NEW ANIMAL MODELS TO EVALUATE THERAPEUTIC TARGETS FOR PAIN, COGNITIVE AND EATING DISORDERS

A thesis presented by **S.Andreea Bura** in partial fulfilment of the requirements for the degree of *Doctor of Philosophy* in Biomedicine at the Department of Experimental and Health Sciences, Faculty of Health and Life Sciences

Universitat Pompeu Fabra

Thesis director: **Prof. Rafael Maldonado López,**

Universitat Pompeu Fabra

Barcelona, 2010

Pentru Mircea si Dica,

pentru parintii mei

Acknowledgements

It is said that "Acknowledgements" is the most widely read chapter in a thesis, because there always are so many people that deserve gratitude, and that is why, I would like to thank you, who reads these lines. All these years while writing this thesis I learned so much! Apart from the scientific knowledge I accumulated, I think I've learned first of all how to live far from home, from family and friends but at the same time I've learned that it is possible to call a place elsewhere –HOME. All these would not have been possible without the wonderful people I've always had around me or in my soul.

I first of all would like to thank my tutor, Rafael Maldonado, to whom I am grateful that he trusted me and gave me the opportunity to become part of his research group, for his unique capacity of being able to find the ideal solution for any problem. I also thank you, Rafa, that you allow me to borrow your characters and use them on the cover of this thesis!

I thank all people from Neuropharm

Miguel Ángel, for your work energy, diplomacy and force you use on our behalf.

For "el grupo técnico": Dulce, the two Cristinas, Neus, Raquel, Roberto, Marta, Alicia, Ismael. Also for Maria and Begonia. Without your help it would have probably last some years more until I had finished my thesis! You are the best!

For the soul of this laboratory, the predocs: Emma, Ainhoa, Aurelijus, Thomas, Juliana, Xevi, Arnau, Laura, Xavi, Africa, Marta. You have always treated me so nicely, deserving or not, you have always given me so much energy (that is may be because you knew I was the "oldest" of you and I needed it). You are my comrades, my friends and so very often, my family. You are so special so that I can write another thesis about your qualities! Carmen, you have to know that you are my little sister I have never had!

For those that are already postdocs: Miguel, Elena, Javi, Jose, thank you for your friendship and companionship. Javi, thank you for the cover of this thesis. I think you could win a fortune if you put your artistic sense into value!

To the seniours: Fernando, Andres, Blanca, Pato, because you have always had time to give me a helping hand when I was in need.

I also thank those that have once been predocs in Neuropharm: Ester, Anna, Lupe, Clara, Lola, and to those postdocs, Murtra, Flavia, Vicky, Ferran. Ester, I hope one day our plan will come true! Graciela, thank you for guiding my first steps into science!

Gracias Chelo y Carmen por cuidarme siempre.

I thank all members of Neurobiología del Comportament

Thank you Olga for the help you gave me throughout this thesis. Neus, Clara, Jessica, Maria Angela, you have always been amiable and we found together solutions to all

problems. I thank especially you, Neus that you had so much patience to listen to me every time I needed!

Outside the laboratory there are also so many people whom I love and to whom I am so grateful!

My dearest Vane! I remember the day when you set by me at the doctoral courses and you introduced yourself and then you asked my name! That moment I felt I knew you for a life time! Thank you for being always near!

Ivan, Nico, Marta Tschon you are special and that is why I love you so much!

To Adela my dearest and life-long friend, I could dedicate hundreds of pages! It is so much important to know that somebody, somewhere is laughing, is crying, is taking decisions, is working together with you, along side with you. I know you suffered together with me so that this thesis became finalized! Thank you for the way you keep our friendship unique!

Familiei mele, pentru sprijinul neconditionat pe care mi l-a acordat mereu. Tata, tie pentru ca ai stiut sa imi trasmiti mereu optimismul tau si ca m-ai invatat ca la orice problema se poate gasi o solutie! Mama... nu sunt destule superlative ca sa iti multumesc! Mereu m-ai facut sa te simt atat de aproape chiar daca in realitate suntem atat de departe! Dica ..la tine m-am blocat! Esti cea mai speciala persoana din viata mea si te iubesc enorm!

To my cousins Bogdan and Andrea, the support I found in you was like a key point and with you the start of this thesis was much easier!

Tibi si Mariana, va multumesc pentru tot ajutorul acordat in acesti ani, pentru ca ati stiut cum sa ma incurajati de fiecare data cand eram in impas.

Parintilor lui Mircea, socrilor mei, “multumesc pentru tot”.

To you, Mircea, to you, Dica and to my parents I dedicate this thesis. Mircea, your unconditioned love, your way of being always gave me the energy to go on. I thank you for your understanding me the so many times I put this thesis beyond us!

Abstract

Animal models are crucial to improve the knowledge of the mechanisms underlying the different pathological processes. These models are also excellent tools to facilitate the research of new targets for the treatment of different diseases and to evaluate the benefit/risk ratio of the potential new treatments. We have focussed this research work in the study of a new potential targets for pain, cognitive and eating disorders using new animal models developed in our laboratory. We first investigated the effects of the interaction between cannabinoids and nicotine on cognitive processes and metabolism using different behavioural models and new experimental devices. In a second part of this work, we investigated new therapeutic targets for neuropathic pain and for this purpose we developed a new behavioural model to improve the study of the therapeutic potential and possible side-effects of novel compounds.

Resumen

Los modelos animales son cruciales para mejorar el conocimiento sobre los mecanismos que constituyen la base de los diversos procesos patológicos. Estos modelos representan también excelentes herramientas para facilitar la investigación de nuevas dianas para el tratamiento de estas enfermedades y para evaluar el cociente beneficio/riesgo de los nuevos tratamientos potenciales. Este trabajo de investigación se encuentra centrado en el estudio de nuevos dianas terapéuticas para el dolor, los procesos cognitivos y los desórdenes alimentarios utilizando nuevos modelos animales desarrollados en nuestro laboratorio. En primer lugar, hemos investigado los efectos de la interacción entre los cannabinoinoides y la nicotina a nivel los procesos cognitivos y del metabolismo usando diversos modelos comportamentales y nuevos dispositivos experimentales. En una segunda parte de este trabajo, hemos estudiado nuevas dianas terapéuticas para el dolor neuropático y hemos desarrollado para este propósito un nuevo modelo comportamental que permite evaluar el potencial terapéutico y los posibles efectos secundarios de nuevos compuestos.

Abbreviations list

- 2-AG: 2-arachidonoylglycerol
- Ach: acetylcholine
- AR: adenosine receptor
- nAChRs: nicotinic acetylcholine receptors
- CNS: central nervous system
- CCI: chronic constriction injury
- DA: dopamine
- DRG: dorsal root ganglion
- FAAH: fatty acid amide hydrolase
- GABA: γ-aminobutyric acid
- 5-HT: serotonin
- MAPK: mitogen-activated protein kinase
- NA: noradrenaline
- NMDA: N-methyl- D- aspartate
- PSNL: partial sciatic nerve ligation
- THC: Δ^9- tetrahidrocannabinol
- TRPV1: vanilloid receptors type 1
- σ1Rs: Sigma 1 receptor

Index

INTRODUCTION

1. Nicotine and cannabinoids are consumed in combination and produce common effects

Drug abuse and dependence, and their consequences represent an important worldwide social and health problem. In Spain, cannabis is the most used illicit drug, particularly in teenagers and young adults. This drug is consumed alone or in combination with alcohol or tobacco. Hashish is the most common used form of cannabis in Spain and it is always mixed with tobacco. Nicotine is the main psychoactive component of tobacco and is a highly addictive substance (Bruijnzeel and Gold 2005; Castañé *et al.* 2005; Cohen *et al.* 2005). Both cannabis and nicotine consumption can affect similar brain and peripheral physiological functions. Learning and memory impairments are among the most commonly reported acute behavioural side-effects of cannabinoids. Thus, acute consumption of marijuana is associated with several subjective effects including impaired memory, altered time sense as well as decrements in various tasks such as reaction time, learning perception, motor coordination and attention (Ameri 1999; Lichtman *et al.* 2002; Sullivan 2000). In addition, chronic marijuana smoking may cause persistent memory and cognitive deficiencies. Otherwise, nicotine has been frequently shown to improve performance in tests of sustained attention (Levin and Rezvani 2000). However, nicotine withdrawal is associated with an impairment of attention (Levin *et al.* 2006).

