSB task failed to show any effect to restore hedonic-like behaviours in AA-treated mice. Moreover, duloxetine administration to control mice (mice administered with AA vehicle) decreased the hedonic responses in the RSB task in a dose-dependent manner (Table 1). The latency to eat evaluated in normal animals (animals administered with the AA vehicle) was increased in a dose-dependent manner after duloxetine administration, in a way similar to the decrease of the amount consumed, which was also significantly

different as compared to vehicle administration at this dose. Previous studies have shown that 5-HT and noradrenaline reuptake inhibition by duloxetine markedly reduces food intake in rats (Jackson *et al*., 1997) and humans (Bernardi and Pallanti, 2010). Therefore, the effects of duloxetine on the RSB task in control mice can be explained by its inhibition of food intake.

Finally, as expected, the non-analgesic stimulant caffeine had no significant effects on any pain-elicited and pain-depressed behavioural responses measured, at any dose tested, thus suggesting that despite the fact that caffeine normalized LMA of AA-treated mice to the level of non-caffeine-treated control animals (de la Puente *et al*., 2015), the pain-induced deficit in RSB was insensitive to the behavioural arousal induced by caffeine.

In summary, a reward seeking behaviour (RSB) paradigm was used where not only consumption but also appetitive, goal directed behaviour have been reliably disrupted by pain and sensitive to analgesics but not to stimulant drugs. However, analgesic side effects such as rewarding properties or food intake impairment would interfere to show positive results in this paradigm. We found that NSAIDs were much more potent to restore AA-related depression of RSB than AA-related increase in writhing, thus suggesting that the affective-motivational dimension was more sensitive than the sensory-discriminative dimension of pain in this visceral pain model. The RSB task can be used as a primary outcome measure to evaluate pain in mice and may complement the more traditional procedures used to assess candidate analgesics.

References

Abramson, LY, Metalsky, GI; Alloy, LB. Hopelessness depression: A theory-based subtype of depression. Psychological Review, Vol 96(2), Apr 1989; 358-372.

Backonja MM, Stacey B. Neuropathic pain symptoms relative to overall pain rating. J Pain. 2004 Nov;5(9):491-7.

Bai Y, Li Y, Lv Y, Liu Z, Zheng X. Complex motivated behaviors for natural rewards following a binge-like regimen of morphine administration: mixed phenotypes of anhedonia and craving after short-term withdrawal. Front Behav Neurosci. 2014 Feb;8:23.

Barbano MF, Cador M. Differential regulation of the consummatory, motivational and anticipatory aspects of feeding behavior by dopaminergic and opioidergic drugs. Neuropsychopharmacology. 2006 Jul;31(7):1371-81.

Barnett SA. Experiments on neophobia in wild and laboratory rats. Br J Psychol. 1958 Aug;49(3):195 201.

Barron S, Razani LJ, Gallegos RA, Riley EP. Effects of neonatal ethanol exposure on saccharin consumption. Alcohol Clin Exp Res. 1995 Feb;19(1):257-61.

Bennett GJ. What is spontaneous pain and who has it? J Pain. 2012 Oct;13(10):921-9.

Bernardi S, Pallanti S. Successful duloxetine treatment of a binge eating disorder: a case report. J Psychopharmacol. 2010 Aug;24(8):1269-72.

Chopra K, Arora V. An intricate relationship between pain and depression: clinical correlates, coactivation factors and therapeutic targets. Expert Opin Ther Targets. 2014 Feb;18(2):159-76.

Cobos EJ, Portillo-Salido E. "Bedside-to-Bench" Behavioral Outcomes in Animal Models of Pain: Beyond the Evaluation of Reflexes. Curr Neuropharmacol. 2013 Dec;11(6):560-91.

Cowan A, Lewis JW, Macfarlane IR. Agonist and antagonist properties of buprenorphine, a new antinociceptive agent. Br J Pharmacol. 1977 Aug; 60(4): 537–545.

de la Puente B, Romero-Alejo E, Vela JM, Merlos M, Zamanillo D, Portillo-Salido E. Changes in saccharin preference behaviour as a primary outcome to evaluate pain and analgesia in acetic acid-induced visceral pain in mice. J Pain Res. 2015 Oct;8:663-673.

Eccleston C, Crombez G. Pain demands attention: a cognitive-affective model of the interruptive function of pain. Psychol Bull. 1999 May;125(3):356-66.

Ettenberg A. The runway model of drug self-administration. Pharmacol Biochem Behav. 2009 Jan;91(3):271-7.

Farmer MA, Leja A, Foxen-Craft E, Chan L, MacIntyre LC, Niaki T, Chen M, Mapplebeck JC, Tabry V, Topham L, Sukosd M, Binik YM, Pfaus JG, Mogil JS. Pain reduces sexual motivation in female but not male mice. J Neurosci. 2014 Apr;34(17):5747-53.

Fürst S. (1991) Pharmacological interaction of opiates with various classes of centrally acting dopaminergic drugs. Drug Metabol Drug Interact. 1991;9(2):77-102.

Gatchel RJ, Peng YB, Peters ML, Fuchs PN, Turk DC. The biopsychosocial approach to chronic pain: scientific advances and future directions. Psychol Bull. 2007 Jul;133(4):581-624.

Ghiglieri O, Gambarana C, Scheggi S, Tagliamonte A, Willner P, De Montis MG (1997) Palatable food induces an appetitive behaviour in satiated rats which can be inhibited by chronic stress. Behav Pharmacol. 1997 Nov;8(6-7):619-28.

Gilron I, Tu D, Holden RR. Sensory and affective pain descriptors respond differentially to pharmacological interventions in neuropathic conditions. Clin J Pain. 2013 Feb;29(2):124-31.

Huang AC, Shyu BC, Hsiao S. Dose-dependent dissociable effects of haloperidol on locomotion, appetitive responses, and consummatory behavior in water-deprived rats. Pharmacol Biochem Behav. 2010 May;95(3):285-91.

Hummel M, Lu P, Cummons TA, Whiteside GT. The persistence of a long-term negative affective state following the induction of either acute or chronic pain. Pain. 2008 Dec;140(3):436-45.

Jackson HC, Needham AM, Hutchins LJ, Mazurkiewicz SE, Heal DJ. Comparison of the effects of sibutramine and other monoamine reuptake inhibitors on food intake in the rat. Br J Pharmacol. 1997 Aug;121(8):1758-62.

Jensen TS. Opioids in the brain: supraspinal mechanisms in pain control. Acta Anaesthesiol Scand. 1997 Jan;41(1 Pt 2):123-32.

Jones CK, Peters SC, Shannon HE. Efficacy of duloxetine, a potent and balanced serotonergic and noradrenergic reuptake inhibitor, in inflammatory and acute pain models in rodents. J Pharmacol Exp Ther. 2005 Feb;312(2):726-32.

Keen-Rhinehart E, Ondek K, Schneider JE. Neuroendocrine regulation of appetitive ingestive behavior. Front Neurosci. 2013 Nov;7:213.

Krebs H, Weyers P, Macht M, Weijers HG, Janke W. Scanning behavior of rats during eating under stressful noise. Physiol Behav. 1997 Jul;62(1):151-4.

Kronenberger JP, Medioni J (1985) Food neophobia in wild and laboratory mice (Mus musculus domesticus). Behav Processes. 1985 Jun;11(1):53-9.

Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, Lacroix-Fralish ML, Matsumiya L, Sorge RE, Sotocinal SG, Tabaka JM, Wong D, van den Maagdenberg AM, Ferrari MD, Craig KD, Mogil JS. Coding of facial expressions of pain in the laboratory mouse. Nat Methods. 2010 Jun;7(6):447-9

Langford DJ, Crager SE, Shehzad Z, Smith SB, Sotocinal SG, Levenstadt JS, Chanda ML, Levitin DJ, Mogil JS. Social modulation of pain as evidence for empathy in mice. Science. 2006 Jun;312(5782):1967-70

Li JX. Pain and depression comorbidity: a preclinical perspective. Behav Brain Res. 2015 Jan;276:92-8.

Low LA, Fitzgerald M. Acute pain and a motivational pathway in adult rats: influence of early life pain experience. PLoS One. 2012 Mar;7(3):e34316.

Maier C, Baron R, Tölle TR, Binder A, Birbaumer N, Birklein F, Gierthmühlen J, Flor H, Geber C, Huge V, Krumova EK, Landwehrmeyer GB, Magerl W, Maihöfner C, Richter H, Rolke R, Scherens A, Schwarz A, Sommer C, Tronnier V, Uçeyler N, Valet M, Wasner G, Treede RD. Quantitative sensory testing in the German Research Network on Neuropathic Pain (DFNS): somatosensory abnormalities in 1236 patients with different neuropathic pain syndromes. Pain. 2010 Sep;150(3):439-50.

Martin TJ, Zhang Y, Buechler N, Conklin DR, Eisenach JC. Intrathecal morphine and ketorlac analgesia after surgery: Comparison of spontaneous and elicited responses in rats. Pain. 2005 Feb;113(3):376-85.

Matson DJ, Broom DC, Carson SR, Baldassari J, Kehne J, Cortright DN. Inflammation-induced reduction of spontaneous activity by adjuvant: A novel model to study the effect of analgesics in rats. J Pharmacol Exp Ther. 2007 Jan;320(1):194-201.

Merali Z, Levac C, Anisman H. Validation of a simple, ethologically relevant paradigm for assessing anxiety in mice. Biol Psychiatry. 2003 Sep;54(5):552-65

Meymandi MS, Sepehri G. Gabapentin action and interaction on the antinociceptive effect of morphine on visceral pain in mice. Eur J Anaesthesiol. 2008 Feb;25(2):129-34.

Miller LL, Picker MJ, Schmidt KT, Dykstra LA. Effects of morphine on pain-elicited and pain-suppressed behaviour in CB1 knockout and wildtype mice. Psychopharmacology (Berl). 2011 Jun;215(3):455-65

Miranda HF, Prieto JC, Puig MM, Pinardi G. Isobolographic analysis of multimodal analgesia in an animal model of visceral acute pain. Pharmacol Biochem Behav. 2008 Feb;88(4):481-6.

Miranda HF, Noriega V, Zepeda RJ, Sierralta F, Prieto JC. Systemic synergism between codeine and morphine in three pain models in mice. Pharmacol Rep. 2013;65(1):80-8.

Mogil JS. The etiology and symptomatology of spontaneous pain. J Pain. 2012 Oct;13(10):932-3.

Negus SS, Morrissey EM, Rosenberg M, Cheng K, Rice KC. Effects of kappa opioids in an assay of pain-depressed intracranial self-stimulation in rats. Psychopharmacology (Berl). 2010 Jun;210(2):149-59.

Negus SS, Neddenriep B, Altarifi AA, Carroll FI, Leitl MD, Miller LL. Effects of ketoprofen, morphine, and kappa opioids on pain-related depression of nesting in mice. Pain. 2015 Jun;156(6):1153-60.

Pereira Do Carmo G, Stevenson GW, Carlezon WA, and Negus SS (2009) Effects of pain- and analgesia-related manipulations on intracranial self-stimulation in rats: further studies on pain-depressed behavior. Pain. 2009 Jul;144(1-2):170-7.

Roberts JD, Gennings C, Shih M. Synergistic affective analgesic interaction between delta-9-tetrahydrocannabinol and morphine. Eur J Pharmacol. 2006 Jan;530(1-2):54-8.

Rutten K, De Vry J, Robens A, Tzschentke TM, van der Kam EL. Dissociation of rewarding, anti-aversive and anti-nociceptive effects of different classes of anti-nociceptives in the rat. Eur J Pain. 2011 Mar;15(3):299-305.

Schwartz N, Temkin P, Jurado S, Lim BK, Heifets BD, Polepalli JS, Malenka RC. Chronic pain. Decreased motivation during chronic pain requires long-term depression in the nucleus accumbens. Science. 2014 Aug;345(6196):535-42.

Stevenson GW, Bilsky EJ, Negus SS. Targeting pain-suppressed behaviors in preclinical assays of pain and analgesia: effects of morphine on acetic acid-suppressed feeding in C57BL/6J mice. J Pain. 2006 Jun;7(6):408-16.

Stevenson GW, Cormier J, Mercer H, Adams C, Dunbar C, Negus SS, Bilsky EJ. Targeting pain-depressed behaviors in preclinical assays of pain and analgesia: drug effects on acetic acid-depressed locomotor activity in ICR mice. Life Sci. 2009 Aug;85(7-8):309-15.

Turk DC, Dworkin RH, Allen RR, Bellamy N, Brandenburg N, Carr DB, Cleeland C, Dionne R, Farrar JT, Galer BS, Hewitt DJ, Jadad AR, Katz NP, Kramer LD, Manning DC, McCormick CG, McDermott MP, McGrath P, Quessy S, Rappaport BA, Robinson JP, Royal MA, Simon L, Stauffer JW, Stein W, Tollett J, Witter J. Core outcome domains for chronic pain clinical trials: IMMPACT recommendations. Pain. 2003 Dec;106(3):337-45.

Valenstein ES, Kakolewski JW, Cox VC. Sex differences in taste preference for glucose and saccharin solutions. Science. 1967 May;156(3777):942-3.

Van Damme S, Crombez G, Eccleston C. Coping with pain: a motivational perspective. Pain. 2008 Sep;139(1):1-4.

Van Damme S, Legrain V, Vogt J, Crombez G. Keeping pain in mind: a motivational account of attention to pain. Neurosci Biobehav Rev. 2010 Feb;34(2):204-13.

Ventura R, Latagliata EC, Morrone C, La Mela I, Puglisi-Allegra S. Prefrontal norepinephrine determines attribution of "high" motivational salience. PLoS One. 2008 Aug;3(8):e3044.

Whishaw IQ, Dringenberg HC, Comery TA.Rats (Rattus norvegicus) modulate eating speed and vigilance to optimize food consumption: effects of cover, circadian rhythm, food deprivation, and individual differences. J Comp Psychol. 1992 Dec;106(4):411-9.

Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain. 1983 Jun;16(2):109-10.

2.2.1. Annex IV

Effect of genetic and pharmacological blockade of the sigma-1 receptor on pain-related depression of reward seeking behaviour after acetic acid-induced visceral pain in mice.

The present Annex explored the role of σ_1R on the affective-motivational component of acute pain using the previously reported model of reward seeking behaviour (RSB) in mice (Article 3). For this purpose, we analysed whether the loss of σ_1R function (through either gene deletion or pharmacological antagonism by BD-1063 and E-52862) could attenuate acetic acid (AA)-induced RSB deficits. We also included a comparison between the effects of these σ_1R antagonists on AA-induced depression of RSB in what we have denominated the **"affective-related phase",** where mice did not show any writhing reflex (from 120 to 130 minutes after AA, same protocol as in Article 3) and the **"sensory-related phase"**, where mice were actively writhing (from 5 to 15 minutes after AA). In addition, we included the dose response effects of diclofenac and ibuprofen in the "sensory-related phase" in order to compare with the dose response effects in the "affective-related phase" (reported in Article 3).

After a baseline evaluation of the RSB (day 1) according to the method previously reported in Article 3, an AA concentration of 0.9% was intraperitoneally injected to induce RSB deficits in female CD-1 wild-type and $\sigma_1R^{-/-}$ mice at 2 hours (day 2) and 24 hours (day 3) after its administration (Fig. 1A).

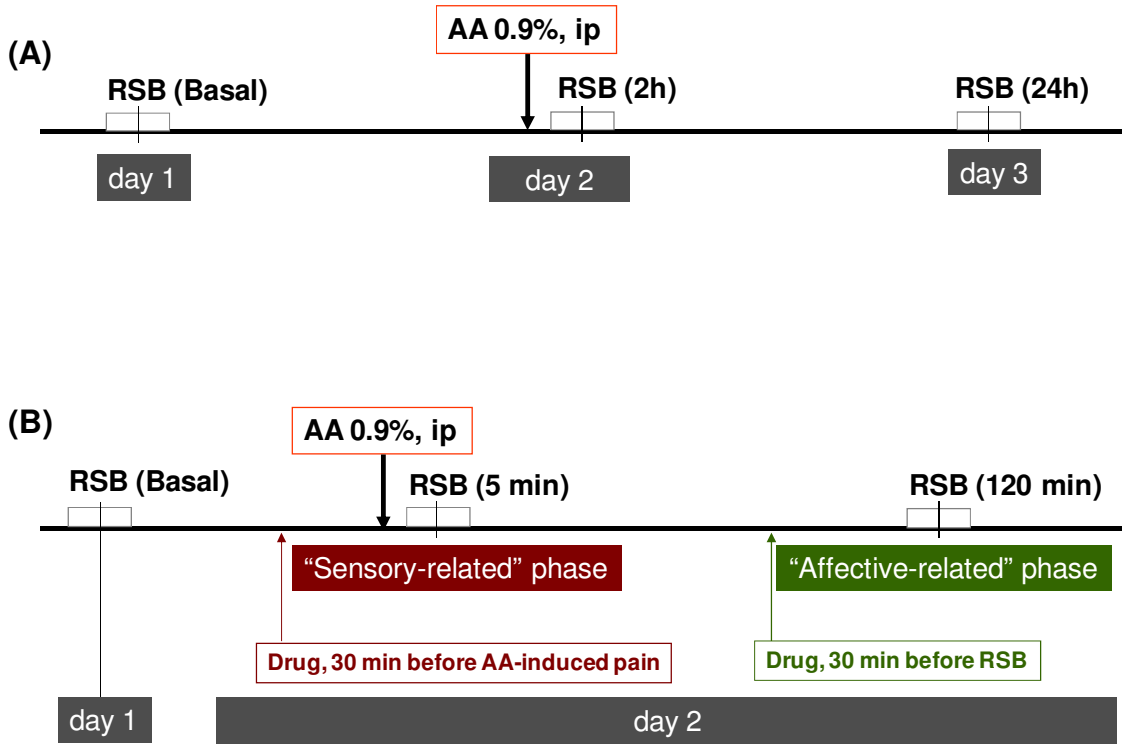


Fig. 1. Schematic representation of the study protocol. (A) Experimental protocol to study AA-induced RSB deficits in wild-type and $\sigma_1R^{-/-}$ mice. After a baseline evaluation of RSB (day 1), 0.9% AA was intraperitoneally injected to each experimental group to induce RSB deficits. The effects of AA on RSB were evaluated at 2 h (day 2) and 24 h (day 3) after its administration. (B) Experimental protocol to evaluate, in two separate studies, the pharmacological effect on the "sensory-related phase", where mice were actively writhing (5 min after AA), and the "affective-related phase" where mice did not show writhing reflex but a RSB deficit was evident (120 min after AA). In the "sensory-related phase", drug administration was performed 30 min before AA; in the "affective-related phase", drug administration was performed 30 min before RSB test.

After the baseline evaluation of RSB (day 1), the pharmacological effects on AA-related depression of RSB were evaluated (day 2) in different groups of mice at two AA-pretreatment times: 5 min ("sensory-related phase" where mice showed writhing behaviour) and 120 min ("affective-related phase" where the writhing behaviour was no longer apparent) (Fig. 1B).

Different doses of E-52862 (10-80 mg/kg), BD-1063 (20-80 mg/kg), ibuprofen (5-320 mg/kg) and diclofenac (2.5-160 mg/kg) were administered. Mice received the drug or its vehicle 30 min before AA injection in the "sensory-related phase", or 30 min before testing RSB in the "affective-related phase" (Fig. 1B).

Data are expressed as means ± SEM. Two-way repeated measures ANOVA was used to analyse behavioural data obtained in the comparative studies of wild-type and $\sigma_1R^{-/-}$ mice (pain and time as main factors), followed by Bonferroni's *post hoc* test. A value of $P < 0.05$ was considered to be statistically significant. Statistical analyses were carried out with the GraphPad Prism 5.00 program (GraphPad Software, San Diego, CA, USA). ED_{50} was calculated according to the statistical methods detailed in Article 3.

RSB measured at baseline, 2 h and 24 h after AA administration or its vehicle on the latency to eat white chocolate in wild-type and $\sigma_1R^{-/-}$ mice is shown in Fig. 2A. Control wild-type and $\sigma_1R^{-/-}$ mice treated with the AA vehicle showed a similar latency to eat at all time points analysed. The statistical analysis in this parameter indicated a significant effect of AA after 2 h of its administration in wild-type ($P < 0.001$) and $\sigma_1R^{-/-}$ mice ($P < 0.01$). The latency to eat observed 24 h after AA administration was similar to that of their respective baseline values for both genotypes ($P > 0.05$).

Similarly, the statistical analysis of the number of approaches indicated a significant effect of AA 2 h after its administration (Fig. 2B) in wild-type ($P < 0.001$) and $\sigma_1R^{-/-}$ ($P < 0.01$) mice.

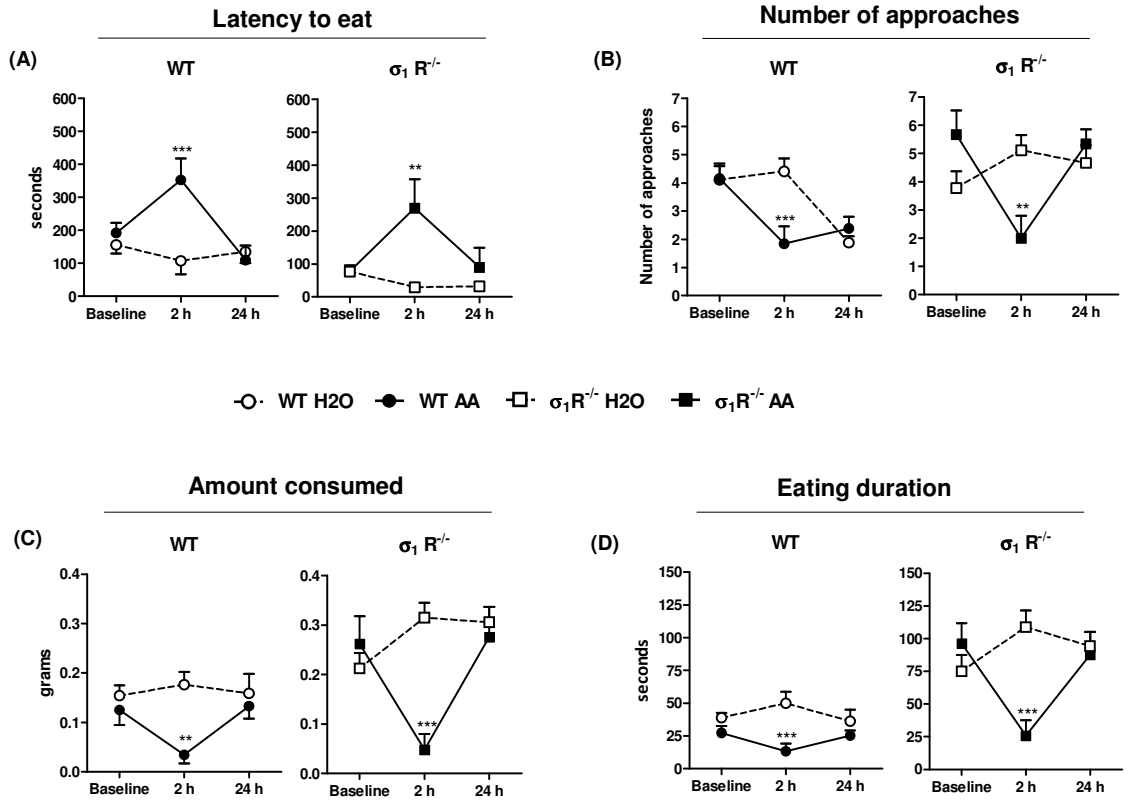


Fig. 2. Comparative effect of AA-induced pain on RSB in wild-type (WT) and $\sigma_1R^{-/-}$ mice. The baseline behaviour was measured the day before AA administration. Two-way RM ANOVA showed a significant effect of pain 2 h after AA administration in both genotypes (**$P < 0.01$; ***$P < 0.001$). The hedonic response was fully recovered 24 h after AA administration. (A) Latency to eat, (B) number of approaches, (C) amount consumed and (D) eating duration.

Fig 2C shows that the amount consumed was highly depressed after the administration of AA in both wild-type and $\sigma_1R^{-/-}$ mice. Two way ANOVA indicated significant effects of AA 2 h after administration in wild-type ($P < 0.01$) and $\sigma_1R^{-/-}$ ($P < 0.001$) mice. Both wild-type and $\sigma_1R^{-/-}$ mice restored the amount consumed to their respective control levels by 24 h after pain induced by AA.

Control mice treated with the AA vehicle showed similar eating duration at all time points analysed in both genotypes (Fig. 2D). The eating duration was strongly reduced

2h after AA administration as compared to control levels (from 27.3 ± 5.3 to 13.3 ± 5.5 seconds and from 96.4 ± 15.5 to 25.9 ± 11.7 seconds for wild-type and $\sigma_1R^{-/-}$ mice, respectively), thus showing a significant effect of AA in both genotypes ($P < 0.001$) at this time. The eating duration was restored to control levels 24 h after AA administration in both genotypes.

Finally, significant differences were also found between wild-type and $\sigma_1R^{-/-}$ mice during the baseline RSB test and 24 h after AA for eating duration and amount consumed. Because previous studies suggest several potential mechanisms through which σ_1R may modulate dopamine and opioid systems (Fu *et al.*, 2010; Navarro *et al.*, 2010), σ_1R could be involved in the processing of natural rewards (Le Merrer *et al.*, 2009; Baik, 2013). $\sigma_1R^{-/-}$ mice could develop compensatory changes which could explain these significant differences in the consummatory response between both genotypes. However, the objective of this study was to evaluate whether AA administration could also induce the RSB deficits in $\sigma_1R^{-/-}$ mice. Therefore, these findings do not affect the main conclusion of this study.

The effects of the pharmacological blockade of σ_1R on AA-depression in reward seeking behaviour were evaluated and the pharmacological sensitivity between "sensory-related" and "affective-related phase" for each drug was compared. Only the latency to eat and the amount consumed as measures of the appetitive-approach and consummatory dimensions of motivated behaviour, respectively, are shown. The maximum effect (E_{max}) and the ED_{50} for each drug evaluated are shown in Table 2.

The NSAID diclofenac showed a clear dose-response effect to attenuate the AA-induced increase in the latency to eat and the AA-induced decrease in the amount

consumed in both "sensory" and "affective-related" phases. However, the dosage range used to restore RSB in the "affective-related phase" of AA-treated mice was much lower (0.001-0.6 mg/kg) than that used in the "sensory-related phase" (20-160 mg/kg) (Fig. 3).

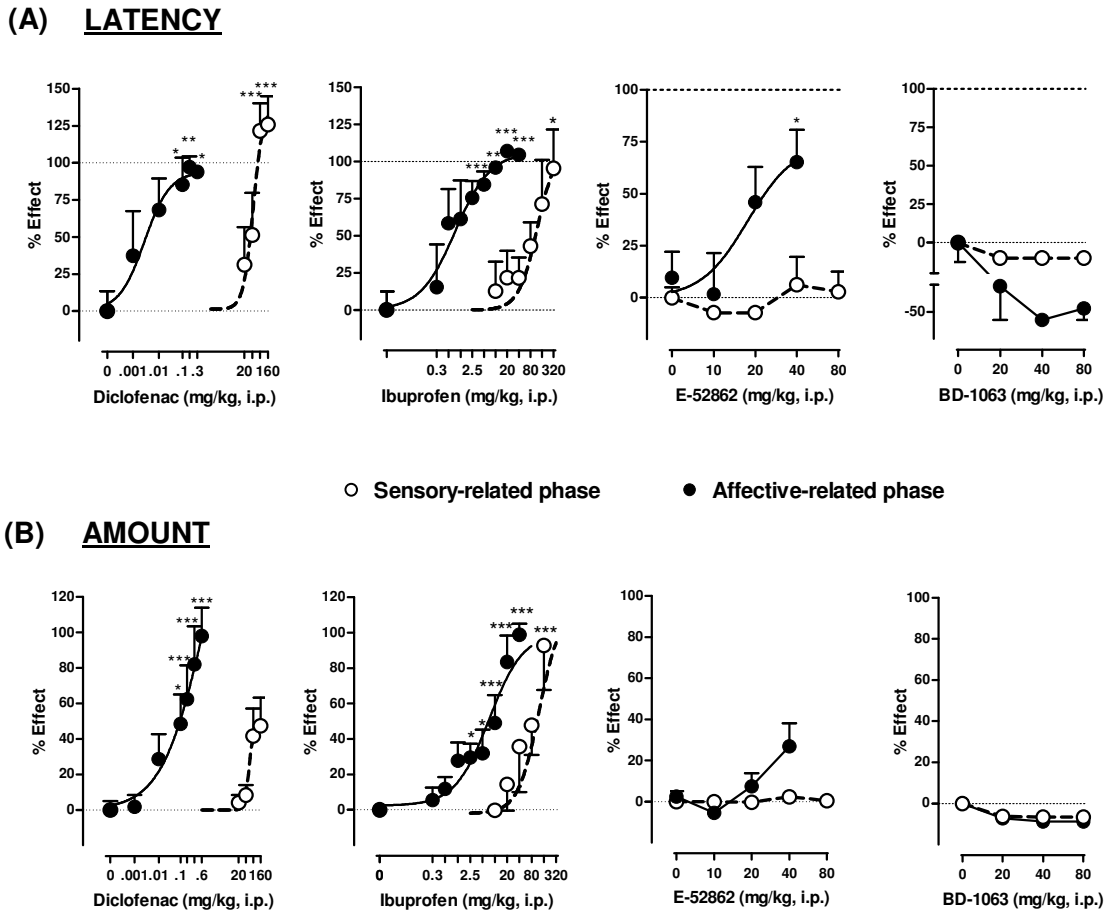


Fig. 3. Effect of drugs on AA-related depression of RSB in the "sensory-related phase" and "affective-related phase". In order to compare the pharmacological sensitivity of both phases, the RSB test was performed 5 min after AA administration, where drugs should block writhing and restore RSB ("sensory-related phase"), and 120 min after AA administration, where drugs should only restore RSB ("affective-related phase"). Pharmacological studies were performed with the NSAIDs diclofenac and ibuprofen and the σ_1R antagonists E-52862 and BD-1063 on latency to consume (A) and amount consumed (B) as quantifiable measures of the motivational and consummatory aspects of hedonic response to palatable food, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, one way-ANOVA, Bonferroni's *post hoc* test.

In the "affective-related phase", a significant effect as compared to the vehicle control group at the doses of 0.08 and 0.3 ($P < 0.05$) and 0.16 mg/kg ($P < 0.01$) in the latency to eat and at the doses of 0.08 ($P < 0.05$), 0.16, 0.3 and 0.6 mg/kg ($P < 0.001$) in the amount consumed parameters was found. In the "sensory-related phase", however, the doses of 80 and 160 mg/kg showed a significant effect as compared to the vehicle control group in the latency to eat parameter ($P < 0.001$), but failed to show any significant effect on the amount consumed parameter. In the latency to eat parameter, the maximum effect of diclofenac was seen at a dose of 0.16 mg/kg in the "affective-related phase" while a dose of 80 mg/kg was necessary to completely abolished pain-depressed RSB in the "sensory-related phase". Similarly, in the amount consumed parameter, diclofenac obtained the maximum effect at 0.6 mg/kg in the "affective-related phase"; however, no significant effect was observed in the "sensory-related phase" at this dose.

Similarly, ibuprofen also showed a clear dose-response effect to attenuate the AA-induced increase in the latency to eat and the AA-induced decrease in the amount consumed in both the "sensory" and "affective-related" phases. The dosage range used to restore RSB in the "affective-related phase" of AA-treated mice was also lower (0.3-40 mg/kg) than that used in the "sensory-related phase" (10-320 mg/kg) (Fig. 3). In the "affective-related phase", a significant effect as compared to the vehicle control group at the doses of 5 ($P < 0.01$), 2.5, 10, 20 and 40 mg/kg ($P < 0.001$) in latency to eat and at the doses of 2.5 ($P < 0.05$), 10, 20 and 40 mg/kg ($P < 0.001$) in the amount consumed parameters was found, while a dose of 320 mg/kg was necessary to observe a significant effect versus the vehicle control group in the "sensory-related phase" ($P < 0.05$ and $P < 0.001$ for latency to eat and amount consumed, respectively). Ibuprofen

induced the maximum effect at 20 mg/kg in the "affective-related phase" while a dose of 160 mg/kg was needed to obtain the same effect in the "sensory-related phase".

Table 1. Maximum effect (E_{max}) and ED_{50} of drugs on AA-related depression of RSB in the "sensory- and affective-related phase".

	Latency to eat (s)			
	Sensory-related phase		**Affective-related phase**	
	ED 50	***E max***	***ED 50***	***E max***
Diclofenac	43.6 ± 14.92	139	0.004 ± 0.003	107.3
Ibuprofen	87.2 ± 24.0	100	0.9 ± 0.5	102.8
E-52862	N.D.	N.D.	17.7 ± 11.5	74.1
BD-1063	N.D.	N.D.	N.D.	N.D.

	Amount consumed (g)			
	Sensory-related phase		**Affective-related phase**	
	ED 50	***E max***	***ED 50***	***E max***
Diclofenac	54.5 ± 17.8	47.9	0.06 ± 0.03	114.9
Ibuprofen	69.9 ± 16.8	100	6.8 ± 1.3	123.1
E-52862	N.D.	N.D.	N.D.	26.9
BD-1063	N.D.	N.D.	N.D.	N.D.

N.D.: not determined

The σ_1R antagonist E-52862 exhibited a partial effect to restore latency to eat in the "affective-related phase" of AA-induced pain. The dose of 40 mg/kg produced a significant effect on the latency to eat parameter as compared to the vehicle ($P < 0.05$). The E_{max} for E-52862 was 74.1% and 26.9% for latency to eat and amount consumed, respectively. However, E-52862 was ineffective to restore hedonic behaviour in the "sensory-related phase". The selective σ_1R antagonist BD-1063 did not show any effect in any "sensory-" and "affective-related" phase of AA-induced pain (Fig. 3).

In summary, the results obtained when the effect of σ_1R deletion on the visceral pain-related depression of RSB was studied showed that, similarly to wild-type mice, AA administration to $\sigma_1R^{-/-}$ mice produced profound behavioural changes in the four RSB-related endpoints analysed. Therefore, the absence of σ_1R failed to modify the changes in RSB as a consequence of AA-induced visceral pain, thus suggesting that σ_1R deletion had no impact on the behavioural manifestations of the affective-motivational component of acute pain.

The results of these pharmacological experiments also showed that the range of doses was different for each phase, with the "affective-related phase" being more sensitive to the analgesic-like effect than the "sensory-related phase".

The pharmacological blockade of σ_1R with the σ_1R antagonist E-52862 partially restored the latency to eat, but not the amount consumed, in wild-type mice. However, the pharmacological blockade of σ_1R with the σ_1R antagonist BD-1063 was ineffective to alter the AA-induced deficits in both parameters. These results suggested that acute modulation of σ_1R by drugs did not significantly alter the affective-motivational component of acute visceral pain.

2.3. ARTICLE 4

Effect of genetic and pharmacological blockade of the sigma-1 receptor on pain-related depression of the reward seeking behaviour after peripheral nerve injury in mice

Beatriz de la Puente, Daniel Zamanillo, José Miguel Vela, Manuel Merlos, Enrique Portillo-Salido

Department of Pharmacology, Drug Discovery and Preclinical Development, ESTEVE

Manuscript to be submitted

Effect of genetic and pharmacological blockade of the sigma-1 receptor on pain-related depression of the reward seeking behaviour after peripheral nerve injury in mice

Abstract

Pharmacological blockade or genetic ablation of σ_1R improved behavioural hypersensitivity in several preclinical models of chronic/neuropathic pain. Since chronic pain is often accompanied by mood complications we explored whether neuropathic mice lacking σ_1R ($\sigma_1R^{-/-}$ mice) developed depressive-like behaviours and whether the blockade of σ_1R by the selective antagonist E-52862, could reverse them in neuropathic wild-type (WT) mice. Neuropathic pain was evoked by partial sciatic nerve ligation (PSNL) in female CD-1 mice. Mechanical hypersensitivity, evaluated using von Frey filaments, and depressive-like behaviour were measured for 10 weeks in nerve-injured, sham-operated, and naïve mice. Depressive-like behaviour was evaluated through a reward seeking behaviour (RSB) task in which both appetitive (latency to eat and number of approaches to eat) and consummatory (amount consumed and eating duration) behaviours to a strongly rewarding stimulus (chocolate) were assessed. WT mice exposed to PSNL developed maximum mechanical hypersensitivty 3 weeks after nerve injury and recovered thereafter (full recovery at week 10). Deficits on both appetitive and consummatory behaviours of RSB were not present 3 weeks after nerve injury but reached the maximum effect at week 6, being still present at week 10. No deficits in RSB and no development of mechanical hypersensitivity were found in nerve-injured $\sigma_1R^{-/-}$ mice. The acute administration of E-52862 (20 and 40 mg/kg ip, week 6) to nerve-injured WT mice reversed the RSB deficits. In summary, depressive-like symptoms were evidenced even after mechanical hypersensitivity recovery in the PSNL model. Moreover, σ_1R blockade could have beneficial effect not only in the sensory component of pain (mechanical hypersensitivity) but also on the mood complications associated to neuropathic pain.

Introduction

Neuropathic pain is a clinical manifestation characterized by spontaneous ongoing or shooting pain and evoked amplified pain responses after noxious or non-noxious stimuli (Baron *et al.*, 2010). Neuropathic pain is often accompanied by mood, sleep and cognitive complications that affect the patient's quality of life (Yalcin and Barrot, 2014). The current therapy for neuropathic pain is not satisfactory and new drugs and targets to design optimal pharmacological treatments are being pursued (Kinloch and Cox, 2005; Burgess and Williams, 2010; McDougall, 2011).

σ_1R is an intracellular chaperone protein that interacts with other proteins, including plasma membrane and endoplasmic reticulum receptors and ion channels. In the context of pain, σ_1R modulates central sensitization phenomena (Drews and Zimmer, 2009; de la Puente et al., 2009; Romero *et al.*, 2012) that are responsible for many of the temporal, spatial and threshold changes in pain sensitivity in acute and chronic pain (Latremoliere and Woolf, 2009). Multiple genetic and pharmacological preclinical studies have given abundant information on the role of the sigma-1 receptor in reflex measures of stimulus-evoked pain. Pain behaviours elicited by intraplantar administration of formalin and capsaicin are attenuated in $\sigma_1R^{-/-}$ mice (Cendán *et al.*, 2005; Entrena *et al.*, 2009). In neuropathic pain conditions, either induced by partial sciatic nerve ligation (PSNL) or after treatment with the antineoplastic agent paclitaxel, cold and mechanical allodynia were strongly attenuated or even not developed in $\sigma_1R^{-/-}$ mice (de la Puente *et al.*, 2009; Nieto *et al.*, 2012). In contrast, thermal (heat) hyperalgesia developed to the same extent as in WT mice following nerve injury. Unlike neuropathic pain models, the genetic inactivation of σ_1R failed to prevent the development of carrageenan-induced and CFA-induced mechanical allodynia when von

Frey filaments were applied (Gris *et al.*, 2014). The selective pharmacological blockade of σ_1R has been effective in a broad range of acute and chronic pain models using different reflexive related readouts (Nieto *et al.*, 2012; Romero *et al.*, 2012; Gonzalez-Cano *et al.*, 2013; Gris *et al.*, 2015).

However, preclinical studies focusing on the role of σ_1R in neuropathic pain-evoked comorbidities are lacking. In this study, the effects of the pharmacological and genetic blockade of σ_1R on both nociceptive reflex behaviours and depression-related behaviours were investigated in a neuropathic pain model in mice.