Both nicotine and cannabinoids have important effects on food intake and metabolism. Indeed, chronic cigarette smoking influences the body weight by decreasing the appetite and/or increasing the metabolic rate (Andersson and Arner 2001; Perkins 1992; Winders and Grunberg 1990). Nevertheless, smoking cessation is associated with an increase in body weight. On the other hand, cannabis also modulates food intake and

metabolism. To date, the best established therapeutic applications of cannabinoids together with the antiemetic action is the amelioration of appetite and weight gain which may provide benefits in patients with cancer cachexia or AIDS (Di Marzo V and Matias 2005; Kirkham *et al.* 2002), and the treatment of obesity using cannabinoids antagonist (Di, V 2008). In addition, the activation of cannabinoid receptors produces other metabolic effects at the central and peripheral levels.

Nicotine and cannabinoids have also important effects on nociception. Due to the widespread distribution of nicotinic acetylcholine receptors (nAChRs), nicotine exerts its antinociceptive effects by acting at both central and peripheral levels. Besides, the presence of cannabinoid receptors in the different peripheral and central structures involved in the transmission of nociceptive messages explains the analgesic properties of cannabis.

In the following chapters, we will focus on the endocannabinoid system and nicotine and on their role in the regulation of cognitive functions, metabolism and pain.

1.1 Nicotine

Tobacco addiction is one of the most important heath problems with major social-economical consequences in developed countries, its relevance being now significantly higher in developing countries. According to World Health Organisation, more than 1,000 million persons smoke tobacco and this phenomenon causes more than 5 million deaths per year and, if the present trend continues, 10 million smokers per year are predicted to die by 2025 (Hatsukami *et al.* 2008). Nicotine is the main psychoactive compound of tobacco and is the main responsible for its addictive properties (Berrendero *et al.* 2010).

1.1.1 General characteristics

Nicotine is named based on the tobacco plant *Nicotiana tabacum*, which in turn is named after Jean Nicot de Villemain, French ambassador in Portugal, who sent tobacco and seeds from Brazil to Paris in 1560 and promoted their medicinal use. Nicotine was isolated for the first time from the tobacco plant in 1828 by the German chemists Posselt and Reimann (Figure 1).

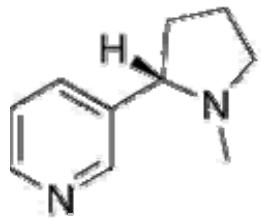


Figure 1 Chemical structure of nicotine ($C_{10}H_{14}N_2$,3-(1-metilpirrolidin-2-il) pyridine). (Hohh et al, 2003)

Nicotine is a tertiary amine that has two isomers. Tobacco contains the most active pharmacological isomer, the L-nicotine. The absorption of this weak base depends of the pH. Thus, nicotine from cigarettes has a pH of 5.5 and is absorbed in the lungs in a percentage that varies from 79% to 90%. It is also absorbed in a small amount through oral mucous, sublingual plexus and skin. Nicotine from pipes, cigars, snuffed and chewing tobacco has a pH of 8.5 and is absorbed mainly by oral and nasal mucous. After absorption in the organism, nicotine is rapidly distributed in the peripheral tissues, it takes 10-19 seconds to reach the brain (Benowitz 1996) and its half-life is estimated to be in the range of 2-3 hours. 80% of the drug is metabolized in the liver where it is converted in an inactive metabolite, cotinine, the main nicotine inactive metabolite. A small percentage (5%) of the drug is excreted through the kidneys.

CYP2A6 is the enzyme that is primarily responsible for the oxidation of nicotine but other enzymes like CYP2B6, UDP-glucuronosyltransferases or FMO3 (Flavin-Containing Monooxygenase 3) are also involved in nicotine metabolism (Hukkanen *et al.* 2005). One important aspect is that genetic variations in the enzymes involved in nicotine metabolism can profoundly affect the rates of metabolism of this drug, which in turn can influence nicotine-taking behaviours (Wall *et al.* 2007).

1.1.2 Nicotinic receptors, classification, structure and distribution

The primary targets of nicotine are the nAChRs, which are highly conserved across species (Le and Changeux 1999). They are expressed in most tissues and organs, including the brain. The endogenous ligand for nAChRs is acetylcholine (ACh) (Hogg *et al.* 2003), and the activation of nAChRs mainly enhances neurotransmitter release and neuronal excitability throughout the brain. As a result, nAChRs modulate a large number of behaviours, ranging from basic physiological functions such as pain sensation, sleep pattern and feeding, to more complex processes involved in learning, emotional responses and reward (Gotti and Clementi 2004; Hogg *et al.* 2003; Hogg and Bertrand 2004; Picciotto *et al.* 2000; Picciotto *et al.* 2002; Picciotto and Zoli 2002; Wonnacott *et al.* 2000). Moreover, nAChRs affect brain development through their effects on synaptic transmission and plasticity (Berg *et al.* 2006), as well as aging, through their neuroprotective effects (Picciotto and Zoli 2002). NAChRs have a pentameric structure with different subunits (α, β, γ, δ) organised around a central channel and can be homo-or hetero-oligomeric receptors. Nowadays, there have been described 10 α subunits (α_1-α_{10}), three β subunits (β2-β4), one γ subunit and one δ subunit. The possible different combinations of these subunits and their extensive location in the organism are responsible for the multitude of physiological functions of

nAChRs (Gotti and Clementi 2004) (Figure 2). On the basis of their different phylogenetic, functional and pharmacological properties, the heterogeneous family of nAChR subtypes have been divided into two main classes: sensitive to α-bugarotoxin, (αBgtx-nAChRs), which may be homomeric (made up of α7–α9 subunit homo-pentamers) or heteromeric (made up of α7, α8 or α9, α10 subunit hetero-pentamers) with a low affinity for nicotine, and α-bugarotoxin no sensitive nAChRs, which contain the α2–α6 and β2–β4 subunits, and only form heteromeric receptors that bind with high affinity to nicotine (Lindstrom 1997).