Material and methods

Animals

Female wild-type (WT) and homozygous knock-out ($\sigma_1R^{-/-}$) mice backcrossed (N10 generation) to a CD1 albino genetic background (Harlan Ibérica, Barcelona, Spain) and aged 6 to 8 weeks at the beginning of the experiments, were used. Null mutant mice were generated on a CD1 background as described previously (de la Puente *et al.*, 2009).

Mice were housed in groups of five in 25 x 50 x 15 cm transparent polypropylene cages with suitable beddings (wood shavings) and food and water *ad libitum*. Animals were left undisturbed for two weeks to acclimatize to laboratory conditions in a testing room with light and temperature controlled at 20-22 ºC with a 12 h light/12 h dark cycle (lights on at 06:00 and off at 18:00). All trials were performed between 8.00 am and 13:00 pm. All animal research was conducted in accordance with protocols approved by the local Committee of Animal Use and Care of our Institution and in accordance with

the Care and Use of Laboratory Animals Guidelines of the European Community (Council Directive of 22 September 2010, 2010/63/EU) and with the International Association for the Study of Pain guidelines on ethical standards for investigation in animals (Zimmermann, 1983).

Neuropathic pain model: partial sciatic nerve ligation.

The partial sciatic nerve ligation model was used to induce neuropathic pain according to the protocol previously described (Malmberg and Basbaum, 1998; de la Puente *et al.*, 2009). Briefly, mice were anesthetized with isoflurane (Abbott–Esteve, Spain) (induction: 5%; surgery: 2.5%) and the common sciatic nerve was exposed at the level of the mid-thigh of the right hind paw according to our previous experimental procedure (de la Puente *et al.*, 2009). Control sham-operated mice underwent the same surgical procedure and the sciatic nerve was exposed, but not ligated. Control naïve mice were not operated or anesthetized.

Evaluation of neuropathic pain-induced mechanical hypersensitivity.

Hypersensitivity to mechanical stimuli was used as a sensory outcome measure of neuropathic pain in sham and nerve-injured mice. The time-course of the neuropathy development was assessed by testing animals 1 day before and every week after surgery, for 9 weeks. Mechanical hypersensitivity was quantified by measuring the hindpaw withdrawal response to von Frey filament stimulation according to the same previous experimental procedure (de la Puente *et al.*, 2009) and the threshold of response was calculated by using the up–down method (Chaplan, 1994).

Evaluation of depressive-like behaviour after nerve injury in mice.

The changes in the reward seeking behaviour (RSB) when mice were offered palatable food, thoroughly described in Article 3, were used to evaluate the affective consequences of neuropathic pain. The RSB task is based on the approach and consumption of white chocolate, which allowed analysing the appetitive and consummatory aspects of motivational behaviour. Individual groups of PSNL, sham or naïve WT mice were evaluated at week 1, 3, 6 or 10 post-injury for five consecutive days, as previously described in Article 3. Mice were weighed before each RSB test. Briefly, the mouse was placed in one corner of its home cage for 10 min providing white chocolate (~2 g) in a glass dish. A total of four dependent measures were used: latency to eat, duration of eating behaviour, number of approach to eat, and amount of chocolate consumed. The latency to eat was defined as the time between the placement of animal and the beginning of eating. The number of times and the time taken to eat chocolate were chosen because they had been shown to be affected by a number of experimental manipulations (Whishaw *et al.*, 1992; Krebs *et al.*, 1997). The amount consumed was assessed by weighing the piece of chocolate before and after testing. The consumption rate was determined by dividing grams of chocolate per second, and palatable food intake was expressed as gram of chocolate consumed per gram of body weight. To study the differences between genotypes, a parallel study with WT and $\sigma_1R^{-/-}$ mice was performed to maintain the same experimental conditions in both genotypes and to avoid the interference of factors such as time of animal manipulations and others.

Pharmacological treatment with E-52862.

E-52862, purchased by Laboratories Esteve (Barcelona, Spain), was dissolved in 0.5% HPMC (Sigma-Aldrich, Co.) and administered intraperitoneally 30 min before the RSB test at a volume of 10 mL/kg. Mice received 3 doses of E-52862 (10, 20 and 40 mg/kg) or its vehicle, administered in a 4x4 Latin-square design for four consecutive days and followed by a 24-h washout period. The responses were considered as internal controls to confirm that baseline thresholds were not influenced by previous treatments.

In mechanical hypersensitivity, E-52862 evaluation was conducted starting at 2 weeks after injury in WT PSNL and sham-operated mice. The effects of single doses of E-52862 on the mechanical threshold were evaluated on days 15-17 post-surgery, according to a 3x3 Latin square design. All animals received the 3 doses of E-52862 (20, 40 and 80 mg/kg at a dose volume of 10 mL/kg) by intraperitoneal route. E-52862 was given intraperitoneally and the hindpaw withdrawal threshold was evoked 30 min after drug administration. Finally, on day 18 post-surgery, the mechanical nociceptive threshold was evaluated after a 24-h drug washout period, and the responses were considered as an internal control to confirm that baseline thresholds were not influenced by previous treatments. In all cases both ipsilateral and contralateral paws were tested.

Statistical analysis

Data obtained from mechanical hypersensitivity test were compared by two-way repeated measures ANOVA with surgical condition and time as main factors of variation. For drug treatment study, the data obtained as baseline mechanical thresholds were analysed with the Student's two-independent-sample *t* test. The data obtained from the Latin square design were analysed with two-way repeated measures ANOVA

(surgery and paw as main factors). To calculate the ED_{50}, data were converted to percentage of antihypersensitivity effect based on the following formula: % antihypersensitivity effect = [(PW– PWi)/(PWc–PWi)] x 100, where PW is the paw withdrawal threshold (g) of the ipsilateral paw in drug treatment, PWi is the paw withdrawal threshold (g) of the ipsilateral paw at pre-treatment day, and PWc is the paw withdrawal threshold (g) of the contralateral paw at pre-treatment day. One way analysis of variance determined the differences between doses.

RSB responses were compared on each experimental week by using a two-way repeated measures ANOVA where the between-group statistical factors were the surgical condition (PSNL-operated, sham-operated and un-manipulated/naive mice) and repeated measure (one test a day for five consecutive days). For drug treatment study, a one-way ANOVA with repeated measures was used.

Data are expressed as mean ± SEM. Bonferroni's *post hoc* analyses were performed in all cases for assessing specific group comparison. A P value of $P < 0.05$ was considered to be statistically significant. Statistical analyses were carried out with the GraphPad Prism 5.00 program (GraphPad Software, San Diego, CA, USA).

Results

Development of mechanical hypersensitivity following partial sciatic nerve ligation injury was observed in female WT mice but not in female $\sigma_1R^{-/-}$ mice.

RSB studies were performed using female mice, thus it makes sense to evaluate mechanical hypersensitivity also in female mice. Consistent with our previous studies

performed in male mice (de la Puente *et al*., 2009) mechanical hypersensitivity was clearly observed in female WT mice but not in female $\sigma_1R^{-/-}$ mice (Fig. 1).

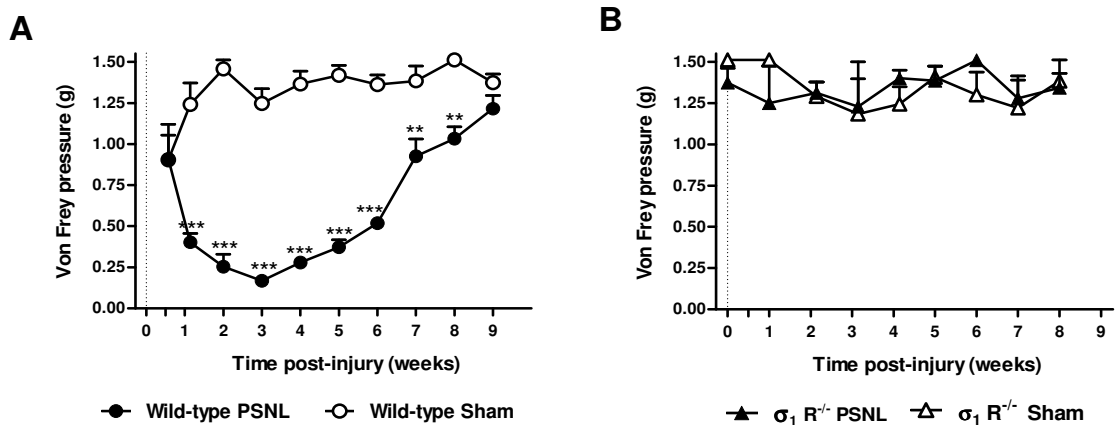


Fig. 1. Development of mechanical hypersensitivity following nerve injury in WT and $\sigma_1R^{-/-}$ deficient female mice. Mechanical hypersensitivity defined as decreased threshold to von Frey filament stimulation was the nociceptive response evaluated in WT and $\sigma_1R^{-/-}$ mice with PSNL and sham-operated mice. Partial sciatic nerve ligation induced long-lasting mechanical hypersensitivity in WT mice but not in $\sigma_1R^{-/-}$ mice. WT PSNL mice developed ipsilateral mechanical hypersensitivity lasting over 8 weeks. The right hind-paw surgical procedure did not affect the left hind-paw withdrawal thresholds. The data presented are mechanical thresholds in gram force and are expressed as means ± SEM. The responses of ipsilateral paws of the PSNL group were compared to sham-operated mice for each genotype and time point. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, two-way RM ANOVA, Bonferroni's *post hoc* test.

The statistical analysis revealed significant effects of surgical condition in WT mice ($F_{2,17}$ = 85.15, *P* < 0.001) but not in $\sigma_1R^{-/-}$ mice ($F_{1,47}$ = 0.41, *P* > 0.05). *Post hoc* tests revealed that WT PSNL mice significantly increased responses to von Frey hair stimulation at weeks 2 - 6 (*P* < 0.001), week 7 (*P* < 0.05) and week 8 (*P* < 0.01) as compared to sham-operated mice, with normal sensitivity being completely restored

within 9 weeks. Mice lacking σ_1R did not exhibit mechanical hypersensitivity following partial sciatic nerve ligation at any time point evaluated. A significant effect of time was observed in WT ($F_{8,136}$ = 8.55, $P < 0.001$) and interaction between surgery and time factors ($F_{16,136}$ = 8.36, $P < 0.001$), thus suggesting that PSNL mice showed significant changes over time —unlike sham-operated mice, whose behavioural responses last for weeks.

Effect of pharmacological blockade of sigma-1 receptors on mechanical hypersensitivity after peripheral nerve injury.

Mechanical hypersensitivity, defined as a significant reduction in the pressure evoking withdrawal of the nerve-injured hindpaw, was clearly evidenced in PSNL mice ($P < 0.001$). The withdrawal threshold value for the right (ipsilateral) paw in PSNL and sham-operated mice was 0.18 ± 0.04 g and 1.43 ± 0.05 g, respectively (Fig. 2). However, the responses to mechanical stimuli in sham-operated mice remained unchanged after surgery ($P > 0.05$). Withdrawal threshold values of 1.5 ± 0.03 g and 1.4 ± 0.04 g were obtained for the contralateral and ipsilateral paw, respectively.

Pharmacological experiments showed that E-52862 had no effect on the responses of sham-operated mice after treatment ($F_{4,64}$ = 2.27, $P > 0.05$), thus suggesting that normal transmission and perception of sensory and nociceptive inputs remain intact following pharmacological antagonism of σ_1R. However, nerve injury-induced mechanical hypersensitivity was dose-dependently inhibited by the acute systemic administration of E-52862, with E_{max} = 100 % and ED_{50} = 35.8 ± 3.4 mg/kg. A statistically significant dose effect was obtained in PSNL mice ($F_{4,64}$ = 23,2; $P < 0.001$). Bonferroni's *post hoc* test revealed that E-52862 significantly increased the paw withdrawal threshold at 40

and 80 mg/kg ($P < 0.001$) as compared to the baseline nerve-injured hindpaw value, while 20 mg/kg of E-52862 had no significant effect ($P > 0.05$) (Fig. 2). The antinociceptive effect of E-52862 was consistent with the observation that $\sigma_1R^{-/-}$ mice failed to develop mechanical hypersensitivity (Fig. 1).

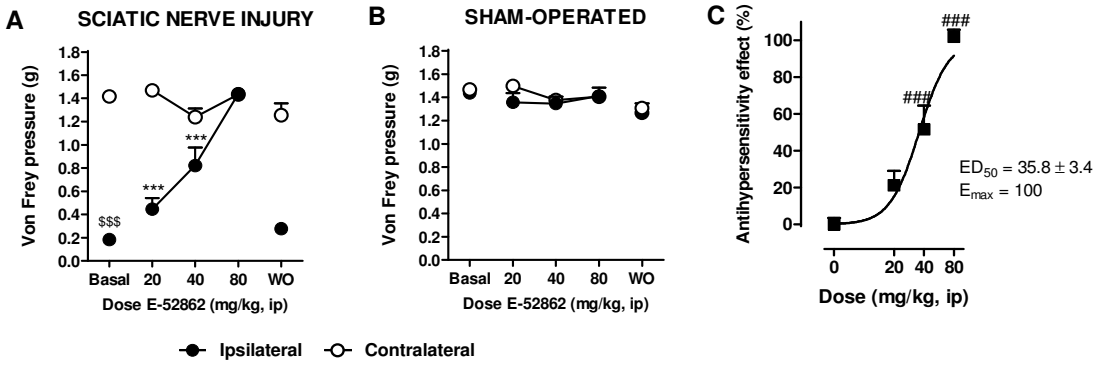


Fig. 2. Effects of the pharmacological blockade of σ_1R on the hind paw withdrawal reflex after mechanical stimulation in mice exposed to peripheral nerve injury. Threshold mechanical sensitivity was assessed with Von Frey filaments in PSNL (A) and sham-operated (B) mice in the ipsilateral and contralateral sides. PSNL surgery induced marked mechanical hypersensitivity in the ipsilateral hind paw 14 days after injury as compared to sham surgery (Basal in the graph). PSNL and sham-operated mice were daily administered with the σ_1R antagonist E-52862 (20, 40 and 80 mg/kg) on days 15−17 post-surgery according to a Latin Square design, and paw withdrawal threshold measurements were performed 30 min after drug administration. After a 24-h drug washout period (WO in the graph), the hindpaw withdrawal threshold was measured at day 18 post-injury. (C) Dose-response curve of the antihypersensitivity effect of E-52862 (E_{max}=100; ED_{50} = 35.8 ± 3.4 mg/kg). Each point and vertical line represent the mean ± SEM. $^{\$\$\$}P < 0.001$, Student's two-sample *t* test. ***$P < 0.001$, two-way RM ANOVA, Bonferroni's *post hoc* test. ###$P < 0.001$, one-way ANOVA, Bonferroni's *post hoc* test.

The development of depressive-like behaviours after nerve injury was evidenced by deficits in the reward-seeking behaviour in mice.

In order to test whether a PSNL injury evoked anhedonic-like behaviours in mice, the RSB test was performed at different time points in both nerve injury and sham-operated mice. Naïve mice were also evaluated as controls for the normal behaviour of non-manipulated mice. In PSNL mice, all behavioural parameters evidenced a decreased hedonic response to palatable food, specifically at week 6 post-injury (see Table 1 for statistical details). No significant interaction involving surgery condition and repeated measures was found in any parameter evaluated. These results indicate that the effect produced by repeated measures on the hedonic response was similar in all experimental groups. Therefore, this effect is not specific to surgery condition.

Latency to eat was altered by surgical condition (Fig 3A). Statistical studies showed that surgery condition failed to significantly modify this parameter at week 1 post-injury ($F_{2,37} = 1.87$, $P > 0.05$). However, it was affected at week 3 ($F_{2,37} = 3.64$, $P < 0.05$), week 6 ($F_{2,72} = 20.20$, $P < 0.001$) and week 10 ($F_{2,34} = 5.74$, $P < 0.01$) post-injury. PSNL mice showed statistical differences as compared to sham-operated mice ($P < 0,05$, trial 4; $P < 0.01$, trials 1 and 3; $P < 0.001$, trials 4 and 5) or naïve mice ($P < 0.01$, trials 2 and 3; $P < 0.001$, trials 1, 4 and 5) at week 6 post-injury.

Like latency to eat, the number of approaches was considered to be an indication of the appetitive motivation to palatable food (Fig. 3B). No effect of surgery condition was observed at weeks 1 and 3 post-injury. However, a significant effect of this factor was observed at week 6 ($F_{2,72} =8.75$, $P < 0.001$) and week 10 ($F_{2,34} =3.39$, $P < 0.05$) when

PSNL mice showed statistical differences as compared to naïve mice ($P < 0.01$, trial 2; $P < 0.05$, trial 4), but not when PSNL mice were compared to sham-operated mice.

The amount consumed seemed to be unaffected by the surgery condition at week 1 and 3 post-injury ($F_{2,37} = 2.12$, $P > 0.05$, $F_{2,37} = 0.0$, $P > 0.05$, respectively). This parameter was affected by the surgery condition at week 6 ($F_{2,72} = 12.47$, $P < 0.001$) when PSNL mice showed a significantly decreased consumption as compared to sham-operated mice ($P < 0.05$, trials 1, 2; $P <0.01$, trial 5) or as compared to naïve mice ($P < 0.05$, trial 5; $P < 0.01$, trial 2; $P < 0.001$, trial 1). These differences were also observed at week 10 ($F_{2,136} = 6.23$, $P < 0.01$). At this time, PSNL mice showed significant differences as compared to naïve mice ($P < 0.05$, trials 2 and 4) but not as compared to sham-operated mice, which showed a decrease in the amount consumed similar to that observed in PSNL mice.

Like the amount consumed, the eating duration was measured as an indication of the consummatory response to palatable food. Experimental groups showed similar hedonic responses as compared to those obtained in the amount consumed parameter. The surgery condition was found to have a significant effect on eating duration at week 6 ($F_{2,288}=9.92$, $P < 0.001$), when PSNL mice showed a shorter eating time as compared to sham-operated ($P < 0.01$, trial 5) or naïve mice $P < 0.05$, trial 2; $P < 0.001$, trial 1). However, this factor had no effect at weeks 1, 3 and 10 post-injury (Table 1).

Body weight changes over time for the different experimental groups are shown in Fig. 4A. No effect of surgery condition on weight was observed at any week evaluated, except at week 10, ($F_{1,120}=4.30$, $P < 0.05$), when PSNL mice showed a body weight decrease that was significantly different from that of sham-operated mice.

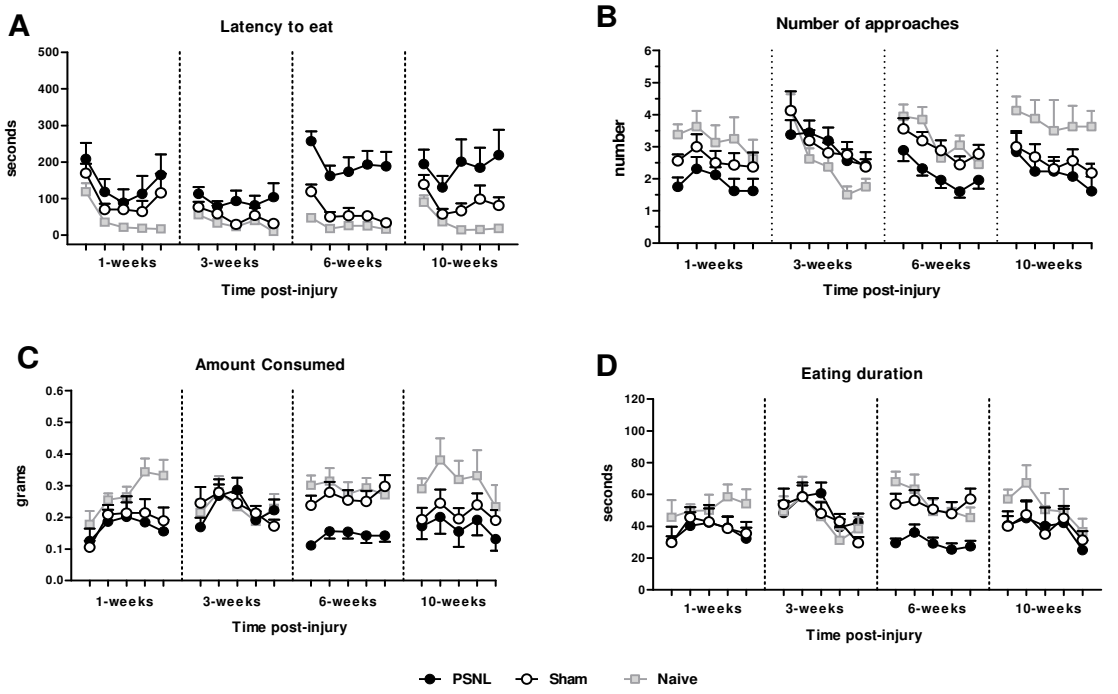


Fig. 3. Development of depressive-like behaviours following nerve injury in mice. The RSB of each experimental group was measured in separate groups of mice at weeks 1, 3, 6 and 10 post-surgery, once a day for five consecutive days. Values are means ± SEM. A two-way RM ANOVA was conducted to explore the effects of surgery condition (PSNL, sham-operated or naïve mice) and repeated measures at each post-injury week on the RSB. The surgery condition factor showed a significant effect on the motivational aspect (latency to eat and Number of approaches parameters) and also on the consummatory aspect (amount consumed and eating duration parameters) of hedonic response to palatable food, at week 6 post-injury. (A) Latency to eat, (B) number of approaches, (C) amount consumed and (D) eating duration.

	Latency to eat (s)			Number of approaches			Amount consumed			Eating duration			Body weight			Intake			Consumption		
	d.f.	*F-value*	*P*	*d.f.*	*F-value*	*P*	*d.f.*	*F-value*	*P*	*d.f.*	*F-value*	*P*	*d.f.*	*F-value*	*P*	*d.f.*	*F-value*	*P*	*d.f.*	*F-value*	*P*
Week-1																					
Surgical condition	2.148	1.87	0.1678	2.148	3.74	< 0.05	2.148	2.12	0.1345	2.148	1.20	0.3125	2.148	0.44	0.8942	2.148	1.65	0.2064	2.148	0.71	0.4968
Repeated measure	4.148	8.23	< 0.001	4.148	3.15	< 0.05	4.148	6.60	< 0.001	4.148	1.43	0.2273	4.148	0.37	0.8309	4.148	5.83	< 0.001	4.148	6.19	< 0.001
condition x measure	8.148	0.37	0.9344	8.148	0.39	0.9267	8.148	1.01	0.4278	8.148	0.34	0.9493	8.148	0.14	0.8683	8.148	0.70	0.6896	8.148	1.03	0.4125
Week-3																					
Surgical condition	2.148	3.64	< 0.05	2.148	0.97	0.3871	2.148	0.0	0.9961	2.148	0.24	0.7906	2.148	0.55	0.5810	2.148	0.01	0.9925	2.148	3.80	< 0.05
Repeated measure	4.148	1.73	0.1470	4.148	10.55	<0.001	4.148	4.22	< 0.01	4.148	6.48	< 0.001	4.148	1.66	0.1634	4.148	4.26	< 0.01	4.148	18.75	< 0.001
condition x measure	8.148	0.65	0.7383	8.148	1.12	0.3529	8.148	1.24	0.2783	8.148	0.82	0.5829	8.148	0.77	0.6301	8.148	1.27	0.2619	8.148	1.52	0.1553
Week-6																					
Surgical condition	2.288	20.20	< 0.001	2.288	8.75	< 0.001	2.288	12.47	< 0.001	2.288	9.92	< 0.001	2.288	0.10	0.9040	2.288	12.21	< 0.001	2.288	3.53	< 0.05
Repeated measure	4.288	7.49	< 0.001	4.288	11.61	< 0.001	4.288	1.06	0.3758	4.288	3.95	< 0.01	4.288	4.15	< 0.01	4.288	1.21	0.3074	4.288	10.81	< 0.001
condition x measure	8.288	0.81	0.5961	8.288	1.05	0.3980	8.288	0.88	0.5351	8.288	1.43	0.1832	8.288	1.01	0.4291	8.288	0.80	0.6011	8.288	0.59	0.7829
Week-10																					
Surgical condition	2.136	5.74	< 0.01	2.136	3.39	< 0.05	2.136	6.23	< 0.01	2.136	3.07	0.0593	1.120	2.90	< 0.05	2.136	5.50	< 0.01	2.136	7.72	< 0.01
Repeated measure	4.136	2.40	0.0530	4.136	3.03	< 0.05	4.136	3.80	< 0.01	4.136	4.29	0.0027	4.120	4.62	< 0.001	4.136	3.91	< 0.01	4.136	1.69	0.1567
condition x measure	8.136	0.96	0.4691	8.136	0.26	0.9769	8.136	0.62	0.7604	8.136	0.48	0.8694	4.120	0.37	0.6467	8.136	0.65	0.7358	8.136	0.54	0.8229

Intake (amount consumed/body weight); Consumption (amount consumed /eating duration)

Two-Way ANOVA test with Bonferroni post-test; not significant differences when P value >0.05; d.f.= degrees of freedom;

Table 1. Statistic analysis for the development of depressive-like behavior following nerve injury in mice. RSB of each experimental group were measured in separate groups of mice at week 1, 3, 6 and 10 post-surgery one a day for five consecutive days. Values are means ± SEM. A two-way ANOVA RM was conducted to explore the effects of surgery condition (PSNL, sham-operated or naïve mice) and repeated measures for each post-injury week on the RSB. Surgery condition factor showed a significative effect on the motivational aspect (Latency to eat and Number of approaches parameters) and also on the consummatory aspect (Amount consumed and Eating duration parameters) of hedonic response to palatable food, at week 6 post-injury. (A) Latency to eat, (B) number of approaches, (C) amount consumed and (D) eating duration.

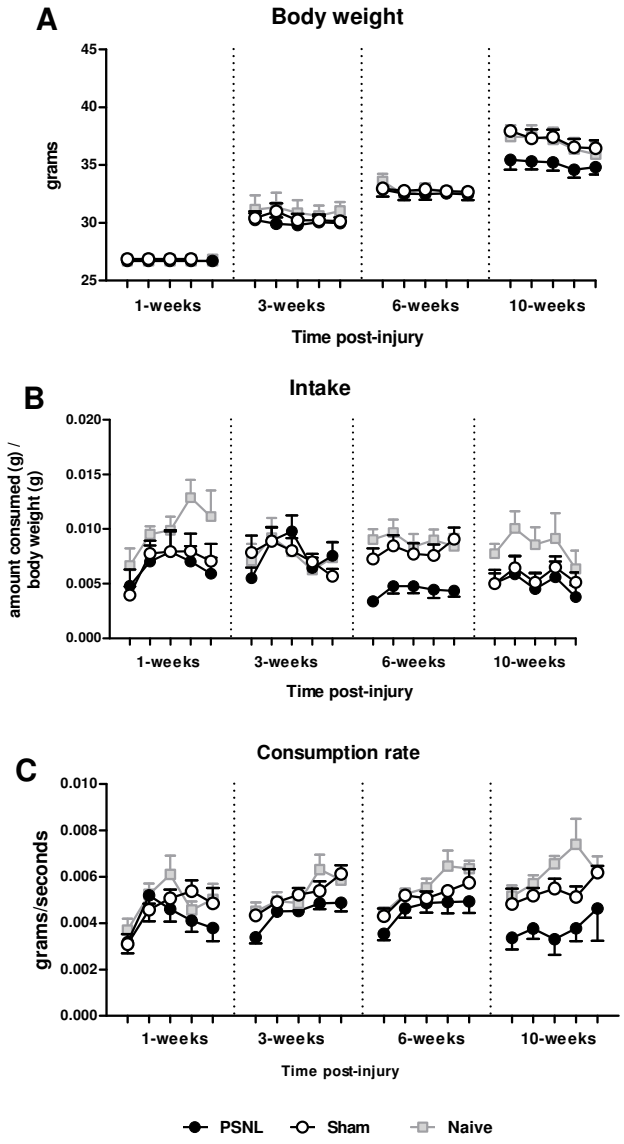


Fig. 4. Effect of neuropathic pain development on the body weight, intake and consumption rate of palatable food. PSNL mice showed a significant weight loss as compared to sham-operated at week 10**.** The decreased palatable food intake in the PSNL mice group at week 6 was significantly lower as compared to sham-operated or naïve mice. At week 10, intake decreased in both PSNL mice and sham-operated mice as compared to naïve mice. In consumption rate, significant differences were observed between PSNL mice and naïve mice only at week 10. Values are means ± SEM. (A) Body weight, (B) intake expressed as grams of white chocolate consumed per animal and (C) consumption rate expressed as grams of white chocolate consumed per second.

Because consumption might be influenced by body size, the amount consumed parameter was normalized per body weight of mouse (grams of chocolate consumed per gram of body weight) in order to obtain the palatable food intake (Fig. 4B). All experimental groups showed similar intakes at week 1 and week 3 post-injury, but the surgery condition significantly altered the intake at week 6 ($F_{2,73} = 11.36$, $P < 0.001$). PSNL mice showed a significantly decreased intake at week 6 as compared to sham-operated (trial 5, $P > 0.01$) or naïve mice (trial 1, $P < 0.001$; trial 2, $P < 0.01$; trials 4 and 5, $P < 0.05$) that was maintained also in week 10. Similarly, at week 10, sham-operated mice showed a decreased intake with values similar to those of PSNL mice ($F_{2,32} = 1.60$, $P > 0.05$) (Fig. 4B).

Consumption rate was expressed as grams of white chocolate consumed per second (Fig. 4C). No obvious differences were observed between groups during week 1 post-injury, as three groups showed similar consumption rates ($F_{2,37} = 0.71$, $P > 0.05$). During weeks 3, 6 and 10 post-injury, however, an effect of the surgery condition was observed at week 3 ($F_{2,185} = 10.96$, $P < 0.001$), week 6 ($F_{2,360} = 8.05$, $P < 0.001$) and week 10 ($F_{2,185} = 12.84$, $P < 0.001$). PSNL mice showed a decreased consumption rate at week 10 as compared to sham-operated or naïve mice, and *post hoc* tests showed significant differences in trial 4 ($P < 0.05$, compared to naïve mice).

Sigma-1 receptor knock-out mice did not develop depressive-like behaviours following partial sciatic nerve ligation injury.

The effect of σ_1R deletion on the development of depressive-like behaviours as a consequence of neuropathic pain was studied (Fig. 5). Based on the previous results, the week 6 after injury was selected to evaluate the depressive-like behaviours. A significant effect of surgery condition factor was observed in the latency to eat ($F_{2,18} =$

14.96, $P < 0.001$), with significant differences between PSNL mice and sham-operated mice or naïve mice ($P < 0.05$ in all trials, except trial 2). The surgery condition also displayed a significant effect on the number of approaches and on the amount consumed ($F_{2,18} = 4.24$, $P < 0.05$; ($F_{2,18} = 4.95$, $P < 0.05$, respectively) but not on the eating duration ($F_{2,72} = 2.43$, $P > 0.05$) in WT mice. Unlike in WT mice, this surgery condition factor failed to show statistical significant effects on any of the RSB-related parameters evaluated in $\sigma_1R^{-/-}$ mice (see Table 2 for detailed statistical results).

Table 2. Statistical analysis of the effect of genetic blockade of σ_1R on the development of depressive-like behaviours following nerve injury in mice. The data from the behavioural studies performed at week 6 post-injury were statistically tested with two-way ANOVA with surgery condition (PSNL, sham-operated or non-manipulated/naïve mice) and repeated measures (one trial a day for five consecutive days) as independent variables. Comparisons were made to identify the effect of injury on the lack of σ_1R. Data show the statistical analysis obtained for the four parameters evaluated in RSB (latency to eat, number of approaches, amount consumed, and eating duration).

	Latency to eat			Number of approaches			Amount consumed			Eating duration		
	d.f.	*F-value*	*P*	*d.f.*	*F-value*	*P*	*d.f.*	*F-value*	*P*	*d.f.*	*F-value*	*P*
Wild-type mice												
Surgical condition	2,72	14.96	< 0.001	2,72	4.24	< 0.05	2,72	4.95	< 0.05	2,72	2.43	0.1160
Repeated measure	4,72	2.30	0.0673	4,72	4.42	< 0.01	4,72	0.22	0.9273	4,72	3.22	< 0.05
Condition x measure	8,72	0.65	0.7323	8,72	0.61	0.7669	8,72	0.32	0.9540	8,72	1.43	0.1972
$\sigma_1R^{-/-}$ mice												
Surgical condition	2,64	0.67	0.5259	2,64	0.88	0.4328	2,64	0.44	0.6497	2,64	0.13	0.8808
Repeated measure	4,64	5.42	< 0.001	4,64	15.29	< 0.001	4,64	9.45	< 0.001	4,64	16.40	< 0.001
Condition x measure	8,64	1.34	0.2389	8,64	1.04	0.4151	8,64	0.80	0.6079	8,64	0.75	0.6465

Two-Way ANOVA test with Bonferroni post-test; not significant differences when P value >0.05; d.f.= degrees of freedom.

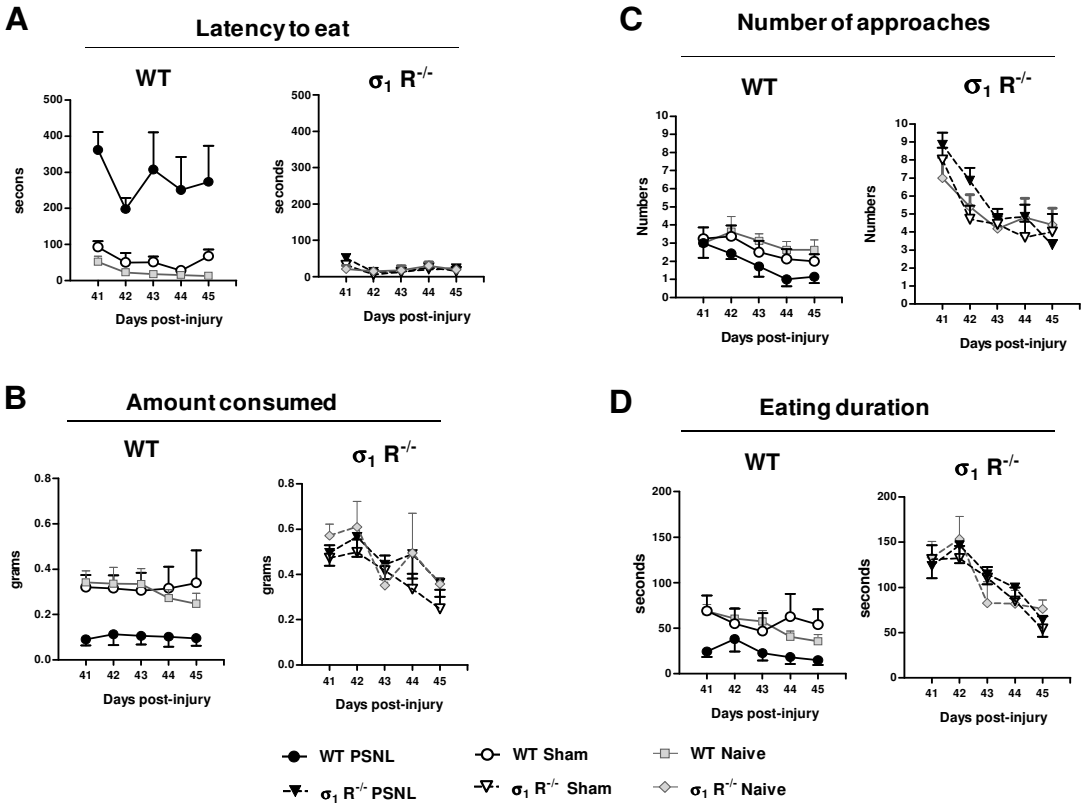


Fig. 5. Effect of genetic blockade of σ_1R on the development of depressive-like behaviours following nerve injury in mice. PSNL-related depression of RSB was not observed in the $\sigma_1R^{-/-}$ genotype. WT mice showed significant differences as compared to PSNL mice and sham-operated mice or naïve mice in the four parameters evaluated. Anhedonic-like behaviours, defined as higher latency to eat, lower number of approaches, lower amount consumed and shorter time spent on consuming palatable food as compared to naïve mice, were not observed in $\sigma_1R^{-/-}$ mice. Therefore, no differences between PSNL mice and sham-operated mice or naïve mice were observed in any behavioural parameter evaluated in this genotype. (A) Latency to eat, (B) amount consumed, (C) number of approaches, and (D) eating duration.

In summary, the absence of the gene for σ_1R resulted in no differences between the experimental groups evaluated (PSNL, sham and naïve mice). Unlike WT mice, PSNL, sham and naïve mice showed the same hedonic response.

The sigma-1 antagonist reversed the anhedonic-like behaviour elicited by neuropathic pain.

The same Latin square design used to test E-52862 in the sensory-discriminative component of chronic neuropathic pain (mechanical hypersensitivity) was used to test E-52862 in the affective-motivational component of chronic neuropathic pain. A group of PSNL mice showed values similar to sham-operated mice, thus suggesting that they did not develop this depressive-like behaviour (Fig. 6). Therefore, only PSNL mice showing a consumption below 0.2 g were included in the analysis.

The results of the administration of E-52862 to prevent PSNL-related depression of RSB in WT mice are shown in Fig. 7. WT PSNL mice administered with vehicle showed a higher latency to eat as compared to sham-operated mice ($P < 0.01$). The administration of E-52862 to PSNL mice dose-dependently restored the latency to eat to sham-operated mice levels ($P < 0.05$, 20 mg/kg vs. vehicle) (Fig. 7A). PSNL mice administered with vehicle consumed a lower amount of white chocolate as compared to sham-operated mice ($P < 0.05$). The administration of E-52862 to PSNL mice also exerted a dose-dependent effect on this parameter and restored chocolate consumption at the dose of 40 mg/kg ($P < 0.01$, vs. vehicle) (Fig. 7C). The analgesic-like effects of E-52862 calculated with regard to sham-vehicle mice in the latency to eat were 96.9% at the dose of 20 mg/kg, and 80.0% effect at the dose of 40 mg/kg in the amount consumed.

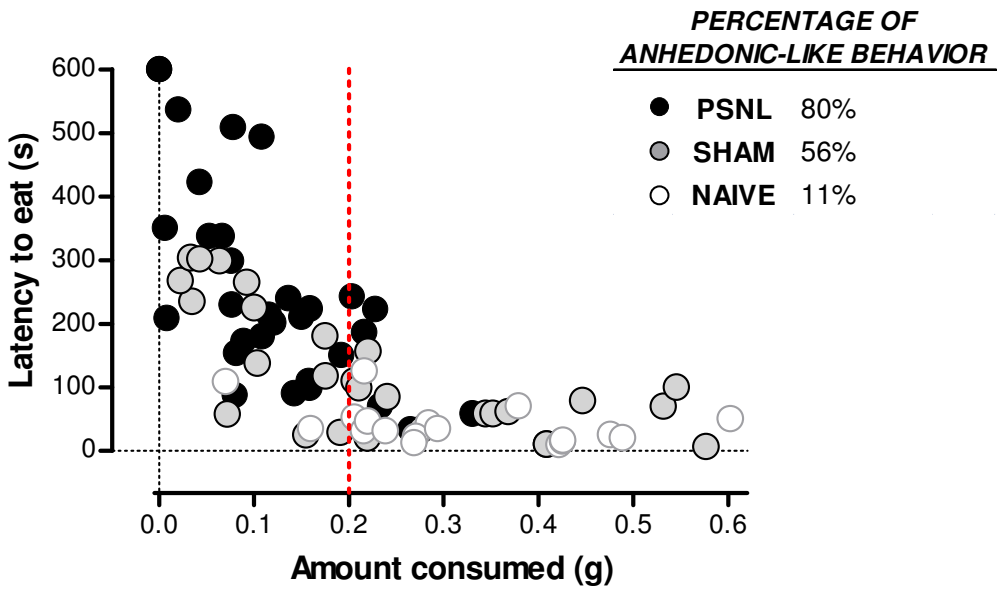


Fig. 6. Correlation data of latency to eat and amount consumed for each experimental group tested for the first time in the RSB test at week 6. Naïve mice consumed an average of 0.3 g of white chocolate and the mean consumption for PSNL mice was 0.14 g. However, a small group of PSNL mice showed values similar to naïve mice, thus suggesting that they did not develop this depressive-like behaviour. The figure also shows that only 2 out of 18 naïve mice consumed less than 0.2 g (11%). We therefore considered that a consumption of less than 0.2 g was an adequate criterion to define anhedonia in each mouse. The figure also shows the percentage of anhedonic mice (mice that ate less than 0.2 g) observed in the different experimental groups evaluated. The red dashed vertical line marks this value.