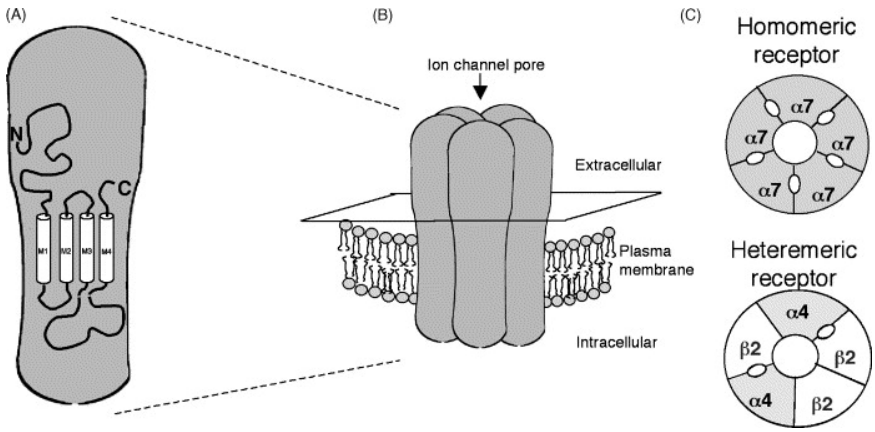


Figure 2: (A–C) Organisation and structure of nAChRs. (A) Schematic representation of nAChR subunits. (B) Pentameric arrangement of nAChR subunits in an assembled receptor. (C) Subunit arrangement and the localisation of the binding site (modified, by Gotti and Clementi, 2004)

The nAChRs are expressed in central nervous system (CNS), autonomic nervous system, skeletal muscles, lymphoid tissue, macrophages, skin, lung cells, vascular tissue, astrocytes, chromaffin cells of adrenal medulla and sensory organs (Gotti *et al.* 2006). In the CNS, the nAChRs are widely expressed and at this level, they are composed exclusively of α (α_2-α_{10}) and β (β2-β4) subunits, forming heteropentameric receptors, or only

of α_7 subunits creating homopentomeric receptors (Figure 3). These receptors are highly expressed in the cortex, hippocampus and subcortical limbic regions, and show low levels in the thalamic regions and basal ganglia (Gotti *et al.* 2006). Anatomical and functional evidence suggest that nAChRs are preferentially located in the CNS at presynaptic sites where they modulate neurotransmitter release, although some few nAChRs have also been found on cell bodies or dendrites where they mediate postsynaptic effects (Gotti *et al.* 2006). In particular, presynaptic nAChRs have been implicated in the release of several neurotransmitters, including ACh (Wilkie *et al.* 1993) noradrenaline (NA) (Clarke and Reuben 1996) dopamine (DA) (Grady *et al.* 1992; Wonnacott *et al.* 2000), glutamate (Alkondon *et al.* 1997; McGehee and Role 1995), and γ-aminobutyric acid (GABA) (Yang *et al.* 1996).

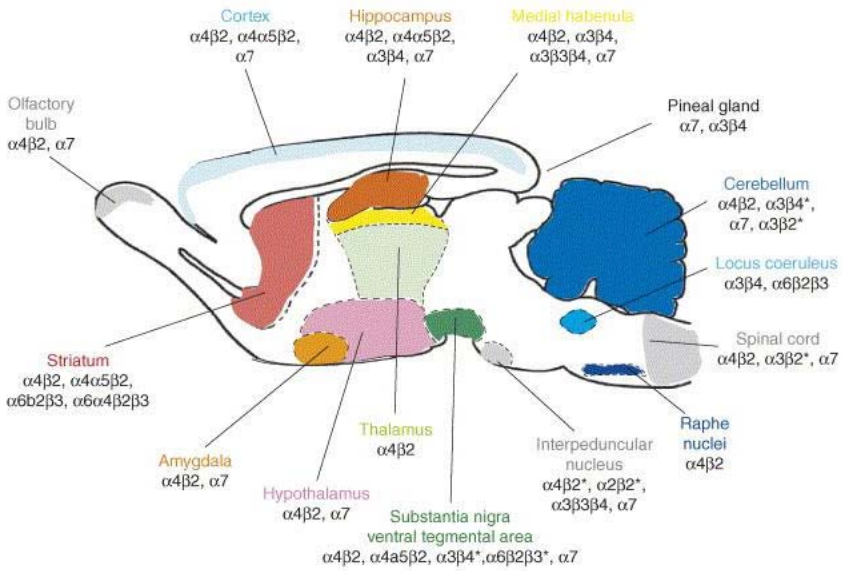


Figure 3: Regional distribution and subunit organization of the nAChRs in the CNS of rodents (modified, Gotti et al, 2006)

Functionally, the different nAChR subtypes can exist in four distinct conformations: resting, open, desensitised and inactive channel states (Gotti and Clementi 2004). In general, nAChRs are found in a closed (resting) state before agonist arrives. They remain briefly in an open state when the agonist binds the receptor, and while the channel is conducting cations during this state. Immediatly after the open state, nAChRs are in desensitized or inactive states while unresponsive to agonist. The likelihood of being in a particular state depends on many factors, including the nAChR subtype, the agonist concentration, and the rate of agonist application. For instance, rapid pulse of agonists causes synchronized activation of nAChRs followed by inactive state whereas long-term exposure to an agonist causes desensitization, loss of functional response, followed by a long-lasting inactive state of the nAChRs (Dani and Heinemann 1996). A slow application of a low agonist concentration can cause desensitization without activation because the desensitized receptor has a higher affinity for agonists than the resting or open receptor (Dani and Heinemann 1996). The higher affinity of the desensitized receptor for agonists and the changing distribution of nAChRs among the various functional states must be considered to understand the processes that occur during sustained nicotine use (Dani and Heinemann 1996).

1.1.3 Pharmacological effects of nicotine

Due to the wide distribution of nAChRs receptors, nicotine produces a large range of pharmacological effects including tachycardia, peripheral vasoconstriction, hypertension and activation/inhibition of CNS among other effects. Table 1 resumes the main pharmacological effects of nicotine. In the next paragraphs, we will focus on the effects induced by nicotine in memory and cognition, food intake and energy balance, and pain.

Table 1 Pharmacological effects of nicotine

Physiological and pathologycal processes	Effects of nicotine	Recent reviews
1. Learning and memory	Improve memory and attention Attention impairments during wihdrawal	Levin et al, 2000; Young et al 2004
2. Food intake	Decrease/increase (withdrawal)	Shoaib and Bizzaro , 2005, Levin et al, 2006
3. Pain	Antinociception	Flores, 2000
4. Motor control	Hypo/ Hyperlocomotion	Yoo et al, 2004, Picciotto , 2003
5. Anxiety	Anxyogenesis, anxyolisis	Balerio et al, 2005
6. Motivation	Euphoria	Pormeleu C, et al 1992
7. Corporal temperature	Hypothermia	Zarrindast et al, 2001
8. Inflamation	Antiinflamatory	Kalra R et al, 2004
9. Neurodegeneration	Neuroprotection	Gotti and Clementi , 2004
10. Immune system	Immunosuppresant	Kalra R et al, 2004
11. Cardiovascular system	Tachycardia, hypertension,	Wang et al, 2001
12. Sleep	Arousal, rapid eye movement sleep	Lena et al, 2004

1.1.3.1 Nicotine and cognitive processes

The effects of nicotine on learning and memory have been recognized for several decades (Levin *et al.* 2006; Rezvani and Levin 2001; Stolerman *et al.* 2000). Cognitive improvement induced by nicotine and nicotinic agonists has been documented in rats (Levin *et al.* 1997), rabbits (Woodruff-Pak 2003), monkeys (Buccafusco *et al.* 1999), mice (Picciotto *et al.* 1995), zebra fishes (Levin and Chen 2004) and also in humans (Levin *et al.* 2006). The different cognitive models available in animals allow the evaluation of working and reference memory. Working memory is defined as memory with changing contents, as opposed to reference memory, which is defined as memory with fixed contents (Decker *et al.* 1995; Levin and Simon 1998). Acute and chronic treatment with nicotine or nicotine agonists, such as dimethylaminoethanol, epibatidine, ABT-418, TC-1734 and lobeline have been shown to significantly improve working memory function in several models in rodents (Levin *et al.* 2006). Thus, a single dose of nicotine significantly improved working memory in rats in the eight-arm maze(Levin *et al.* 1997). Another task for assessing working memory is the object recognition paradigm. In this

model, rodents are able to discriminate between a familiar object and a new object for a short period of time after initial presentation of the familiar object. Acute nicotine administration enhanced acquisition, consolidation, and restitution of the information in the object recognition task in rats (Puma *et al.* 1999). Chronic treatment with nicotine agonists has also been shown to improve memory performance in other memory tasks such as the Morris water maze (Attaway *et al.* 1999), one-way avoidance, Lashley III maze (Arendash *et al.* 1995) and passive avoidance task (Ciamei *et al.* 2001). Thus, chronic nicotine improved working but not reference memory in the Morris water maze. Chronic nicotine pretreatment induced an enhancement in overall learning and reference memory, but did not affect working memory in the 17-arm radial maze (Arendash *et al.* 1995) and improved acquisition in the active avoidance and the Lashley III maze (Arendash *et al.* 1995). The Lashley III maze consists of a start box, four interconnected alleys, and a goal box containing a food reward and the animals placed in the start compartment are allowed to traverse the maze to obtain a food pellet located in the goal box.