Finally, while there is a tendency to a shorter time spent on consuming chocolate in PSNL mice as compared to sham-operated mice, no significant differences were observed in eating duration in PSNL mice and sham-operated mice administered with the vehicle ($P > 0.05$). E-52862 also showed a tendency to restore the eating duration to control values, but no significant differences were detected for this parameter at any dose tested (Fig. 7D). While no statistical differences in the number of approaches were obtained between these groups when the vehicle was administered, a statistical

significance between PSNL and sham-operated groups was observed after the washout period ($P < 0.01$) (Fig. 7B).

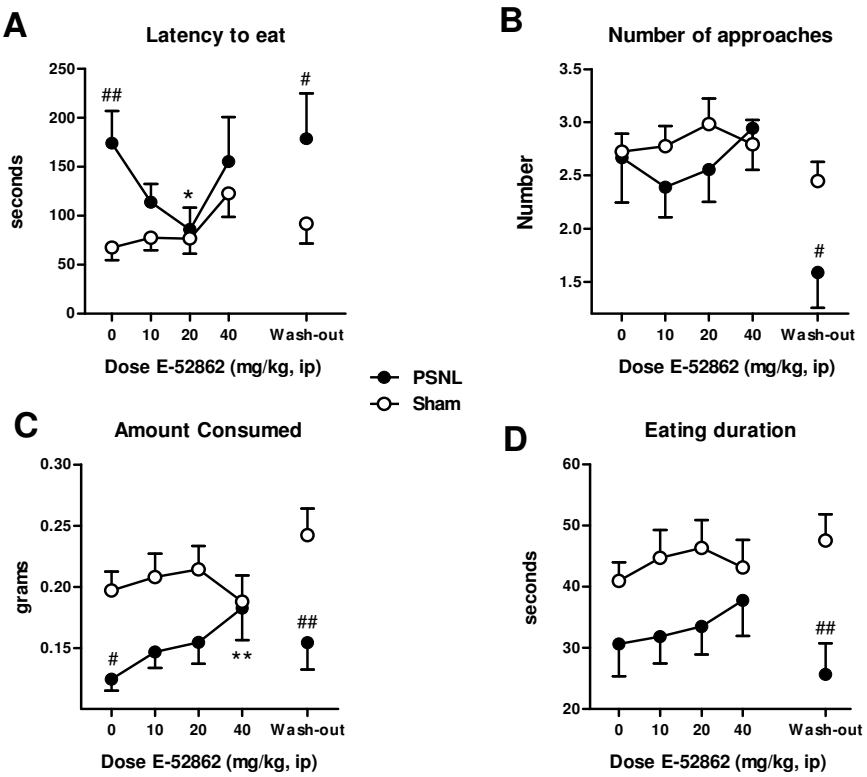


Fig. 7. Effect of the σ_1R antagonist E-52862 on neuropathic pain-induced depression in mice. E-52862 restored RSB deficits exhibited in neuropathic mice. The latency to eat was more sensitive to the σ_1R antagonist effect than the amount consumed. Deficits observed in these parameters were restored by E-52862 to sham-operated mice values at 20 mg/kg and 40 mg/kg, respectively. E-52862 was administered intraperitoneally 30 minutes prior to the RSB test in a Latin-square dose design. Values are shown as means ± SEM. *$P < 0.05$, **$P < 0.01$ vs. vehicle, #$P < 0.05$, ##$P < 0.01$, vs. sham-operated mice. Bonferroni's *post hoc* test. (A) Latency to eat, (B) amount consumed, (C) number of approaches, and (D) eating duration.

Discussion

In this study we have compared the involvement of σ1R on the sensory-discriminative and the affective-motivational component of neuropathic pain evidenced by sensitivity to a non-noxious stimulus (allodynia) and the depression of reward seeking behaviour in mice. We have evaluated the time course of both paw hypersensitivity (sensory) and anhedonia (affective). Anhedonia was evidenced by changes in a reward seeking behaviour (RSB) paradigm previously used in an AA-induced visceral pain model (de la Puente et al, 2015). The main findings of this study were: (i) peripheral nerve injury produced a depressive-like behaviour as evidenced by the long-lasting depression of both the appetitive and consummatory components of RSB in mice; (ii) this depressive-like behaviour was present beyond the resolution of mechanical hypersensitivity; (iii) σ1R-/- mice did not develop depressive like behaviours; (iv) both mechanical hypersensitivity and depressive-like behaviours were dose-dependently attenuated by treatment with E-52862 in WT mice; and (v) E-52862 was more potent in reversing PSNL-induced changes in RSB than in PSNL-induced mechanical hypersensitivity.

The sensory-discriminative (mechanical hypersensitivity) studies were performed in female mice for comparison with the affective-motivational studies where females were used on the basis of studies of reports which indicate that females are more susceptible to psychological distress and severe pain than their male counterparts (Mogil and Chanda, 2005; Breivik *et al.*, 2006; Theis *et al.*, 2007; Fillingim *et al.*, 2009).

Mechanical hypersensitivity was clearly observed in female WT mice but not in female $\sigma_1R^{-/-}$ mice. The absence of mechanical hypersensitivity in PSNL $\sigma_1R^{-/-}$ mice remained stable over time. These results are consistent with previous results where the lack of σ_1R

failed to induce mechanical hypersensitivity after partial sciatic nerve injury in male mice (de la Puente *et al*., 2009). However, mechanical hypersensitivity was progressively increased (decreased nociceptive threshold), beginning on day 4 after injury in PSNL WT mice reaching the lowest value at 3 weeks post-injury. A gradual reduction of injury-induced mechanical hypersensitivity over time until total recovery at week 9 post-injury was observed. The results were also similar to data previously obtained by Rácz and co-workers (Rácz *et al*., 2015), where PSNL-induced mechanical allodynia was undetectable 8 weeks after nerve ligation.

The role of σ_1R in the sensory-discriminative component of chronic pain was also analysed by evaluating the effect of the pharmacological antagonism of σ_1R on the nociceptive reflex responses exhibited in PSNL WT mice. The efficacy and potency of the σ_1R antagonist E-52862 to reduce mechanical hypersensitivity secondary to neuropathic pain was maximum and well within the range of doses where E-52862 was active in other pain models, including chemical sensitization with capsaicin (ED_{50} = 26.3 ± 5.5 mg/kg), formalin (ED_{50} = 40.0 ± 5.2 mg/kg), CFA (42.1± 7.1 mg/kg) or carrageenan (35.9 ± 9.8 mg/kg) (Gris *et al.*, 2014), and PSNL-induced mechanical allodynia (ED_{50} = 23.4 ± 0.9 mg/kg) (Romero *et al.*, 2012). Our results showed that the ED_{50} of E-52862 was higher than that obtained by Romero and co-workers in the same model of neuropathic pain in male mice, thus suggesting the possibility of gender influences.

Interestingly, a different time profile between paw hypersensitivity (sensory) and anhedonia (affective) was found. Mice developed mechanical hypersensitivity from 1 to 8 weeks after neuropathy induction, and depression-related behaviours were observed from 6 to 10 weeks after injury. Surprisingly, 10 weeks post-injury, sham-operated mice

displayed a decreased hedonic response to palatable food as compared to naïve mice. In fact, they were indistinguishable from PSNL mice. These results suggest that sham-operated mice suffer some affective consequences caused by surgery with no obvious sensory abnormalities. During the sham surgical procedure the sciatic nerve is not ligated but a wound is performed on the skin and on the right leg biceps femoris which is closed with a permanent single muscle suture. Both wound pain and chronic muscle pain are significant clinical problems affecting many people and have been related to poor adaptation to living with a wound, such as depression and low self-esteem (Kehlet *et al*., 2006; Solowiej *et al*., 2009). Peripheral muscle alterations are parts of the activated pain mechanisms in common chronic pain conditions such as fibromyalgia syndrome, chronic widespread pain, or post-operative neuropathic pain (Gerdle *et al*., 2014). Thus, it is likely that sham-operated mice were suffering a chronic pain condition from a muscle and/or wound-related origin (Arendt-Nielsen and Graven-Nielsen, 2011). To our knowledge this is the first time that affective consequences caused by this surgical procedure has been reported in mice. This finding could be useful to model chronic muscle pain or post-operative neuropathic pain. Further studies are needed to determine whether sham-operated mice can be considered a new model of chronic pain.

Globally, our time course experiments show that the sensory and affective consequences of neuropathic pain evolve differently over time, and that affective alterations can persist with no obvious sensory abnormalities. Similar results have been previously observed under acute visceral and chronic neuropathic pain conditions (de la Puente, *et al*, 2015; Yalcin *et al*., 2011).

A significantly decreased body weight of PSNL mice at week 10 together with a decreased in their consumption rate were observed. These results suggest that PSNL mice showed modifications in their appetitive behaviour to standard food as an indication of depressive-like behaviour. These alterations in PSNL mice may mimic the weight loss or appetite changes modifications observed in depressed patients (DSM-IV).

In the present study we analysed whether the lack of σ_1R in $\sigma_1R^{-/-}$ mice could protect from development of pain-induced mood alterations as reported for pain-induced sensory hypersensitivity. Interestingly, the deficits in the hedonic responses to palatable food observed in WT mice were not observed in $\sigma_1R^{-/-}$ mice. PSNL, sham-operated and naïve $\sigma_1R^{-/-}$ mice exhibited similar hedonic behaviour in all parameters evaluated in RSB. These results suggest that σ_1R was necessary for the development of pain-related anhedonia. However, significant differences were also found between wild-type and $\sigma_1R^{-/-}$ mice during the baseline RSB test for eating duration and amount consumed. Because previous studies suggest several potential mechanisms through which σ_1R may modulate dopamine and opioid systems (Fu *et al.*, 2010; Navarro *et al.*, 2010), σ_1R could be involved in the processing of natural rewards (Le Merrer *et al.*, 2009; Baik, 2013). $\sigma_1R^{-/-}$ mice could develop compensatory changes which could explain these significant differences in the consummatory response between both genotypes. However, the objective of this study was to evaluate whether AA administration could induce the RSB deficits found in WT mice, in $\sigma_1R^{-/-}$ mice. Therefore, these findings should not affect the main conclusion of this study.

We also found that pharmacological treatment with the σ_1R antagonist E-52862 dose-dependently reversed the anhedonic-like behaviour shown after partial sciatic nerve injury. However, the efficacy of E-52862 in mechanical hypersensitivity

following PSNL was only partial at the dose of 40 mg/kg (52%). Moreover, the dose of 20 mg/kg only showed a low non significant antihypersensitivity effect (21%). E-52862, in contrast, was able to fully restore the normal RSB of PSNL mice at doses of 20 and 40 mg/kg for latency to eat and amount consumed, respectively. These pharmacological experiments showed that the affective-motivational component of neuropathic pain was more sensitive to the analgesic-like effect of E-52862 than the sensory-discriminative component of neuropathic pain: it is four times more potent in the affective-motivational than in the sensory-discriminative component.

In summary, a different time profile for the sensory and the affective consequences of neuropathic pain has been shown. The genetic and pharmacological study showed that σ_1R blockade suppresses nociceptive reflex behaviours in a peripheral nerve injury model in mice, thus supporting the hypothesis that σ_1R plays a role in the expression and development of sensory-discriminative component of chronic neuropathic pain. Furthermore, the genetic and pharmacological results also suggest that σ_1R plays an important role in the expression and development of anhedonic-like behaviours causally associated with neuropathic pain. Therefore, the results of the present study support the pharmacological modulation of σ_1R as useful strategy to manage both the sensory and mood alterations of neuropathic pain.

References

Baron R, Binder A, Wasner G. Neuropathic pain: diagnosis, pathophysiological mechanisms, and treatment. Lancet Neurol. 2010 Aug;9(8):807-19.

Breivik H, Collett B, Ventafridda V, Cohen R, Gallacher D. Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. Eur J Pain. 2006 May;10(4):287-333.

Burgess G, Williams D. The discovery and development of analgesics: new mechanisms, new modalities. J Clin Invest. 2010 Nov;120(11):3753-9.

Cendán CM, Pujalte JM, Portillo-Salido E, Montoliu L, Baeyens JM. Formalin-induced pain is reduced in sigma(1) receptor knockout mice. Eur J Pharmacol. 2005 Mar;511(1):73-4.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods. 1994 Jul;53(1):55-63.

de la Puente B, Nadal X, Portillo-Salido E, Sánchez-Arroyos R, Ovalle S, Palacios G, Muro A, Romero L, Entrena JM, Baeyens JM, López-García JA, Maldonado R, Zamanillo D, Vela JM. Sigma-1 receptors regulate activity-induced spinal sensitization and neuropathic pain after peripheral nerve injury. Pain. 2009 Oct;145(3):294-303.

de la Puente B, Romero-Alejo E, Vela JM, Merlos M, Zamanillo D, Portillo-Salido E. Changes in saccharin preference behaviour as a primary outcome to evaluate pain and analgesia in acetic acid-induced visceral pain in mice. J Pain Res. 2015 Oct;8:663-673.

Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. OJ L. 2010;276:33–79. The European Parliament and the Council of the European Union. [Internet]. 2010. [Cited 19 June 2014]. Available at: <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF>

DMM-IV. Diagnostic and Statistical Manual of Mental Disorders, 4th Edition. Washington, D.C., American Psychiatric Press, 2000.

Drews E, Zimmer A. Central sensitization needs sigma receptors. Pain. 2009 Oct;145(3):269-70.

Entrena JM, Cobos EJ, Nieto FR, Cendán CM, Gris G, Del Pozo E, Zamanillo D, Baeyens JM. Sigma-1 receptors are essential for capsaicin-induced mechanical hypersensitivity: studies with selective sigma-1 ligands and sigma-1 knockout mice. Pain. 2009 Jun;143(3):252-61.

Fillingim RB, King CD, Ribeiro-Dasilva MC, Rahim-Williams B, Riley JL 3rd. Sex, gender, and pain: a review of recent clinical and experimental findings. J Pain. 2009 May;10(5):447-85.

González-Cano R, Merlos M, Baeyens JM, Cendán CM. σ1 receptors are involved in the visceral pain induced by intracolonic administration of capsaicin in mice. Anesthesiology. 2013 Mar;118(3):691-700.

Gris G, Cobos EJ, Zamanillo D, Portillo-Salido E. Sigma-1 receptor and inflammatory pain. Inflamm Res. 2015 Jun;64(6):377-81.

Gris G, Merlos M, Vela JM, Zamanillo D, Portillo-Salido E. S1RA, a selective sigma-1 receptor antagonist, inhibits inflammatory pain in the carrageenan and complete Freund's adjuvant models in mice. Behav Pharmacol. 2014 Jun;25(3):226-35.

Kinloch RA, Cox PJ. New targets for neuropathic pain therapeutics. Expert Opin Ther Targets. 2005 Aug;9(4):685-98.

Krebs H, Weyers P, Macht M, Weijers HG, Janke W. Scanning behavior of rats during eating under stressful noise. Physiol Behav. 1997 Jul;62(1):151-4.

Latremoliere A, Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J Pain. 2009 Sep;10(9):895-926.

Malmberg AB, Basbaum AI. Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. Pain. 1998 May;76(1-2):215-22.

McDougall JJ. Peripheral analgesia: Hitting pain where it hurts. Biochim Biophys Acta. 2011 Apr;1812(4):459-67.

Mogil JS, Chanda ML. The case for the inclusion of female subjects in basic science studies of pain. Pain. 2005 Sep;117(1-2):1-5.

Nieto FR, Cendán CM, Sánchez-Fernández C, Cobos EJ, Entrena JM, Tejada MA, Zamanillo D, Vela JM, Baeyens JM. Role of sigma-1 receptors in paclitaxel-induced neuropathic pain in mice. J Pain. 2012 Nov;13(11):1107-21.

Rácz I, Nent E, Erxlebe E, Zimmer A. CB1 receptors modulate affective behaviour induced by neuropathic pain. Brain Res Bull. 2015 May;114:42-8.

Romero L, Zamanillo D, Nadal X, Sánchez-Arroyos R, Rivera-Arconada I, Dordal A, Montero A, Muro A, Bura A, Segalés C, Laloya M, Hernández E, Portillo-Salido E, Escriche M, Codony X, Encina G, Burgueño J, Merlos M, Baeyens JM, Giraldo J, López-García JA, Maldonado R, Plata-Salamán CR, Vela JM. Pharmacological properties of S1RA, a new sigma-1 receptor antagonist that inhibits neuropathic pain and activity-induced spinal sensitization. Br J Pharmacol. 2012 Aug;166(8):2289-306.

Theis KA, Helmick CG, Hootman JM. Arthritis burden and impact are greater among U.S. women than men: intervention opportunities. J Womens Health (Larchmt). 2007 May;16(4):441-53.

Whishaw IQ, Dringenberg HC, Comery TA. Rats (Rattus norvegicus) modulate eating speed and vigilance to optimize food consumption: effects of cover, circadian rhythm, food deprivation, and individual differences. J Comp Psychol. 1992 Dec;106(4):411-9.

Yalcin, I., Bohren, Y., Waltisperger, E., Sage-Ciocca, D., Yin, J.C., Freund-Mercier, M.J., Barrot, M., 2011. A time-dependent history of mood disorders in a murine model of neuropathic pain. Biol Psychiatry. 2011 Nov;70(10):946-53.

Yalcin I, Barrot M. The anxiodepressive comorbidity in chronic pain. Curr Opin Anaesthesiol. 2014 Oct;27(5):520-7.

Zimmermann M (1983). Ethical guidelines for investigations of experimental pain in conscious animals. Pain. 1983 Jun;16(2):109-10.

VI. General Discussion

The overall purpose of this Doctoral Thesis was to explore the role of σ_1R in the sensory-discriminative and affective-motivational components of pain. To this end, acetic acid-induced visceral pain and partial sciatic nerve ligation in mice were used to model an acute and chronic pain state, respectively. The sensory-discriminative and affective-motivational components of pain in these models were infered by changes in reflexive and non-reflexive behavioural outcomes, respectively. Abdominal contractions (writhing) and paw behavioural hypersensitivity to mechanical and thermal stimuli were analysed as reflexive outcomes. On the other hand, pain-related changes of sweet preference, locomotor activity and reward-seeking behaviour were analysed as non-reflexive outcomes. These models and behavioural outcomes were used to evaluate the effects of pharmacological and genetic blockade of σ_1R and the effects of some reference compounds. Furthermore, electrophysiological and molecular studies in $\sigma_1R^{-/-}$ mice were used to understand the role of σ_1R in the sensory-discriminative component of chronic pain.