Nicotine can also counteract working memory deficits induced by lesions of the forebrain cholinergic projection systems in the water maze task, (Decker and Majchrzak 1992; Decker *et al.* 1992; Grigoryan *et al.* 1994; Riekkinen, Jr. *et al.* 1993) and both working and reference memory in the radial maze task (Hodges, H *et al.* 1992). Furthermore, chronic nicotine infusion has been shown to reverse working memory deficits due to lesions of the fimbria or medial basal cortical projection (Levin *et al.* 1993). In rats, learning impairments caused by AF64A, a neurotoxic derivative of choline that produces long-lasting cholinergic deficits, were attenuated by nicotine in the passive avoidance (Hiramatsu *et al.* 2002). In addition, the deficits caused by the muscarinic cholinergic antagonist scopolamine in the passive avoidance paradigm were decreased by oral

administration of the nicotinic agonist TC-1734 (Gatto *et al.* 2004). In the eye-blink conditioning, a classical test for associative learning and memory evaluation, nicotine and GTS-21 α7 nicotinic receptor partial agonist and α4β2 nicotinic receptors antagonist reversed mecamylamine-induced deficits in rabbits (Woodruff-Pak 2003).

Studies evaluating the effects of nicotine on working memory and learning in young versus old animals provided a mixed picture of age-related effects. In young rats, nicotine improved the cognitive acquisition in the Morris water maze procedure which suggests an improvement in working memory. However, no beneficial effects of nicotine in reference memory were found in either age group (Attaway *et al.* 1999). In contrast with these findings, the nicotine agonist, SIB-1553A, improved performances in working memory in a delayed matching to sample task in aged mice, but was less effective in improving the performance in reference memory tasks (Bontempi *et al.* 2001).

In spite of these studies showing positive effects of nicotine in learning and memory, some few animal studies did not show cognitive improvement after nicotine administration or found the opposite effect. Thus, it has been reported that acute administration of nicotine did not improve the cognitive acquisition in the water maze in group-housed mice and even impaired it in individually housed mice (Moragrega *et al.* 2003), whereas chronic administration of nicotine in NMRI mice did not significantly change performance in the water maze tested at any age (Vicens *et al.* 2003).

Specific nAChRs subunits are responsible for the modulation of cognitive processes as revealed by studies using knockout mice. These studies have demonstrated that the receptors composed by $\alpha_4\beta_2$ subunit combinations and by α_7 subunit are specifically involved in the regulation of memory (Levin *et al.* 2006) and attention (Wilens and Decker 2007). Besides, the β_2 subunit of nAChRs has been selectively involved in neuronal survival

and in the maintenance of cognitive performance during aging (Gotti *et al.* 2006). In addition, the α_7 subunit has been correlated with attention deficit from schizophrenia (Freedman *et al.* 1995; Leonard *et al.* 1998; Young *et al.* 2007). Thus, patients with schizophrenia have an impaired ability for sensory gating that may result in the flooding of information and it has been shown that smoking improves sensory gating in overnight abstinent smokers with schizophrenia. These improvements in auditory sensory gating may result from stimulation of α7 nicotinic receptors by nicotine, as altered number and function of α7 nicotinic receptor were related to schizophrenia (Court *et al.* 1999; Guan *et al.* 1999).

The effects of nicotine on cognitive processes have also been investigated in other animal species, different from rodents including pigeons, zebra fish and monkeys. Thus, low doses of nicotine improved memory performance in zebrafish, while high doses produced the opposite effect (Levin and Chen 2004). In monkeys, nicotine and GTS-21 reversed the working memory impairment induced by ketamine in the delayed matching-to-sample task (Buccafusco and Terry, Jr. 2009). In contrast with these data, nicotine decreased accuracy in two models of sustained attention in pigeons (Lemmonds *et al.* 2002).

In humans, the effects of nicotine on memory performance have been evaluated in both smokers and non-smokers. It has been proposed that nicotine may enhance performance on tasks requiring primarily left hemisphere resources while impairing right hemisphere-based performance (McClernon *et al.* 2003). The effects of transdermal nicotine administration on lateralized consonant identification and memory interference were examined in dependent smokers and non-smokers in a double-blind, placebo-controlled study (McClernon *et al.* 2003). In this study a lateralized cognitive task was used, in which subjects had to complete a lateralized letter identification task that required them to identify strings of three consonants presented in the left or right visual

field while keeping a word in memory. Nicotine decreased word memory errors on trials where consonants were presented in the right visual field and increased errors on lefts visual field. These findings suggest that nicotine may enhance working memory by decreasing distractibility and that the effects of nicotine on cognition may be lateralized. A recent study, suggests also that acute nicotine administration may exert direct beneficial effects on novelty detection and subsequent memory recognition in both smokers and nonsmokers (Froeliger *et al.* 2009). All these studies underline the important effects induced by nicotine in memory and learning processes in both experimental animals and humans.

The stimulation of nAChRs has also other behavioural effects that can be related to the improvement of learning and memory. Indeed, nicotine and nAChRs agonists have been reported to improve attentional function in both animals and humans (Rezvani and Levin 2001). Thus, a low-dose range of nicotine improves attention in an operant visual signal detection task in rats, as reflected in the increase in choice accuracy (Rezvani *et al.* 2002). This effect was blocked by the nAChRs antagonist mecamylamine (Rezvani *et al.* 2002). Mecamylamine significantly antagonised the effects of nicotine on correct response latency and on anticipatory responses in the five-choice serial reaction time task (Grottick and Higgins 2000; Mirza and Stolerman 1998), while the nicotine agonists ABT-418 and SIB-1553A improved the correct responses in the same task (Terry, Jr. *et al.* 2002). This task is used to assess both selective and sustained attention and requires that the rat responds with a nose-poke following the presentation of a brief visual stimulus in one of five locations (Blondel *et al.* 2000). In addition, nicotine reversed also attentional impairments in rats caused by basal forebrain or septohippocampal pathways lesions (Levin *et al.* 1993; Mirza and Stolerman 1998; Stolerman *et al.* 2000).

Several studies have also revealed an improvement of attentional function in humans after nicotine administration. Thus, nicotine given in

transdermally patches improved attention in non-smoking subjects who had no preexisting attentional deficits (Levin *et al.* 2006). Subcutaneous nicotine administration improved also both working memory and attention in non-smoking healthy volunteers, as revealed in a functional magnetic resonance imaging study (Kumari *et al.* 2003). In addition, young female smokers manifest poorer performance than non-smokers on attention-related tasks (Greenbaum *et al.* 2009). Controlled nicotine administration in the smoker group had stronger short-term facilitating effects on attention in women then in men. In this study women had higher number of attention deficit symptoms and consumed more nicotine and caffeine (Rigbi *et al.* 2010).