To study the affective-motivational component of pain we have used some innovative methodological approaches which are a step forward to improve the modelling of pain phenomena in rodents. The analysis of pain-induced changes in a non-operant motivational task like the reward-seeking behaviour has allowed us to find a differential sensitivity to pain and analgesics depending on the different phases of the motivated behaviour. Approach motivation was more sensitive to noxious stimulus and to the analgesic-like effects than consummatory behaviours as evidenced by the dose response and time course studies on AA-induced changes in RSB (Article 3). AA dose response study found that while the approach-related parameters were not significantly affected by the dose of 0.6% AA, the consummatory-related parameters were already reduced by

approximately 50%. The time-course study found a partial recovery after 3 hours of the approach-related parameters of AA-treated mice whereas the amount consumed was totally absent at that time point. Finally, we have also found that active drugs were more potent in restoring the approach-motivation aspect of the hedonic response than in restoring the consummatory aspect of it. A possible evolutionary explanation for these results is suggested since strong impediments in approach motivation —which occur before the consummatory-related behaviours— could seriously affect body survival. At the same time, a faster recovery of motivation for natural rewards likely improves body survival.

In addition, we found that the affective-motivational component was more sensitive to the analgesic-like effect than the sensory-discriminative component. In fact, we have clearly showed that inactive doses of analgesics in the sensory component were actually active in the affective component. Similar results have also been obtained in preclinical and clinical research (see Cobos and Portillo-Salido, 2013 for review and Table 2 from the Introduction of this Doctoral Thesis). The RSB endpoint was extraordinarily more sensitive to the analgesic-like effects than the classic sensory-related endpoint in the acute pain model. This was clearly evident for ibuprofen and diclofenac which were much more potent to restore the deficits in the approach-motivation parameter in the RSB task than to inhibit the reflexive endpoint of writhing in the visceral pain model. This particular resistance to drug-induced inhibition of noxious-induced reflexive behaviours could make sense again, from an evolutionary perspective. Reflexive responses to noxious stimulation could occur in virtually all living species possessing nervous systems. Thus, some of these mechanisms may be quite primitive and drug-induced impediments in the expression of sensory-reflexive behaviours can be hard to

achieve. In fact, reflexive behaviour can often be observed in decerebrated animals (Vierck *et al*., 2008) and humans with extensive damage of the cerebral hemispheres can still respond to noxious stimuli (however they are unconscious and unable to experience pain) (Sneddon *et al*., 2003).

Table 1. Role of σ_1R in the sensory-discriminative component of pain (A) and the affective-motivational component of pain (B). The effects of the reference drugs used to validate some methods developed for this Doctoral Thesis are also showed.

(A) Sensory-discriminative component of pain

Injury	Endpoint	WT mice	$\sigma_1R^{-/-}$ mice	Pharmacological blockade of σ_1R (E-52862)	Pharmacological effects of reference drugs
AA	Writhing reflex	Increased	Similar to WT	100% Effect	All 100%, except caffeine = no effect
PSNL	Mechanical sensitivity	Increased	No developed	100% Effect	N.T
	Thermal (hot) sensitivity	Increased	Similar to WT	N.T	N.T
	Thermal (cold) sensitivity	Increased	Attenuated	N.T	N.T

(B) Affective-motivational component of pain

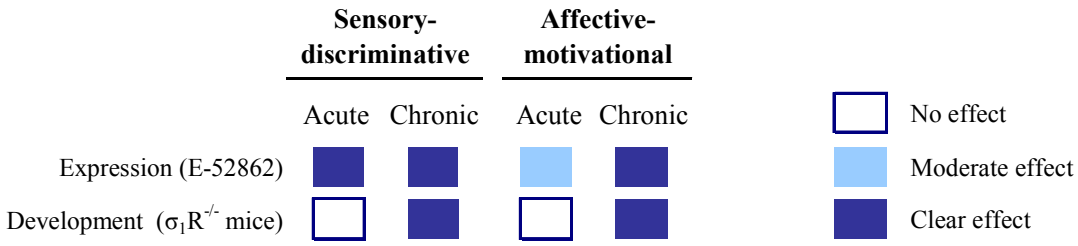
Injury	Endpoint	WT mice	$\sigma_1R^{-/-}$ mice	Pharmacological blockade of σ_1R (E-52862)	Pharmacological effects of reference drugs
AA	Sweet preference	Decreased	N.T.	N.T.	100% Ibuprofen
	Locomotor activity	Decreased	N.T.	N.T.	100% Ibuprofen
	Reward-seeking behaviour	Decreased	Similar to WT	Effective on approach motivation but not on consumption	More potent on approach motivation than consumption
PSNL	Sweet preference	No changes	N.T.	N.T.	N.T.
	Locomotor activity	N.T.	N.T.	N.T.	N.T.
	Reward-seeking behaviour	Decreased	No developed	100% Effect	More potent on approach motivation than consumption

N.T.: not tested

All together these results showing that inactive doses of analgesics in the sensory component were actually active in the affective component are important in a drug discovery context because possible analgesics may be currently being discarded based on a lack of efficacy on sensory-based pain screening experimental models. Finally, a further interesting finding was that the sensory and affective consequences of neuropathic pain evolve differently over time, and that affective alterations can persist with no obvious sensory abnormalities. Interestingly, we found that sham-operated mice showed depressive-like behaviours as compared to naïve mice 10 weeks after the surgery which could be considered as a new model of chronic post-operative pain.

The use of $\sigma_1R^{-/-}$ mice showed that σ_1R was necessary for the development of both behavioural hypersensitivity and the depressive-like phenotype observed after partial sciatic nerve ligation (Tables 1 and 2). $\sigma_1R^{-/-}$ mice also showed an attenuation in the number of action potentials induced by repeated electrical C-fibres stimulation on the dorsal root and in the increased phosphorylation of ERK in the spinal cord of mice exposed to partial sciatic nerve injury. These molecular and electrophysiological studies performed in the chronic neuropathic pain model provided, for the first time, a mechanistic explanation for the "pain-resistant" behavioural phenotype of $\sigma_1R^{-/-}$ mice. Overall, these results strongly support, for the first time, that σ_1R plays a key role in both the sensory-discriminative and affective-motivational components of chronic pain.

Table 2. Schematic representation of the overall effect of σ_1R in the sensory-discriminative and affective-motivational components of acute and chronic pain based on the results of this Doctoral Thesis.



In contrast, the genetic absence of σ_1R failed to modify the writhing behaviours and the depressed RSB of the acute visceral pain model, thus suggesting that σ_1R was not necessary for the expression of the sensory-discriminative and affective-motivational component of pain. Because neuropathic pain and visceral pain are known to involve different pathways (Ness and Gebhart, 1990; Sikandar and Dickenson, 2012), the different phenotypes observed in $\sigma_1R^{-/-}$ mice suggest —depending on the pain model— a distinct involvement of the σ_1R system in the mechanisms underlying each type of pain.

The use of the selective σ_1R antagonist E-52862 showed that the pharmacological blockade of σ_1R fully inhibited writhing in the acute visceral pain model, and also behavioural hypersensitivity to mechanical stimuli induced by sciatic nerve injury with similar potency (Table 1). These results suggest that acute pharmacological modulation of σ_1R was sufficient to have an impact on the sensory-discriminative component of both acute and chronic pain. Interestingly, we also found that E-52862 induced a recovery of the normal emotional functioning of neuropathic mice by restoring the

reward-seeking behaviour —the "affective outcome" being 4 times more sensitive than the "sensory outcome" (paw hypersensitivity).

In summary, all these results suggest that affective outcomes were more sensitive to analgesics than sensory outcomes, as well as a prominent role of σ_1R in both the sensory-discriminative and affective-motivational components of pain (particularly in chronic pain). Finally, the present results support moving E-52862 forward into clinical development as a new therapeutic approach to neuropathic pain and its mood-related comorbidities.

VII. Conclusions

1. Both sensory-discriminative and affective-motivational components of acute pain were evidenced by the appearance of abdominal contractions (pain-stimulated behaviour) and decreases in the expression of sweet preference behaviour or novelty-induced locomotor activity (pain-depressed behaviours), respectively.

2. Pain-related depression of sweet saccharine preference failed to detect the affective-motivational component of chronic neuropathic pain.

3. Pain-depressed behaviours are much more sensitive to the analgesic effects of drugs that pain-stimulated behaviour.

4. The new reward-seeking behaviour (RSB) task performed in our laboratory may be useful as a sensitive behavioural test for preclinical studies to assess both pain-related functional impairment of motivated behaviour and restoration of pain-depressed behaviour by candidate analgesic drugs.

5. The sensory-discriminative component of chronic neurophatic pain is functionally dissociated from its affective-motivational component as evidenced by the different time-course of the behavioural expression of both components.

6. The σ_1R is necessary for the development of the behavioural manifestations of both the sensory-discriminative and the affective-motivational component of chronic neuropathic pain, but not for acute visceral pain.

7. The σ_1R play a key role in the modulation of physiological and molecular mechanism involved in neuropathic pain.

8. The pharmacological blockade of σ_1R prevent the expression of behavioural manifestations of the sensory-discriminative and the affective-motivational components of both acute (partially) and chronic pain (fully).

9. σ_1R blockade could have beneficial effect on both behavioural hypersensitivity and mood complications of neuropathic pain.

The σ_1R plays a significant role in both the affective-motivational and sensory-discriminative components of pain and σ_1R blockade could have beneficial effect on behavioural hypersensitivity and the mood complications of neuropathic pain.

VIII. References

Abadias M, Escriche M, Vaqué A, Sust M, Encina G. Safety, tolerability and pharmacokinetics of single and multiple doses of a novel sigma-1 receptor antagonist in three randomized phase I studies. Br J Clin Pharmacol. 2013; 75:103-117.

Albe-Fessard D, Berkley KJ, Kruger L, Ralston III HJ, Willis Jr WD. Diencephalic mechanisms of pain sensation. Brain Res. 1985; 356:217-296.

Almansa C, Vela JM. Selective sigma-1 receptor antagonists for the treatment of pain. Future Med Chem. 2014; 6:1179-99.

Alonso G, Phan VL, Guillemain I, Saunier M, Legrand A, Anoal M, Maurice T. Immunocytochemical localization of the sigma1 receptor in the adult rat central nervous system. Neuroscience 2000; 97:155-70.

Alvarez-Leefmans FJ. Chloride transporters in presynaptic inhibition, pain and neurogenic inflammation. In: Alvarez-Leefmans FJ, Delpire E (Eds). Physiology and Pathology of Chloride Transporters and Channels in the Nervous System. 2009; pp. 439-470. New York, New York, USA: Academic Press.

Amir R, Devor M. Electrical excitability of the soma of sensory neurons is required for spike invasion of the soma, but not for through-conduction. Biophys J. 2003; 84:2181-2191.

Andrews PW, Thomson JA. The bright side of being blue: depression as an adaptation for analyzing complex problems. Psychol Rev. 2009; 116:620-654.

Apkarian AV, Sosa Y, Krauss BR, Thomas PS, Fredrickson BE, Levy RE, Harden RN, Chialvo DR. Chronic pain patients are impaired on an emotional decision-making task. Pain 2004a; 108:129-136.

Apkarian AV, Sosa Y, Sonty S, Levy RM, Harden RN, Parrish TB, Gitelman DR. Chronic back pain is associated with decreased prefrontal and thalamic gray matter density. J Neurosci. 2004b; 24:10410-10415.

Apkarian AV, Bushnell MC, Treede RD, Zubieta JK. Human brain mechanisms of pain perception and regulation in health and disease. Eur J Pain 2005; 9:463-484.

Apkarian AV. Pain perception in relation to emotional learning. Curr Opin Neurobiol. 2008; 18:464-468.

Apkarian AV, Baliki MN, Geha PY. Towards a theory of chronic pain. Prog Neurobiol. 2009; 87:81-97.

Arendt-Nielsen L, Graven-Nielsen T. Translational musculoskeletal pain research. Best Pract Res Clin Rheumatol. 2011; 25:209-26.

Auvray M, Myin E, Spence C. The sensory-discriminative and affective-motivational aspects of pain. Neurosci Biobehav Rev. 2010; 34:214-23.

Aydar E, Palmer CP, Klyachko VA, Jackson MB. The sigma receptor as a ligand-regulated auxiliary potassium channel subunit. Neuron 2002; 34:399-410.

Backonja, MM. Defining neuropathic pain. Anesth Analg. 2003; 97:785-790.

Bahia PK, Suzuki R, Benton DC, Jowett AJ, Chen MX, Trezise DJ, Dickenson AH, Moss GW. A functional role for small-conductance calcium-activated potassium channels in sensory pathways including nociceptive processes. J Neurosci. 2005; 25:3489-98.

Baik JH. Dopamine signaling in reward-related behaviors. Front Neural Circuits 2013; 7:152.

Bair MJ, Robinson RL, Katon W, Kroenke K. Depression and pain comorbidity: a literature review. Arch Intern Med. 2003; 163:2433-45.

Baliki MN, Chialvo DR, Geha PY, Levy RM, Harden RN, Parrish TB, Apkarian AV. Chronic pain and the emotional brain: specific brain activity associated with spontaneous fluctuations of intensity of chronic back pain. J Neurosci. 2006; 26:12165-73.

Baliki MN, Apkarian AV. Nociception, Pain, Negative Moods, and Behavior Selection. Neuron 2015; 87:474-91.

Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and molecular mechanisms of pain. Cell 2009; 139:267-284.

Batmaz I, Sariyildiz MA, Dilek B, Bez Y, Karakoc M, Cevik R. Sleep quality and associated factors in ankylosing spondylitis: relationship with disease parameters, psychological status and quality of life. Rheumatol Int. 2013; 33:1039-45.

Becker S, Kleinböhl D, Baus D, Hölzl R. Operant learning of perceptual sensitization and habituation is impaired in fibromyalgia patients with and without irritable bowel syndrome. Pain 2011; 152:1408-17.

Becker S, Gandhi W, Schweinhardt P. Cerebral interactions of pain and reward and their relevance for chronic pain. Neurosci Lett. 2012; 520:182-187.

Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 1988; 33:87-107.

Berman SM, Naliboff BD, Suyenobu B, Labus JS, Stains J, Ohning G, Kilpatrick L, Bueller JA, Ruby K, Jarcho J, Mayer EA. Reduced brainstem inhibition during anticipated pelvic visceral pain correlates with enhanced brain response to the visceral stimulus in women with irritable bowel syndrome. J Neurosci. 2008; 28:349-359.

Black JA, Nikolajsen L, Kroner K, Jensen TS, Waxman SG. Multiple sodium channel isoforms and mitogen-activated protein kinases are present in painful human neuromas. Ann Neurol. 2008; 64: 644-53.

Blackburn-Munro G, Blackburn-Munro RE. Chronic pain, chronic stress and depression: coincidence or consequence? J. Neuroendocrinol. 2001; 13:1009-1023.

Blackburn-Munro G. Hypothalamo-pituitary-adrenal axis dysfunction as a contributory factor to chronic pain and depression. Curr Pain Headache Rep. 2004; 8:116-24.

Borsook D, Moulton EA, Schmidt KF, Becerra LR. Neuroimaging revolutionizes therapeutic approaches to chronic pain. Mol Pain 2007a; 3:25.

Borsook D, Becerra L, Carlezon WA, Shaw M, Renshaw P, Elman I, Levine J. Reward-aversion circuitry in analgesia and pain: implications for psychiatric disorders. Eur J Pain 2007b; 11:7-20.

Bowen WD, Hellewell SB, McGarry KA. Evidence for a multi-site model of the rat brain sigma receptor. Eur J Pharmacol. 1989; 163:309-318

Boyce-Rustay JM, Zhong C, Kohnken R, Baker SJ, Simler GH, Wensink EJ, Decker MW, Honore P. Comparison of mechanical allodynia and the affective component of inflammatory pain in rats. Neuropharmacology 2010; 58:537-43.

Boyette-Davis J, Fuchs PN. Chronic administration of phencyclidine produces decreased sensitivity to mechanical stimulation in the absence of altered affective behavior: Implications for pain processing in schizophrenia. Neurosci Lett. 2011; 498:153-7.

Breedlove SM and Watson NV. Chapter 8: General Principles of Sensory Processing, Touch, and Pain. Available from: http://7e.biopsychology.com/vs08.html. This website is a companion to the textbook Biological Psychology: An Introduction to Behavioral, Cognitive, and Clinical Neuroscience (7th ed.). Published by Sinauer Associates.

Breivik H, Collett B, Ventafridda V, Cohen R, Gallacher D. Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment. Eur J Pain 2006; 10:287-333.

Burgess G, Williams D. The discovery and development of analgesics: new mechanisms, new modalities. J Clin Invest. 2010; 120:3753-9.

Bushnell MC, Ceko M, Low LA. Cognitive and emotional control of pain and its disruption in chronic pain. Nat Rev Neurosci. 2013; 14:502-11.

Byers MR, Bonica JJ. Peripheral pain mechanisms and nociceptor plasticity. In: Loeser JD, Butler SH, Chapman RC, Turk DC (Eds). Bonica's management of pain (3rd ed.) 2001; pp. 26 72. Philadelphia: Lippincott Williams & Wilkins.

Campbell JN, Meyer RA. Mechanisms of neuropathic pain. Neuron 2006; 52:77-92.

Cannon WB. The Wisdom of the Body. 1939. New York: W.W. Norton.

Carrasquillo Y, Gereau RW. Pain Sensitization. In: Sweatt JD ed. Molecular Mechanisms of Memory. 2008; pp. 65-90, Elsevier, Amsterdam, The Netherlands.

Casey KL. Neural mechanisms of pain: an overview. Acta Anaesthesiol Scand Suppl. 1982; 74:13-20.

Cendan CM, Pujalte JM, Portillo-Salido E, Baeyens JM. Antinociceptive effects of haloperidol and its metabolites in the formalin test in mice. Psychopharmacology (Berlin) 2005a; 182:485-493

Cendan CM, Pujalte JM, Portillo-Salido E, Montoliu L, Baeyens JM. Formalin-induced pain is reduced in sigma-1 receptor knockout mice. Eur J Pharmacol. 2005b; 511:73-74.

Chang L. Brain responses to visceral and somatic stimuli in irritable bowel syndrome: a central nervous system disorder? Gastroenterol Clin North Am. 2005; 34:271-279.

Chapman CR, Tuckett RP, Song CW. Pain and stress in a systems perspective: reciprocal neural, endocrine, and immune interactions. J Pain 2008; 9:122-145.

Chien CC, Pasternak GW. Selective antagonism of opioid analgesia by a sigma system. J Pharmacol Exp Ther 1994; 271:1583-1590.

Chudler EH, Anton F, Dubner R, Kenshalo DR. Responses of nociceptive SI neurons in monkeys and pain sensation in humans elicited by noxious thermal stimulation: effect of interstimulus interval. J Neurophysiol. 1990; 63:559-569.

Cobos EJ, Entrena JM, Nieto FR, Cendan CM, Del Pozo E. Pharmacology and therapeutic potential of sigma-1 receptor ligands. Curr Neuropharmacol. 2008; 6:344-366.

Cobos EJ, Ghasemlou N, Araldi D, Segal D, Duong K, Woolf CJ. Inflammation-induced decrease in voluntary wheel running in mice: a nonreflexive test for evaluating inflammatory pain and analgesia. Pain 2012; 153:876-84.

Cobos EJ, Portillo-Salido E."Bedside-to-Bench" Behavioral Outcomes in Animal Models of Pain: Beyond the Evaluation of Reflexes. Curr Neuropharmacol. 2013; 11:560-91.

Coderre TJ: Contribution of protein kinase C to central sensitization and persistent pain following tissue injury. Neurosci Lett. 1992; 140:181-184.

Costigan M, Scholz J, Woolf CJ. Neuropathic pain: a maladaptive response of the nervous system to damage. Annu Rev Neurosci. 2009; 32:1-32.

Craig AD. A new view of pain as a homeostatic emotion. Trends Neurosci. 2003a; 26:303-7.

Craig AD. Interoception: the sense of the physiological condition of the body. Curr Opin Neurobiol 2003b; 13:500-505.

Craig AD. Pain mechanisms: labeled lines versus convergence. in central processing. Annu. Rev. Neurosci. 2003c; 26:1-30.

Cytawa J, Trojniar W. Hedonesthesia: the nervous process determining motivated ingestive behavior. Acta Neurobiol Exp (Wars). 1978; 38:139-51.

Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 2000; 87:149-58.