It is well recognised that the most common behavioural symptoms that appear after smoking cessation are emotional dysregulation and cognitive deficits (Hughes 2007; Ward *et al.* 2001). Nicotine was able to ameliorate the attention deficit that occurs in smokers who abstained from smoking for at least 10 h prior to testing (Mancuso *et al.* 1999) and varenicline, a partial agonist of the α4β2 nAChRs, improved mood and cognition during smoking abstinence (Patterson *et al.* 2009). Nicotine has also been shown to improve the detrimental effects of cigarette abstinence in basic aspects of cognition (e.g., sustained attention), but may not alleviate higher-level processes such as memory (Kelemen and Fulton 2008).

Another cognitive aspect that deserves a special attention is the potential effect of nicotine and the involvement of nAChRs in Alzheimer's disease. Postmortem assessment of the brains of patients with Alzheimer's disease has shown significant nAChRs loss in cortex and striatum (Court *et al.* 2001) and this loss appears to be less intense in smokers than non-smokers with the Alzheimer's disease (Hellstrom-Lindahl *et al.* 2004). Postmortem evidence also shows lesser plaque formation in smokers than non-smokers with Alzheimer's disease (Hellstrom-Lindahl *et al.* 2004). Acute and chronic nicotine or nicotine agonists administration in Alzheimer's

disease patients has been reported to enhance attention function, but not memory performance (Levin *et al.* 2006). However, another study has reported that transdermal nicotine improved the acquisition of information in Alzheimer's disease patients (Wilson *et al.* 1995). It is important to underline that the most widely used treatments for Alzheimer's disease at the present moment are anticholinesterase inhibitors, which increase brain levels of choline and indirectly stimulate all the cholinergic receptors, including nAChRs. Nevertheless, there are at least four studies that have not found evidence of improvement of memory function by nicotine administration in Alzheimer's disease patients (Snaedal *et al.* 1996; White and Levin 1999; Wilson *et al.* 1995). All these data suggest that the nAChRs could represent interesting targets in the treatment of pathologies related with memory, learning and attention deficits.

1.1.3.2 Nicotine in food intake and energy balance

It is well known that nicotine reduces appetite and alters feeding patterns typically resulting in reduced body weight after chronic exposure (Chiolero *et al.* 2008; Grunberg 1986; Grunberg *et al.* 1986; Miyata *et al.* 1999). The effects of chronic nicotine administration on appetite suppression and body weight have been described in both humans and laboratory animals (Jo *et al.* 2002).

Feeding behaviour gathers complex peripheral and central mechanisms in which the nAchRs are involved. Peripheral tissues implicated in the regulation of food intake receive inputs from sympathetic and/or para-sympathetic neurons that are controlled by cholinergic transmission. All the autonomic neurons, as well as many enteric, and even a subset of sensory neurons express a variety of nAChRs that are activated by nicotine (Cooper 2001; De *et al.* 2000; Picciotto *et al.* 1995). Therefore, the anorexigenic effects of nicotine can be partly mediated by the

activation of peripheral neuronal components of visceral-sensory and visceral-motor pathways involved in the regulation of feeding.

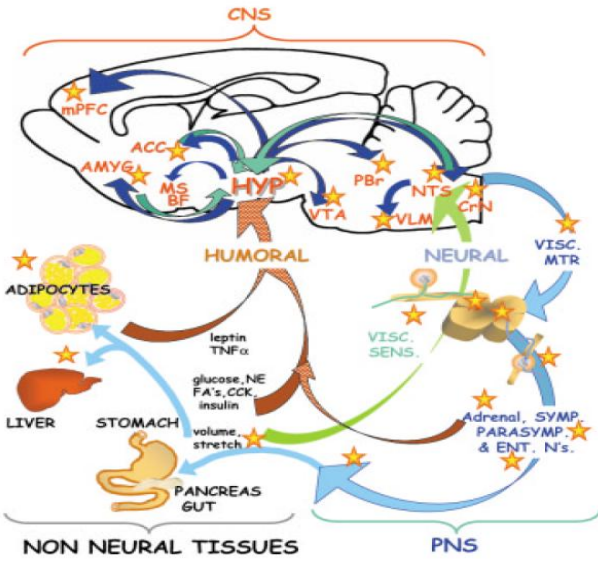


Figure 4. Possible sites of nicotine effects on feeding behaviour (adapted from Jo et al 2002). The brain receives a multitude of signals from the periphery reporting on adequacy of food intake and energy balance. These include humoral signals (red ribbon arrow) such as hormones and cytokines (leptin, TNFα, insulin, cholescystokinin, norepinephrine) and metabolites (glucose and fatty acids) as well as neural signals (green ribbon arrow). Within the hypothalamus (HYP) and the nucleus of the tractus solitarius (NTS) this information is integrated and transmitted to multiple brain regions (dark blue arrows), and the appropriate behaviour is elicited. In addition to these central responses, the PNS (light blue) neurons including sympathetic, parasympathetic, and enteric, innervate the gastrointestinal tract, adipose depots, and endocrine organs. Possible sites at which nicotine might modify feeding behaviour or energy balance are indicated with stars. A number of non-neural tissues express nAChRs, and could respond directly to nicotine. In the CNS, nicotine could act within the hypothalamus, the NTS, and in the regions throughout the neuroaxis to which these structures project (mPFC, medial septal, and basal forebrain nuclei; VTA, ventral tegmental area; NTS, nucleus tractus solitarius; PBr, parabrachial nucleus; VLM, ventrolateral medullary nucleus; CrN, cranial nerve nuclei; VISC. SENS and VISC MTR, visceral sensory and motor neurons; NE, norephnephrine, ENT, N's, enteric neurons; CCK, cholecystokinin; FAs, fatty acids).

In addition to the direct alteration of neuronal excitability, nicotine also modulates the release of several neurotransmitters in the vegetative system. Thus, it enhances the release of both ACh and norepinephrine by

acting on presynaptic nAChRs increasing the sympathetic activity and enhancing therefore the energy expenditure (Bray 2000). In the same line, nicotine administration increases sympathetic activity in liver, adipose tissue and gut (Bray 2000), and alters at this level metabolic processing in hepatocytes and adipocytes (Arai *et al.* 2001; Ashakumary and Vijayammal 1997; Sztalryd *et al.* 1996). In the adipose tissue, nicotine induces lipolysis by the stimulation of both nAChRs and beta-adrenoceptors located on the fat cell via catecholamine release (Andersson and Arner 2001) (Figure 4). In the liver, nicotine increases the synthesis and secretion of triglyceride-rich lipoproteins (Ashakumary and Vijayammal 1997). Nicotine also decreases feeding by increasing leptin levels and/or by enhancing the leptin–receptor-mediated signaling cascade. Leptin is a peptide hormone produced mainly by adipose tissue that decreases appetite and increases energy expenditure.

Besides these peripheral responses, the CNS also plays an important role in the effects of nicotine on food intake and metabolism. Thus, a crucial control site for the anorectic effects of nicotine is the hypothalamus. NAChRs, mainly α7 and α/β containing subunits, are detected throughout the hypothalamus (Britto *et al.* 1992; Hatton and Yang 2002; O'Hara *et al.* 1998; Okuda *et al.* 1993; Pabreza *et al.* 1991). The lateral hypothalamus is considered an important site of nicotine induced appetite suppression. Thus, nicotine administration into the LH decreased food intake (Miyata *et al.* 1999; Miyata *et al.* 2001; Yang *et al.* 1999) and produced both short- and long-term changes in the release of a variety of transmitters (Li *et al.* 2001; Meguid *et al.* 2000a; Miyata *et al.* 1999; Zhang *et al.* 2001). In particular, the hypophagic effect of nicotine was associated with increased serotonin (5-HT) and DA activity in lateral hypothalamus, whereas hyperphagia after nicotine cessation was accompanied by decreased concentrations of these neurotransmitters (Meguid *et al.* 2000b; Miyata *et al.* 1999). Moreover, nicotine enhances GABA release and

potentiates glutamatergic activity within the lateral hypothalamus (Jo *et al.* 2005b). Nicotine also modulates the release of orexin and other orexigenic neuropeptides in the hypothalamus, such as neuropeptide Y and melanin-concentrating hormone (Frankish *et al.* 1995; Jo *et al.* 2005b; Frankish *et al.* 1995). Neuropeptide Y is highly concentrated in the hypothalamus and melanin-concentrating hormone is uniquely expressed in lateral hypothalamus neurons. Nicotine administration decreases NPY and NPY mRNA levels in the arcuate nucleus by 35%, which also correlates with the reduction of food intake (Frankish *et al.* 1995). Nicotine administration or elevated cholinergic tone decreases the activity of melanin-concentrating hormone neurons via activation of presynaptic α7-nAChRs (Jo *et al.* 2005b).

Therefore, the effects of nicotine in the regulation of appetite have been well described in the literature. However, the underling mechanisms implicated in these responses remain to be elucidated and further studies are necessary to clarify these neurobiological mechanisms.

1.1.3.3. Nicotine and pain

Both clinical and preclinical studies have shown that nicotine induces antinociceptive effects in several experimental conditions (Arneric *et al.* 2007; Flores 2000; Pomerleau *et al.* 1984). In animals, nicotine and other nAChRs agonists induce antinociceptive effects in several models of acute, inflammatory and neuropathic pain (Berrendero *et al.* 2002; Castañé *et al.* 2002; Cohen *et al.* 2005; Flores 2000) Studies conducted in humans have shown that the pain threshold and tolerance pain ratings were increased after smoking and transdermal administration of nicotine (Girdler *et al.* 2005; Pauli *et al.* 1993; Pomerleau *et al.* 1984).

Nicotine exerts its antinociceptive effects by acting at both central and peripheral levels. At the peripheral level, nAChRs are located in the

nociceptive terminals and dorsal root ganglion (DRG) neurons of peripheral C fibers (Genzen *et al.* 2001; Young *et al.* 2008). At the spinal level, both the interneurons of substantia gelatinosa (layers II-III) and the inhibitory/excitatory neurons from the ventral layers, express nAChRs (Cordero-Erausquin *et al.* 2000; Rashid *et al.* 2006). At the supraspinal level, the nAChRs are found in areas involved in the integration and transmission of pain, such as thalamus, amygdala, hypothalamus and cortex. Moreover, neurons of structures that belong to the descending pain inhibitory system, such as pedunculopontine tegmental nucleus, rafe magnus nucleus and locus coeruleus, also express nAChRs (Gotti *et al.* 2006) that modulate the activity of other neurotransmitter systems such as noradrenergic, GABAergic, serotoninergic, opioid and endocannabinoid systems (Berrendero *et al.* 2002; Cordero-Erausquin *et al.* 2004; Decker *et al.* 2004; Iwamoto and Marion 1993; Jafari *et al.* 2007; Rashid *et al.* 2006). In this sense, several neurotransmitters have been involved in the antinociceptive effects of nicotine. Thus, the stimulation of the spinal norepinephrine release through the activation of nAChRs in the noradrenergic terminals has been involved in these antinociceptive effects (Li and Eisenach 2002). In addition, nicotine enhances the level of endogenous opioid peptides derived from preproenkephalin through nAChRs activation, which participates in spinal and supraspinal nicotine antinociception by stimulating μ opioid receptors (Maldonado and Berrendero 2010). A tonic nicotinic modulation of 5-HT release has also been demonstrated, that provides an additional argument to support that cholinergic nicotinic transmission participates in the physiological regulation of descending serotoninergic pathways (Cordero-Erausquin and Changeux 2001; Mason 1999). Another system that has been recently demonstrated to play an important role in the modulation of nicotine-induced antinocipetive effects is the endocannabinoid system (Castañé *et al.* 2002; Jafari *et al.* 2007).

Among the different nAChRs subunits, α_4 seems to play a crucial role in nicotine antinociceptive effects. Indeed, lesion of 5-HT-containing neurons from rafe magnus nucleus resulted in a decrease of α_4 subunit and in a subsequent reduction of nicotine antinociception (Bitner *et al.* 1998). In the same direction, studies using knockout mice deficient of α_4 subunit have shown that nicotine antinociception was blocked in these mutants (Marubio *et al.* 1999). Studies using β_2 subunit knockout mice proved that this subunit also participates in the antinociceptive effects of nicotine (Marubio *et al.* 1999). Hence, it seems that the combination of $\alpha_4\beta_2$ subunits plays a crucial role in the mediation of nicotine-induced antinociception.

In humans, nicotine from smoking or transdermic parches increased the pain threshold and tolerance pain ratings (Girdler *et al.* 2005; Jamner *et al.* 1998; Pauli *et al.* 1993; Pomerleau *et al.* 1984). On the other hand, smokers endure better the pain compared with those smoking a nicotine-free cigarette (Pomerleau *et al.* 1984). However, it has been shown that gender plays an important role in the effects of smoking on nociception. Thus, smoking was associated in women with decreased pain sensitivity to ischemic pain, while male smokers had decreased pain sensitivity to cold pressor pain. Nevertheles, smoking did not influence pain perception for either gender in response to thermal heat pain (Girdler *et al.* 2005).

The possible effects of nicotine administration on neuropathic pain are of a particular interest. A higher incidence of smoking is often reported in patients with chronic pain conditions (Goldberg *et al.* 2000). Thus, clinical data support a correlation between the incidence of smoking and the incidence of diabetic neuropathy (Mitchell *et al.* 1990; Muhlhauser *et al.* 1986), but the relationship between nicotine intake and the severity of other neuropathic pain conditions is less clear. When tested directly, pain thresholds are increased immediately after smoking (Fertig and Allen 1996). However, the improvement of symptoms of cold intolerance

following peripheral nerve injury appeared more likely in non-smokers than smokers (Irwin *et al.* 1997)

Preclinical data also gather contradictory evidence regarding the relationship between nicotine and neuropathic pain. Thus, chronic nicotine administration increased mechanical sensitivity to pressure in both normal and nerve-injured rats in a spinal nerve ligation model of neuropathic pain via the prolonged desensitization of α4β2 nAChRs. The degree of mechanical hypersensitivity was reflected in the spinal cord of these chronic nicotine treated rats, by an enhancement of CREB phosphorylation (Josiah and Vincler 2006). In contrast, intrathecal nicotine and intrathecal nicotine agonist epibatidine completely reverse thermal and mechanical hypersensitivity in partial sciatic nerve-ligated mice at doses that have no effect in sham-operated animals(Rashid and Ueda 2002). In addition intrathecal administration of the α3β2/α6β2 nAChR antagonist, α-CTX MII, reduced mechanical hypersensitivity following this model of spinal nerve ligation, suggesting that endogenous ACh within the spinal cord inhibits the transmission of nociceptive mechanical stimuli (Young *et al.* 2008).