Der-Avakian A, Markou A. The neurobiology of anhedonia and other reward related deficits. Trends Neurosci. 2012; 35, 68-77.

Derbyshire SW. A systematic review of neuroimaging data during visceral stimulation. Am J Gastroenterol. 2003; 98:12-20.

Díaz JL, Cuberes R, Berrocal J, Contijoch M, Christmann U, Fernández A, Port A, Holenz J, Buschmann H, Laggner C, Serafini MT, Burgueño J, Zamanillo D, Merlos M, Vela JM, Almansa C. Synthesis and biological evaluation of the 1-arylpyrazole class of σ1 receptor antagonists: Identification of 4-{2-[5-methyl-1- (naphthalen-2-yl)-1H-pyrazol-3-yloxy]ethyl}morpholine (S1RA, E-52862). J Med Chem. 2012; 55:8211-8224.

Dickenson AH, Sullivan AF. Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep rat dorsal horn nociceptive neurones following C fibre stimulation. Neuropharmacology 1987; 26:1235-1238.

Diehl B, Hoheisel U, Mense S. Histological and neurophysiology changes induced by carrageenan in skeletal muscle of cat and rat. Agents Actions 1988; 25:210-213.

Djouhri L, Lawson SN. A[beta]-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. Brain Res Brain Res Rev. 2004; 46:131-145

Dowdall T, Robinson I, Meert TF. Comparison of five different rat models of peripheral nerve injury. Pharmacol Biochem Behav. 2005; 80:93-108.

Drake CL, Roehrs T, Roth T. Insomnia causes, consequences, and therapeutics: an overview. Depress Anxiety 2003; 18:163-176.

Drews E, Zimmer A. Central sensitization needs sigma receptors. Pain 2009; 145:269-70.

Duquette M, Roy M, Leporé F, Peretz I, Rainville P. Cerebral mechanisms involved in the interaction between pain and emotion. Rev Neurol (Paris) 2007; 163:169-79.

Dworkin RH. An overview of neuropathic pain: syndromes, symptoms, signs, and several mechanisms. Clin J Pain. 2002; 18:343-9.

Dworkin RH, Backonja M, Rowbotham MC, Allen RR, Argoff CR, Bennett GJ, Bushnell MC, Farrar JT, Galer BS, Haythornthwaite JA, Hewitt DJ, Loeser JD, Max MB, Saltarelli M, Schmader KE, Stein C, Thompson D, Turk DC, Wallace MS, Watkins LR, Weinstein SM. Advances in neuropathic pain. Diagnosis, mechanisms and treatment recommendations. Arch Neurol. 2003; 60:1524-1534.

Eccleston C, Crombez G. Pain demands attention: a cognitive-affective model of the interruptive function of pain. Psychol Bull. 1999; 125:356-66

El Hage W, Powell JF, Surguladze SA. Vulnerability to depression: what is the role of stress genes in gene x environment interaction? Psychol Med. 2009; 39:1407-11.

Elman I, Borsook D, Volkow ND. Pain and suicidality: insights from reward and addiction neuroscience. Prog Neurobiol. 2013; 109:1-27.

Entrena JM, Cobos EJ, Nieto FR, Cendán CM, Gris G, Del Pozo E, Zamanillo D, and Baeyens JM Sigma-1 receptors are essential for capsaicin-induced mechanical hypersensitivity: studies with selective sigma-1 ligands and sigma-1 knockout mice. Pain 2009; 143:252-261.

Fernandez E, Turk DC. Sensory-discriminative and affecfive components of pain: separation and synthesis. Psychol Bull. 1992; 112:205-17.

Fillingim RB, King CD, Ribeiro-Dasilva MC, Rahim-Williams B, Riley JL 3rd. Sex, gender, and pain: a review of recent clinical and experimental findings. J Pain. 2009; 10:447-85.

Fine PG, Ashburn MA. Functional neuroanatomy and nociception. In: Ashburn MA, Rice LJ (Eds). The management of pain. 1998; pp. 1-16. New York: Churchill Livingstone.

Fregni F, Pascual-Leone A, Freedman SD. Pain in chronic pancreatitis: a salutogenic mechanism or a maladaptive brain response? Pancreatology 2007; 7:411-422.

Friedman DP, Murray EA. Thalamic connectivity of the second somatosensory area and neighboring somatosensory fields of the lateral sulcus of the macaque. J Comp Neurol. 1986; 2523:348-73.

Friedman DP, Murray EA, O'Neill JB, Mishkin M. Cortical connections of the somatosensory fields of the lateral sulcus of macaques: evidence for a corticolimbic pathway for touch. J Comp Neurol. 1986; 252:323-47.

Fu Y, Zhao Y, Luan W, Dong LY, Dong Y, Lai B, Zhu Y, and Zheng P. Sigma-1 receptors amplify dopamine D1 receptor signaling at presynaptic sites in the prelimbic cortex. Biochim Biophys Acta 2010; 1803:1396-1408.

Gatchel RJ, Pen YB, Peters ML, Fuchs PN, Turk DC. The biopsychosocial approach to chronic pain: scientific advances and future directions. Psychol Bull. 2007; 133:581-624.

Gebhart GF, Ness TJ. Central mechanisms of visceral pain. Can J Physiol Pharmacol. 1991; 69:627-34.

Gerdle B, Ghafouri B, Ernberg M, Larsson B. Chronic musculoskeletal pain: review of mechanisms and biochemical biomarkers as assessed by the microdialysis technique. J Pain Res. 2014; 7:313-26.

Gilron I, Tu D, Holden RR. Sensory and affective pain descriptors respond differentially to pharmacological interventions in neuropathic conditions. Cli J Pain 2013; 29:124-31.

Goffer Y, Xu D, Eberle SE, D'amour J, Lee M, Tukey D, Froemke RC, Ziff EB, Wang J. Calcium-permeable AMPA receptors in the nucleus accumbens regulate depression-like behaviors in the chronic neuropathic pain state. J Neurosci. 2013; 33:19034-44.

Gonçalves L, Silva R, Pinto-Ribeiro F, Pêgo JM, Bessa JM, Pertovaara A, Sousa N, Almeida A. Neuropathic pain is associated with depressive behaviour and induces neuroplasticity in the amygdala of the rat. Exp Neurol. 2008; 213:48-56.

Gotlib IH, Joormann J, Minor KL, Hallmayer J. HPA axis reactivity: a mechanism underlying the associations among 5-HTTLPR, stress, and depression. Biol Psychiatry 2008; 63:847-851.

Greenspan JD, Lee RR, Lenz FA. Pain sensitivity alterations as a function of lesion location in the parasylvian cortex. Pain 1999; 81:273-282.

Gris G, Merlos M, Vela JM, Zamanillo D, Portillo-Salido E. S1RA, a selective sigma-1 receptor antagonist, inhibits inflammatory pain in the carrageenan and complete Freund's adjuvant models in mice. Behav Pharmacol. 2014; 25:226-35.

Guitart X, Codony X, Monroy X. Sigma receptors: biology and therapeutic potential. Psychopharmacology (Berl) 2004; 174:301e319.

Gureje O, Simon GE, Von Korff M. A cross-national study of the course of persistent pain in primary care. Pain 2001; 92:195-200.

Gusnard DA, Raichle ME, Raichle ME. Searching for a baseline: functional imaging and the resting human brain. Nat Rev Neurosci. 2001; 2:685-694.

Hains BC, Saab CY, Klein JP, Craner MJ, Waxman SG. Altered sodium channel expression in second-order spinal sensory neurons contributes to pain after peripheral nerve injury. J Neurosci. 2004; 24: 4832-39.

Hammond DL. Inference of pain and its modulation from simple behaviors. in Issues in Pain Measurement: Advances in Pain Research and Therapy, eds Chapman CR, Loeser JD (Raven Press, New York). 1989; 12:69-91.

Hanner M, Moebius FF, Flandorfer A, Knaus HG, Striessnig J, Kempner E, Glossmann H. Purification, molecular cloning, and expression of the mammalian sigma-1 binding site. Proc Nat Acad Sci. 1996; 93:8072-8077.

Harden N, Cohen M. Unmet needs in the management of neuropathic pain. J Pain Symptom Manage. 2003; 25(5 Suppl):S12-7.

Hayashi T, Su TP. Intracellular dynamics of sigma-1 receptors (σ_1 binding sites) in NG108-15 cells. J Pharmacol Exp Ther 2003; 306:726-733.

Hayashi T, Su TP. Sigma-1 receptor chaperones at the ERmitochondrion interface regulate Ca(2+) signaling and cell survival. Cell 2007; 131:596-610.

Hayashi T, Justinova Z, Hayashi E, Cormaci G, Mori T, Tsai SY, Barnes C, Goldberg SR, Su TP. Regulation of sigma-1 receptors and endoplasmic reticulum chaperones in the brain of methamphetamine self-administering rats. J Pharmacol Exp Ther. 2010; 332:1054-1063.

Hayashi T, Tsai SY, Mori T, Fujimoto M, and Su TP. Targeting ligand-operated chaperone sigma-1 receptors in the treatment of neuropsychiatric disorders. Expert Opin Ther Targets. 2011; 15:557-577.

Heidrich G, Slavic-Svircev V, Kaiko R. Efficacy and quality of ibuprofen and acetaminophen plus codeine analgesia. Pain 1985; 22:385-397.

Heinricher MM, Tavares I, Leith JL, Lumb BM. Descending control of nociception: Specificity, recruitment and plasticity. Brain Res Rev. 2009; 60:214-225.

Hellewell SB, Bowen WD. A sigma-like binding site in rat pheochromocytoma (PC12)cells: Decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that of guinea pig brain. Brain Res. 1990; 527:244-253.

Hellewell SB, Bruce A, Feinstein G, Orringer J, Williams W, Bowen WD. Rat liver and kidney contain high densities of sigma 1 and sigma 2 receptors: characterization by ligand binding and photoaffinity labeling. Eur J Pharmacol. 1994; 268:9-18.

Herrero JF, Laird JM, López-García JA. Wind-up of spinal cord neurones and pain sensation: much a do about something? Prog Neurobiol. 2000; 61:169-203.

Hu B, Doods H, Treede RD, Ceci A. Depression-like behaviour in rats with mononeuropathy is reduced by the CB2-selective agonist GW405833. Pain 2009; 143:206-212.

Hunt SP, Mantyh PW. The molecular dynamics of pain control. Nat Rev Neurosci. 2001; 2:83-91.

Hutchinson WD, Davis KD, Lozano AM, Tasker RR, Dostrovsky JO. Pain-related neurons in the human cingulate cortex. Nat Neurosci. 1999; 2:403- 405.

Jackson HC, Needham AM, Hutchins LJ, Mazurkiewicz SE, Heal DJ. Comparison of the effects of sibutramine and other monoamine reuptake inhibitors on food intake in the rat. Br J Pharmacol. 1997; 121:1758-62.

Ji G, Sun H, Fu Y, Li Z, Pais-Vieira M, Galhardo V, Neugebauer V. Cognitive impairment in pain through amygdala-driven prefrontal cortical deactivation. J Neurosci. 2010; 30:5451-5464.

Johansen JP, Fields HL. Glutamatergic activation of anterior cingulate cortex produces an aversive teaching signal. Nat. Neurosci. 2004; 7:398-403.

Jones AK, Kulkarni B, Derbyshire SW. Functional imaging of pain perception. Curr Rheumatol Rep. 2002; 4:329 -333.

Kawasaki Y, Kohno T, Zhuang ZY, Brenner GJ, Wang H, Van Der Meer C, Befort K, Woolf CJ, Ji RR. Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element-binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. J Neurosci. 2004; 24:8310-21.

Kehlet H, Jensen TS, Woolf CJ. Persistent postsurgical pain: risk factors and prevention. Lancet 2006; 367:1618.

Kekuda R, Prasad, PD, Fei YJ, Leibach FH, Ganapathy V. Cloning and functional expression of the human type 1 sigma receptor(hSigmaR1). Biochem. Biophys. Res. Commun. 1996; 229: 553-558.

Kenshalo DR, Isensee O. Responses of primate SI cortical neurons to noxious stimuli. J Neurophysiol. 1983; 50:1479-1496.

Kenshalo DR, Chudler EH, Anton F, Dubner R. SI nociceptive neurons participate in the encoding process by which monkeys perceive the intensity of noxious thermal stimulation. Brain Res. 1988; 454:378-382.

Kim FJ, Kovalyshyn I, Burgman M, Neilan C, Chien CC, Pasternak GW. Sigma 1 receptor modulation of G-protein-coupled receptor signaling: potentiation of opioid transduction independent from receptor binding. Mol Pharmacol. 2010; 77:695-703.

Kim H, Chen L, Lim G, Sung B, Wang S, McCabe MF, Rusanescu G, Yang L, Tian Y, Mao J. Brain indoleamine 2,3-dioxygenase contributes to the comorbidity of pain and depression. J Clin Invest. 2012; 122:2940-54.

Kinloch RA, Cox PJ. New targets for neuropathic pain therapeutics. Expert Opin Ther Targets. 2005; 9:685-98.

Kontinen VK, Kauppila T, Paananen S, Pertovaara A, Kalso E. Behavioural measures of depression and anxiety in rats with spinal nerve ligation-induced neuropathy. Pain 1999; 80:341-346.

Koster R, Anderson M, de Beer EJ. Acetic acid for analgesic screening. Fed Proc 1959; 18:412.

Kuchinad A1, Schweinhardt P, Seminowicz DA, Wood PB, Chizh BA, Bushnell MC. Accelerated brain gray matter loss in fibromyalgia patients: premature aging of the brain? J Neurosci. 2007; 27:4004-4007.

LaBuda CJ, Fuchs PN. Low dose aspirin attenuates escape/avoidance behavior, but does not reduce mechanical hyperalgesia in a rodent model of inflammatory pain. Neurosci Lett. 2001; 304:137-40.

LaGraize SC, Borzan J, Peng YB, Fuchs PN. Selective regulation of pain affect following activation of the opioid anterior cingulate cortex system. Exp Neurol. 2006; 197:22-30.

Lai J, Hunter JC, Porreca F. The role of voltage-gated sodium channels in neuropathic pain. Curr Opin Neurobiol. 2003; 13:291-97.

Langa F, Codony X, Tovar V, Lavado A, Giménez E, Cozar P, Cantero M, Dordal A, Hernández E, Pérez R, Monroy X, Zamanillo D, Guitart X, Montoliu L. Generation and phenotypic analysis of sigma receptor type I (sigma 1) knockout mice. Eur J Neurosci. 2003; 18:2188-96.

Latremoliere A, Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J Pain 2009; 10:895-926.

Le Bars D, Gozariu M, Cadden SW. Animal models of nociception. Pharmacol Rev. 2001; 53:597-652.

Le Merrer J, Becker JA, Befort K, Kieffer BL. Reward processing by the opioid system in the brain. Physiol Rev. 2009; 89:1379-412.

Lee BH, Yoon YW, Chung K, Chung JM. Comparison of sympathetic sprouting in sensory ganglia in three animal models of neuropathic pain. Exp Brain Res. 1998; 120:432-438.

Leknes S, Tracey I. A common neurobiology for pain and pleasure. Nat Rev Neurosci. 2008; 9:314-20.

Lesho EP. When the spirit hurts: an approach to the suffering patient. Arch Intern Med. 2003; 163:2429-32.

Li Y, Zhang S, Zhu J, Du X, Huang F. Sleep disturbances are associated with increased pain, disease activity, depression, and anxiety in ankylosing spondylitis: a case-control study. Arthritis Res Ther. 2012; 14:R215.

Li K, Tan YH, Light AR, Fu KY. Different peripheral tissue injury induces differential phenotypic changes of spinal activated microglia. Clin Dev Immunol. 2013; 2013:901420.

Lombard MC, Nashold Jr BS, Albe-Fessard D, Salman N, Sakr C. Deafferentation hypersensitivity in the rat after dorsal rhizotomy: a possible animal model of chronic pain. Pain 1979; 6:163-174.

Lorenz J, Cross DJ, Minoshima S, Morrow TJ, Paulson PE, Casey KL. A unique representation of heat allodynia in the human brain. Neuron 2002; 35:383-393.

MacLean, P. D. Psychosomatic disease and the “visceral brain.” Recent developments bearing on the Papez theory of emotion. Psychosom. Med. 1949; 11:338-353.

Magni G, Moreschi C, Rigatti-Luchini S, Merskey H. Prospective study on the relationship between depressive symptoms and chronic musculoskeletal pain. Pain 1994; 56:289-97.

Maihöfner C, Handwerker HO. Differential coding of hyperalgesia in the human brain: a functional MRI study. Neuroimage 2005; 28:996-1006.

Marbach JJ, Lund P. Depression, anhedonia and anxiety in temporomandibular joint and other facial pain syndromes. Pain 1981; 11:73-84.

Marbach JJ, Richlin DM, Lipton JA. Illness behavior, depression and anhedonia in myofascial face and back pain patients. Psychother Psychosom. 1983; 39:47-54.

Martin TJ, Buechler NL, Kahn W, Crews JC, Eisenach JC. Effects of laparotomy on spontaneous exploratory activity and conditioned operant responding in the rat: a model for postoperative pain. Anesthesiology 2004; 101:191-203.

Martin WR, Eades CG, Thompson JA, Huppler RE, Gilbert PE. The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal dog. J Pharmacol Exp Ther. 1976; 197:517-32.

Matson DJ, Broom DC, Carson SR, Baldassari J, Kehne J, Cortright DN. Inflammation-induced reduction of spontaneous activity by adjuvant: a novel model to study the effect of analgesics in rats. J Pharmacol Exp Ther. 2007; 320:194-201.

Maurice T, Su TP. The pharmacology of sigma-1 receptors. Pharmacol Ther. 2009; 124:195-206.

McDougall JJ. Peripheral analgesia: Hitting pain where it hurts. Biochim Biophys Acta. 2011; 1812:459-67.

Medford N, Critchley HD. Conjoint activity of anterior insular and anterior cingulate cortex: awareness and response. Brain Struct Funct. 2010; 214:535-549

Mei J, Pasternak GW. Molecular cloning and pharmacological characterization of the rat sigma1 receptor. Biochem Pharmacol. 2001; 62:349-55.

Merskey HM. Pain terms. Pain 1986; suppl. 3:S215-S221.

Mesulam MM, Mufson EJ. Insula of the old world monkey. III: Efferent cortical output and comments on function. J Comp Neurol. 1982; 212:38-52.

Metz AE, Yau HJ, Centeno MV, Apkarian AV, Martina M. Morphological and functional reorganization of rat medial prefrontal cortex in neuropathic pain. Proc Natl Acad Sci USA. 2009; 106:2423-2428.

Meyer RA, Ringkamp M, Campbell JN, Raja SN. Peripheral mechanisms of cutaneous nociception. In: McMahon SB, Koltzenburg M (Eds). Wall and Melzack's Textbook of Pain. 2008; pp. 3-34. Philadelphia: Elsevier.

Millan MJ. The induction of pain: an integrative review. Prog Neurobiol. 1999; 57:1-164.

Miller JM, Vorel SR, Tranguch AJ, Kenny ET, Mazzoni P, van Gorp WG, Kleber HD. Anhedonia after a selective bilateral lesion of the globus pallidus. Am J Psychiatry 2006; 163:786-788.

Miller LL, Picker MJ, Schmidt KT, Dykstra LA. Effects of Morphine on Pain-elicited and Pain-suppressed Behavior in CB1 Knockout and Wildtype Mice. Psychopharmacology 2011; 215:455-465.

Milligan ED, Watkins LR. Pathological and protective roles of glia in chronic pain. Nat Rev Neurosci. 2009; 10:23-36.

Moebius FF, Reiter RJ, Hanner M, Glossmann H. High affinity of sigma1-binding sites for sterol isomerization inhibitors: evidence for a pharmacological relationship with the yeast sterol C8-C7 isomerase. Br J Pharmacol. 1997; 121:1-6.

Mogil JS, Chanda ML. The case for the inclusion of female subjects in basic science studies of pain. Pain 2005; 117:1-5.