Therefore, all these data suggest that compounds targeting neuronal nAChRs may represent a new class of analgesic agents. However, the possible clinical use of nicotine in pain is limited because of several serious limitations: its modest and short duration effect, its possible secondary effects such as the appearance of confusion at high doses, and the development of tolerance after chronic administration. Considering the many nAChRs subtypes that have been identified, the development of new selective agonists of some of these receptors may provide the basis to dissociate the therapeutic and side-effects in order to improve the benefit/risk ratio of this type of compounds (Decker *et al.* 2004)

1.2 Cannabinoid system

1.2.1 Natural and synthetic cannabinoids

The Cannabis plant has been used since antiquity for recreational purposes and as a medicinal herb. Marijuana is the dry shredded mixture of flowers stems, seeds and leaves of the plant *Cannabis sativa.* The dried mixture can be smoked in form of cigarette (named *joint*) or in a pipe. Other preparations include hash or hashish, the dried sticky resin of the flowers of the female plant, or hash oil, a sticky black liquid. In Spain, hashish is the most common used form of cannabis and it is almost always mixed with tobacco.

Cannabis sativa contains more then 400 different compounds of which 60 are psychoactive and are referred to as cannabinoids. The main psychoactive compound of *Cannabis sativa*, Δ^9- tetrahidrocannabinol (THC), was identified and isolated in 1964 by Gaoni and Mechoulam. THC content is the highest in the oil from the flowering tops and the lowest in the seeds. After inhalation, THC is detectable in plasma within seconds, reaching the peak in 3-10 min after smoking. Because of its high lipophilicity, it rapidly enters in the highly vascularized tissues including the liver, heart and brain (Grotenhermen 2003). Significant accumulation of cannabinoids occurs later in less vacularized tissues and body fat. THC is only slowly released back into the bloodstream and other body tissues from these deposits and full elimination from the body is slow (plasmatic half-life 24-36 h) (Grotenhermen 2003). THC is metabolised in the liver and its active and inactive metabolites are excreted through the digestive tract, kidneys and sweat. Other important compounds of *Cannabis sativa* are Δ^8-tetrahidrocannabinol and cannabinol which also show psychoactive properties and cannabidiol that does not have these characteristics.

Studies that link the structure of natural cannabinoids with their pharmacological activity and the cloning of cannabinoid receptors (Matsuda *et al.* 1990; Munro *et al.* 1993) allowed the development of new molecules that selectively bind to the cannabinoid receptors. Nowadays, the synthetic agonists of the cannabinoid receptors can be classified into two main categories: (1) compounds that have similar structure to THC, such as HU-210, CP-55,940 and nabilone (Figure 5), and (2) compounds that have a different chemical structure, such as the aminoalkylindoles, that include WIN 55,212-2.

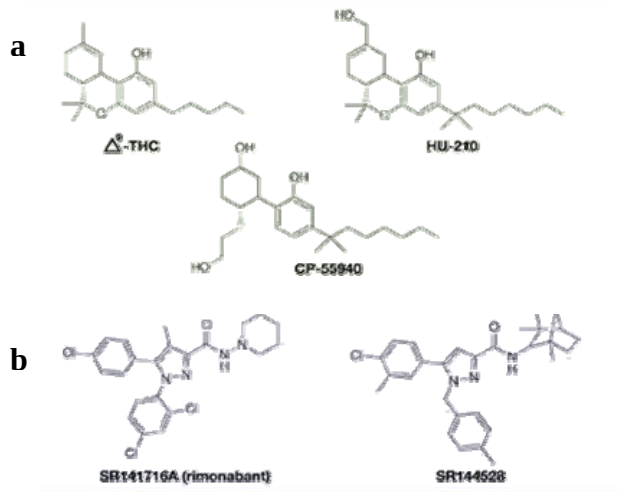


Figure 5. Chemical structure of Δ^9-THC, the most important derivate of *Cannabis Sativa* and synthetic compounds that bind to cannabinoid receptors. a) cannabinoid receptors agonist which activates both CB_1 and CB_2 receptors. b) selective antagonists for CB_1 (SR141716A, rimonabant) and CB_2 (SR144528). (adapted from Piomelli, 2003)

The synthetic compounds have different intrinsic activity and affinity for the cannabinoid receptors (Howlett 2002). Thus, CP-55,940 and WIN 55,212-2 show a higher affinity for CB_1 and CB_2 receptors than THC. In addition, CP-55,940 has the same affinity for both CB_1 and CB_2, while the

affinity of WIN 55,212-2 is slightly higher for CB_2 (Pertwee 2008). Studies about the relationship between structure and activity, gave rise to the development of selective cannabinoid receptor antagonists. Thus, SR141716A (rimonabant) (Rinaldi-Carmona *et al.* 1994) and SR144528 (Rinaldi-Carmona *et al.* 1998) were the first selective antagonists for CB_1 and CB_2 receptors respectively (Figure 5 and table 2).

Table 2 Affinity (Ki, nM) of several agonists and antagonists for CB_1 and CB_2 receptors

AGONISTS	CB1	CB2
Δ9-THC	40,7	36,4
CP55,940	0,58	0,69
HU-210	0,06	0,52
WIN55,212-2	1,89	0,28
ANTAGONISTS		
SR141716A	5,6	>1000
SR144528	437	0,6
AM-281	12	4200
AM-630	5152	31,2

1.2.2 Canabinoid receptors: structure and distribution

The cannabinoids exert their pharmacological actions through the activation of at lest two distinct subtypes of cannabinoid receptors: CB_1 and CB_2 cannabinoid receptors. The first cannabinoid receptor, CB_1, was cloned in the early 1990s (Matsuda *et al.* 1990) and three years later Munro et al. cloned the CB_2 cannabinoid receptor (Munro *et al.* 1993). Both are G-protein coupled receptors with seven trasmembrane domains.

There are considerable differences in the size of CB_1 and CB_2 receptors. While the human CB_1 receptor consists of 472 amino acids and has a molecular weight of 60 kDa, the human CB_2 receptor consists of only 360 amino acids and has a molecular weight of 50 kDa (Matsuda *et al.* 1990) (figure 6). Nevertheless, compelling evidence supports the existence of additional others G protein coupled receptors with cannabinoid activity (Brown 2007). Recently, it has been accepted that the orphan receptor GPR55 could be considered as the third receptor with cannabinoid activity (Baker *et al.* 2006; Ryberg *et al.* 2007).

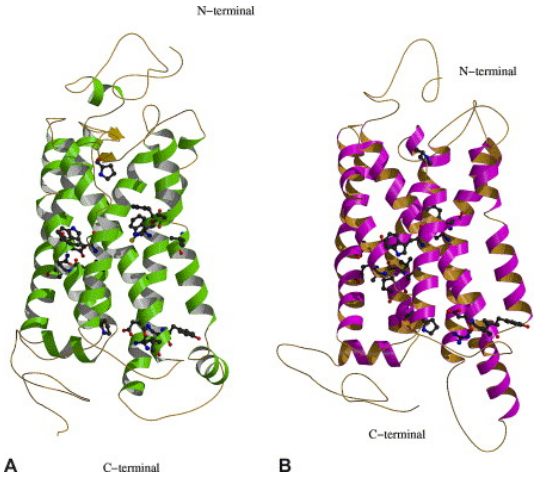


Figure 6 Three-dimensional representation of CB_1 (A) and CB_2 (B) structure

CB_1 receptors are the most abundantly expressed metabotropic receptors in the brain and their distribution has been well characterized both in rodents (Herkenham *et al.* 1991; Tsou *et al.* 1998) and humans (Westlake *et al.* 1994) (figure7). CB_1 receptors are highly expressed in caudate-putamen, globus pallidus, hippocampus, ectopeduncular nucleus, sustansia nigra and cerebellum (Compton *et al.* 1990). They are also found in the amygdala, hypothalamus, nucleus accumbens, thalamus, periapeduncular gray and the spinal cord as well as in other brain areas mainly in the telencephalon and diencephalum (Cota *et al.* 2003a; Tsou *et al.* 1998)

(figure 7). In all these areas CB_1 receptors are mainly expressed in GABAergic and glutamatergic neurons (Rodriguez de *et al.* 2005). CB_1 receptors are also expressed in several peripheral organs. Thus, they are present in adipocytes (Cota *et al.* 2003b), liver (Osei-Hyiaman *et al.* 2005), lungs, smooth muscle, gastrointestinal tract (Calignano *et al.* 1997), pancreatic beta cells (Bermudez-Silva *et al.* 2008), vascular endothelium (Liu *et al.* 2000) and other peripheral tissues.