Moisset X, Bouhassira D. Brain imaging of neuropathic pain. Neuroimage 2007; 37 Suppl 1:S80-8.

Mufson EJ, Mesulam MM, Pandya DN. Insular interconnections with the amygdala in the rhesus monkey. Neuroscience 1981; 67:1231-48.

Mufson EJ, Mesulam MM. Insula of the old world monkey. II: Afferent cortical input and comments on the claustrum. J Comp Neurol. 1982; 212:23-37.

Narita M, Kaneko C, Miyoshi K, Nagumo Y, Kuzumaki N, Nakajima M, Nanjo K, Matsuzawa K, Yamazaki M, Suzuki T. Chronic pain induces anxiety with concomitant changes in opioidergic function in the amygdala. Neuropsychopharmacology 2006; 31:739-50.

Navarro G, Moreno E, Aymerich M, Marcellino D, McCormick PJ, Mallol J, Cortés A, Casadó V, Canela EI, Ortiz J, Fuxe K, Lluís C, Ferré S, Franco R. Direct involvement of sigma-1 receptors in the dopamine D1 receptor-mediated effects of cocaine. Proc Natl Acad Sci U S A. 2010; 107:18676-81.

Ness TJ, Gebhart GF. Visceral pain: a review of experimental studies. Pain 1990; 41:167-234.

Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM.Neurobiology of depression. Neuron 2002; 34:13-25.

Nickel FT, Seifert F, Lanz S, Maihöfner C. Mechanisms of neuropathic pain. Eur Neuropsychopharmacol. 2012; 22:81-91.

Nieto FR, Cendán CM, Sánchez-Fernández C, Cobos EJ, Entrena JM, Tejada MA, Zamanillo D, Vela JM, Baeyens JM. Role of sigma-1 receptors in paclitaxel-induced neuropathic pain in mice. J Pain. 2012;13:1107-21.

Nugent NR, Tyrka AR, Carpenter LL, Price LH. Gene-environment interactions: early life stress and risk for depressive and anxiety disorders. Psychopharmacology (Berl) 2011; 214:175-196.

Ohara PT, Vit JP, Jasmin L. Cortical modulation of pain. Cell Mol Life Sci. 2005; 62:44-52.

Ozaki S, M. Narita, M. Iino, J. Sugita, Y. Matsumura, T. Suzuki, Suppression of the morphine-induced rewarding effect in the rat with neuropathic pain: implication of the reduction in mu-opioid receptor functions in the ventral tegmental area, Journal of neurochemistry. 2002; 82:1192-1198.

Ozaki S, Narita M, Narita M, Iino M, Miyoshi K, Suzuki T. Suppression of the morphine-induced rewarding effect and G-protein activation in the lower midbrain following nerve injury in the mouse: involvement of G-protein-coupled receptor kinase 2. Neuroscience 2003; 116:89-97

Pais-Vieira M, Mendes-Pinto MM, Lima D, Galhardo V. Cognitive impairment of prefrontal-dependent decision-making in rats after the onset of chronic pain. Neuroscience 2009; 161:671-679.

Pasero C, Paice JA, McCaffery M. Basic mechanisms underlying the causes and effects of pain. In: McCaffery M, Pasero C. Pain: clinical manual (2nd ed). 1999; pp. 18-34. St. Louis, MO: Mosby.

Pedersen LH, Blackburn-Munro G. Pharmacological characterisation of place escape/avoidance behaviour in the rat chronic constriction injury model of neuropathic pain. Psychopharmacology (Berl) 2006; 185:208-17.

Penfield W, Boldrey E. Somatic motor and sensory representation in the cerebral cortex of man as studied by electrical stimulation. Brain 1937; 60:389-443.

Peyron R, Laurent B, Garcia-Larrea L. Functional imaging of brain responses to pain. A review and meta-analysis. Neurophysiol Clin. 2000; 30:263-288.

Pfaffmann C. The pleasures of sensation. Psychol Rev. 1960; 67: 253-268.

Pfau DB, Klein T, Putzer D, Pogatzki-Zahn EM, Treede RD, and Magerl W. Analysis of hyperalgesia time courses in humans after painful electrical highfrequency stimulation identifies a possible transition from early to late LTP-like pain plasticity. Pain 2011; 152:1532-1539.

Phelps EA, Delgado MR, Nearing KI, LeDoux JE. Extinction learning in humans: role of the amygdala and vmPFC. Neuron 2004; 43:897-905

Piche M, Arsenault M, Poitras P, Rainville P, Bouin M. Widespread hypersensitivity is related to altered pain inhibition processes in irritable bowel syndrome. Pain 2010; 148:49-58.

Pilowsky I, Crettenden I, Townley M. Sleep disturbance in pain clinic patients. Pain 1985; 23:27-33.

Ploner M, Freund HJ, SchnitzlerA. Pain affect without pain sensation in a patient with a postcentral lesion. Pain 1999; 81:211-214.

Porro CA, Baraldi P, Pagnoni G, Serafini M, Facchin P, Maieron M, Nichelli P. Does anticipation of pain affect cortical nociceptive systems? J Neurosci. 2002; 22:3206-3214.

Portenoy RK, Kanner RM. Definition and assessment of pain. In: Portenoy RK, Kanner RM (Eds). Pain management: theory and practice. 1996; pp. 3-18. Philadelphia: F.A. Davis.

Prasad PD, Li HW, Fei YJ, Ganapathy ME, Fujita T, Plumley LH, Yang-Feng T L, Leibach FH, Ganapathy V. Exon-intron structure, analysis or promoter region, and chromosomal localization of the human type 1 sigma receptor gene. J Neurochem. 1998; 70:443-451.

Price DD, Von der Gruen A, Miller J, Rafii A, Price C. A psychophysical analysis of morphine analgesia. Pain 1985; 22:261-269.

Price DD. Central neural mechanisms that interrelate sensory and affective dimensions of pain. Mol Interv. 2002; 2:392-403, 339.

Puglisi-Allegra S, Imperato A, Angelucci L, Cabib S. Acute stress induces time-dependent responses in dopamine mesolimbic system. Brain Research 1991; 554:217-222.

Rainville P, Duncan GH, Price DD, Carrier B, Bushnell MC. Pain affect encoded in human anterior cingulate but not somatosensory cortex. Science 1997; 277:968 -971.

Rhudy JL, Meagher MW. Fear and anxiety: divergent effects on human pain thresholds. Pain 2000; 84:65-75.

Roberts JD, Gennings C, Shih M. Synergistic affective analgesic interaction between delta-9-tetrahydrocannabinol and morphine. Eur J Pharmacol. 2006; 530:54-58.

Romanelli P, Eposito V, Adler J. Ablative procedures for chronic pain. Neurosurg Clin N Am. 2004; 15:335-342.

Rome HP, Rome JD. Limbically augmented pain syndrome (LAPS): Kindling, corticolimbic sensitization, and the convergence of affective and sensory symptoms in chronic pain disorders. Pain Medicine 2000; 1:7-23.

Romera I, Montejo AL, Caballero F, Caballero L, Arbesu J, Polavieja P, Desaiah D, Gilaberte I. Functional impairment related to painful physical symptoms in patients with generalized anxiety disorder with or without comorbid major depressive disorder: post hoc analysis of a cross-sectional study. BMC. Psychiatry 2011; 11:69.

Romero L, Zamanillo D, Nadal X, Sánchez-Arroyos R, Rivera-Arconada I, Dordal A, Montero A, Muro A, Bura A, Segalés C, Laloya M, Hernández E, Portillo-Salido E, Escriche M, Codony X, Encina G, Burgueño J, Merlos M, Baeyens JM, Giraldo J, López-García JA, Maldonado R, Plata-Salamán CR, Vela JM. Pharmacological properties of S1RA, a new sigma-1 receptor antagonist that inhibits neuropathic pain and activity-induced spinal sensitization. Br J Pharmacol. 2012; 166:2289-306.

Rowbotham MC, Petersen KL. Anticonvulsants and local anesthetic drugs. In: Loeser JD, Butler SH, Chapman RC, Turk DC (Eds). Bonica's management of pain (3rd ed.) 2001; pp. 1727-1735. Philadelphia: Lippincott Williams &; Wilkins.

Saavedra JM, Axelrod J. Psychotomimetic N-methylated tryptamines: formation in brain in vivo and in vitro. Science 1972; 175:1365-1366.

Schlaepfer TE, Cohen MX, Frick C, Kosel M, Brodesser D, Axmacher N, Joe AY, Kreft M, Lenartz D, Sturm V. Deep brain stimulation to reward circuitry alleviates anhedonia in refractory major depression. Neuropsychopharmacology 2008; 33:368-77.

Schwartz N, Temkin P, Jurado S, Lim BK, Heifets BD, Polepalli JS, Malenka RC. Chronic pain. Decreased motivation during chronic pain requires long-term depression in the nucleus accumbens. Science 2014; 345:535-42.

Schweinhardt P, Bountra C, Tracey I. Pharmacological FMRI in the development of new analgesic compounds. NMR Biomed. 2006; 19:702-711.

Seal RP, Wang X, Guan Y, Raja SN, Woodbury CJ, Basbaum AI, and Edwards RH. Injury-induced mechanical hypersensitivity requires C-low threshold mechanoreceptors. Nature 2009; 462:651-655.

Seifert F, Jungfer I, Schmelz M, Maihöfner C. Representation of UV-B-induced thermal and mechanical hyperalgesia in the human brain: a functional MRI study. Hum Brain Mapp. 2008; 29:1327-1342.

Seifert F, Maihöfner. Central mechanisms of experimental and chronic neuropathic pain: findings from functional imaging studies. Cell Mol Life Sci. 2009; 66:375-390.

Seltzer Z, Dubner R, Shir Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. Pain 1990; 43:205-18.

Seminowicz DA, Mikulis DJ, Davis KD. Cognitive modulation of pain-related brain responses depends on behavioral strategy. Pain 2004; 112:48-58.

Seth P, Fei YJ, Huang W, Leiback FH, Ganapathy V. Cloning and functional characterization of a sigma receptor from rat brain. J of Neurochem. 1998; 70:922-931.

Siddall PJ, Cousins MJ. Spine update: Spinal pain mechanisms. Spine 1997; 22:98-104.

Sikandar S, Dickenson AH. Visceral pain: the ins and outs, the ups and downs. Curr Opin Support Palliat Care. 2012; 6:17-26.

Simons LE, Sieberg CB, Carpino E, Logan D, Berde C. The Fear of Pain Questionnaire (FOPQ): assessment of pain-related fear among children and adolescents with chronic pain. J Pain. 2011; 12:677-686.

Simons LE, Elman I, Borsook D. Psychological processing in chronic pain: a neural systems approach. Neurosci Biobehav Rev. 2014; 39:61-78.

Siqueira SR, Alves B, Malpartida HM, Teixeira MJ, Siqueira JT. Abnormal expression of voltage-gated sodium channels Nav1.7, Nav1.3 and Nav1.8 in trigeminal neuralgia. Neuroscience 2009; 164:573-7.

Sneddon LU, Braithwaite VA, Gentle MJ. Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system. Proc Biol Sci. 2003; 270:1115-21.

Solowiej K, Mason V, Upton D. Review of the relationship between stress and wound healing: part 1. J Wound Care 2009; 18:357-66.

Starr CJ, Sawaki L, Wittenberg GF, Burdette JH, Oshiro Y, Quevedo AS, Coghill RC. Roles of the insular cortex in the modulation of pain: insights from brain lesions. J Neurosci. 2009; 29:2684-94.

Stevenson GW, Bilsky EJ, Negus SS. Targeting pain-suppressed behaviors in preclinical assays of pain and analgesia: effects of morphine on acetic acid-suppressed feeding in C57BL/6J mice. J Pain. 2006; 7:408-16.

Stevenson GW, Cormier J, Mercer H, Adams C, Dunbar C, Negus SS, Bilsky EJ. Targeting pain-depressed behaviors in preclinical assays of pain and analgesia: drug effects on acetic acid-depressed locomotor activity in ICR mice. Life Sci. 2009; 85:309-15.

Su TP, Hayashi T, Maurice T, Buch S, Ruoho AE. The sigma-1 receptor chaperone as an inter-organelle signaling modulator. Trends Pharmacol Sci. 2010; 31:557-66.

Su TP, London ED, Jaffe JH. Steroid binding at singma receptors suggests a link between endocrine, nervous, and immune systems. Science 1988; 240:219-221

Tang NKY, Salkovskis PM, Hodges A, Wright KJ, Hanna M, Hester J. Effects of mood on pain responses and pain tolerance: an experimental study in chronic back pain patients. Pain 2008; 138:392-401.

Taylor SE, Way BM, Welch WT, Hilmert CJ, Lehman BJ, Eisenberger NI. Early family environment, current adversity, the serotonin transporter promoter polymorphism, and depressive symptomatology. Biol. Psychiatry 2006; 60:671-676.

Theis KA, Helmick CG, Hootman JM. Arthritis burden and impact are greater among U.S. women than men: intervention opportunities. J Womens Health (Larchmt) 2007; 16:441-53.

Tietjen GE1, Brandes JL, Peterlin BL, Eloff A, Dafer RM, Stein MR, Drexler E, Martin VT, Hutchinson S, Aurora SK, Recober A, Herial NA, Utley C, White L, Khuder SA. Allodynia in migraine: association with comorbid pain conditions. Headache 2009; 49:1333-1344.

Tracey I, Mantyh PW. The cerebral signature for pain perception and its modulation. Neuron 2007; 55:377-391.

Treadway MT, Zald DH. Reconsidering anhedonia in depression: lessons from translational neuroscience. Neurosci Biobehav Rev. 2011; 35:537-55.

Tsai SY, Hayashi T, Mori T, Su TP. Sigma-1 receptor chaperones and diseases. Cent Nerv Syst Agents Med Chem. 2009; 9:184-9.

Turk DC, Okifuji A. Psychological Factors in Chronic Pain: Evolution and Revolution. J Consult Clin Psychol. 2002; 70:678-90

Ultenius C, Linderoth B, Meyerson BA, Wallin J. Spinal NMDA receptor phosphorylation correlates with the presence of neuropathic signs following peripheral nerve injury in the rat. Neurosci Lett. 2006; 399: 85-90.

Urban R, Scherrer G, Goulding EH, Tecott LH, Basbaum AI. Behavioral indices of ongoing pain are largely unchanged in male mice with tissue or nerve injury-induced mechanical hypersensitivity. Pain 2011; 152:990-1000.

Valet M, Sprenger T, Boecker H, Willoch F, Rummeny E, Conrad B, Erhard P, Tolle TR. Distraction modulates connectivity of the cingulo-frontal cortex and the midbrain during pain —an fMRI analysis. Pain 2004; 109:399-408.

van der Kam EL, Vry JD, Schiene K, Tzschentke TM. Differential effects of morphine on the affective and the sensory component of carrageenan-induced nociception in the rat. Pain 2008; 136:373-9.

Veinante P, Yalcin I, Barrot M. The amygdala between sensation and affect: a role in pain. J Mol Psychiatry. 2013; 1:9.

Vela JM, Merlos M, Almansa C. Investigational sigma-1 receptor antagonists for the treatment of pain. Expert Opin Investig Drugs. 2015; 24:883-96.

Vidal-Torres A, de la Puente B, Rocasalbas M, Touriño C, Bura SA, Fernández-Pastor B, Romero L, Codony X, Zamanillo D, Buschmann H, Merlos M, Baeyens JM, Maldonado R, Vela JM. Sigma-1 receptor antagonism as opioid adjuvant strategy: enhancement of opioid antinociception without increasing adverse effects. Eur J Pharmacol. 2013; 711:63-72.

Vierck CJ, Hansson PT, Yezierski RP. Clinical and pre-clinical pain assessment: are we measuring the same thing? Pain 2008; 135:7-10.

Vogt BA, Sikes RW, Vogt LJ. In: Vogt BA, Gabriel M (Eds). Neurobiology of cingulate cortex and limbic thalamus. 1993; pp 19-70, 313-344. Boston: Birkhäuser.

Vogt BA, Sikes RW. The medial pain system, cingulate cortex, and parallel processing of nociceptive information. Prog Brain Res. 2000; 122:223-235.

Vogt BA. Pain and emotion intercations in subregions of the cingulated gyrus. Nat Rev Neurosci. 2005; 6:533-544.

Wager TD, Rilling JK, Smith EE, Sokolik A, Casey KL, Davidson RJ, Kosslyn SM, Rose RM, Cohen JD. Placebo-induced changes in fMRI in the anticipation and experience of pain. Science 2004; 303:1162-1167.

Wall PD, Devor M, Inbal R, Scadding JW, Schonfeld D, Seltzer Z, Tomkiewicz MM. Autotomy following peripheral nerve lesions: experimental anaesthesia dolorosa. Pain 1979; 7:103-113.

Wang J, Goffer Y, Xu D, Tukey DS, Shamir DB, Eberle SE, Zou AH, Blanck TJ, Ziff EB. A single subanesthetic dose of ketamine relieves depression-like behaviors induced by neuropathic pain in rats. Anesthesiology 2011; 115:812-21.

Wilson KG, Eriksson MY, D'Eon JL, Mikail S, Emery PC. Major depression and insomnia in chronic pain. Clin J Pain 2002; 18:77-83.

Wolfe SA, Culp SG, de Souza EB. Sigma-receptors in endocrine organs: identification, characterization, and autoradiographic localization in rat pituitary, adrenal, testis, and ovary. Endocrinology 1989; 124:1160-1172.

Wong GT. Speed congenics: applications for transgenic and knock-out mouse strains. Neuropeptides. 2002; 36:230-6.

Wood PB. Mesolimbic dopaminergic mechanisms and pain control. Pain 2006; 120:230-234.

Wood PB, Schweinhardt P, Jaeger E, Dagher A, Hakyemez H, Rabiner EA, Bushnell MC, Chizh BA. Fibromyalgia patients show an abnormal dopamine response to pain. Eur J Neurosci. 2007; 25:3576-82.

Woolf CJ. Evidence for a central component of post-injury pain hypersensitivity. Nature 1983; 306:686-688.

Woolf CJ, Salter MW. Plasticity and pain: role of the dorsal hon. In: McMahon SB, Koltzenburg M (Eds). Textbook of pain. 5. 2006. pp. 91 105. New York: Churchill-Livingstone.

Woolf CJ, Ma Q. Nociceptors: noxious stimulus detectors. Neuron 2007; 55:353-364

Woolf CJ. Central sensitization: implications for the diagnosis and treatment of pain. Pain 2011; 152(3 Suppl):S2-15.

Yoon YW, Lee DH, Lee BH, Chung K, Chung JM. Different strains and substrains of rats show different levels of neuropathic pain behaviors. Exp Brain Res. 1999; 129:167-71.

Zaghi S, Heine N, Fregni F. Brain stimulation for the treatment of pain: A review of costs, clinical effects, and mechanisms of treatment for three different central neuromodulatory approaches. J Pain Manag. 2009; 23:339-352.

Zamanillo D, Romero L, Merlos M, Vela JM. Sigma 1 receptor: a new therapeutic target for pain. Eur J Pharmacol. 2013; 716:78-93.

Zhang RX, Zhang M, Li A, Pan L, Berman BM, Ren K, Lao L. DAMGO in the central amygdala alleviates the affective dimension of pain in a rat model of inflammatory hyperalgesia. Neuroscience 2013; 252:359-66.

Zhu CZ, Mills CD, Hsieh GC, Zhong C, Mikusa J, Lewis LG, Gauvin D, Lee CH, Decker MW, Bannon AW, Rueter LE, Joshi SK. Assessing carrageenan-induced locomotor activity impairment in rats: comparison with evoked endpoint of acute inflammatory pain. Eur J Pain. 2012; 16:816-26.

Zhuo M. A Synaptic model for pain: long-term potentiation in the anterior cingulate cortex. Mol. Cells. 2007; 23:259-271

Zhuo M. Cortical excitation and chronic pain. Trends Neurosci. 2008; 31:199-207.