The distribution of CB_2 receptors is manly restricted to the periphery in the immune system cells such as, macrophages, neutrofils, monocytes, lymphocytes B-cells and lymphocytes T-cells (Galiegue *et al.* 1995; Matsuda *et al.* 1990; Munro *et al.* 1993). However, the presence of CB_2 receptors has also been demonstrated at the central level, in astrocytes (Sanchez *et al.* 2001), microglial cells (Nunez *et al.* 2004; Walter *et al.* 2003) and brainstein neurons (Van *et al.* 2005). Recently, CB_2 receptor expression has also been shown in bone cells such as osteoblasts, osteocytes and osteoclasts (Ofek *et al.* 2006), liver (Julien *et al.* 2005) and somatostatin secreting cells in pancreas (Bermudez-Silva *et al.* 2008).

So far, few data are available with regards to the distribution of GPR55 receptor. The expression of this receptor has been shown by fluorescence in situ hybridization in the spleen and in some regions of human brain, such as the caudate nucleus and putamen (Sawzdargo *et al.* 1999). An abundant presence of this receptor has also been demonstrated in the neurons of the DRG (Lauckner *et al.* 2008). In rat brain, GPR55 mRNA was detected by in situ hybridization in hippocampus, thalamic nuclei and regions of the mid-brain (Brown 2007) and in the periphery GPR55 mRNA was present in the spleen (Sawzdargo *et al.* 1999).

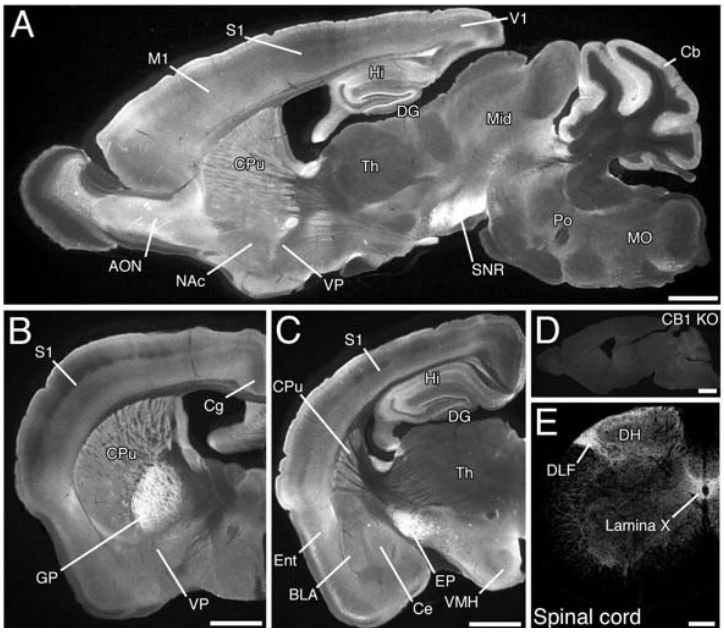


Figure 7 Distribution of CB_1 receptors in the CNS of adult mice. *A–D*: overall distribution in parasagittal (*A* and *D*) and coronal (*B* and *C*) brain sections of wild-type (*A–C*) and CB_1-knockout (*D*) mice immunolabeled with a high-titer polyclonal antibody against the COOH terminus of mouse CB_1. SNR: substantia nigra reticulata, GP: globus pallidus, EP: entopeduncular nucleus, Hi: hippocampus, DG: dentate gyrus, S1: primary somatosensory cortex, M1: primary motor cortex, V1: primary visual cortex, Cg: cingulate cortex, Ent: entorhinal cortex. BLA: basolateral amygdaloid nucleus AON: anterior olfactory nucleus, CPu: caudate putamen, VMH: ventromedial hypothalamus, Cb: cerebellar cortex, DH: dorsal horn, DLF: dorsolateral funiculus, lamina X. (adapted from Kano M et al, 2009)

1.2.3 Canabinoid receptor signalling

Stimulation of CB_1 and CB_2 cannabinoid receptors activates a number of signal transduction pathways mainly via Gi/o family of G proteins. Activation of CB_1 and CB_2 cannabinoid receptors produces the inhibition of adenylate cyclase, and the corresponding decreased activity of the

protein kinase A pathway. The activation of cannabinoid receptors also stimulates the activity of the mitogen-activated protein kinase (MAPK) pathway. Cannabinoid receptor activation, through the stimulation of Gi/o proteins, is also directly coupled to inhibition of voltage-activated Ca2+ channels and stimulation of inwardly rectifying K+ channels in neurons, with subsequent inhibition of neurotransmitter release (Di, V *et al.* 2004) (figure 8).

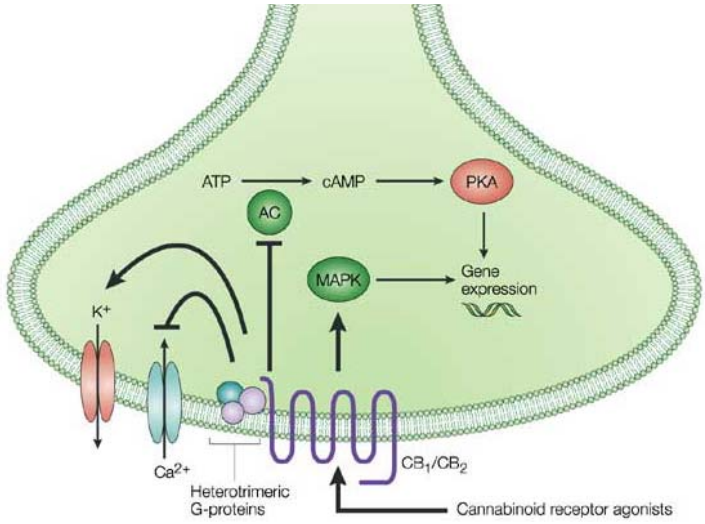


Figure 8. Major signalling pathways associated with cannabinoid receptor activation by agonists (adapted from Di Marzo, 2004); adenylate cyclase (AC), mitogen-activated protein kinase (MAPK), protein kinase A (PKA).

The GPR55 has distinct intracellular signalling responsses in comparison with CB_1 and CB_2 receptors (Lauckner *et al.* 2008). Thus, the activation of this receptor induces the increase of intracellular calcium via Gq and phospholipase C, and the inhibition of potassium current through M-type potassium channels, which includes an increase in neuronal excitability (Lauckner *et al.* 2008)

1.2.4 Endocannabinoids: characteristics and mechanism of action

The first endogenous ligands for CB_1 and CB_2 cannabinoid receptors, the endocannabinoids, were isolated in the early 1990. All endocannabinoids identified so far are derivatives (amides, esters and even ethers) of long chain polyunsaturated fatty acids derived from the membrane phospholipids degradation, specifically the arachidonic acid, and exhibit different selectivity for the two cannabinoid receptors (figure 9).

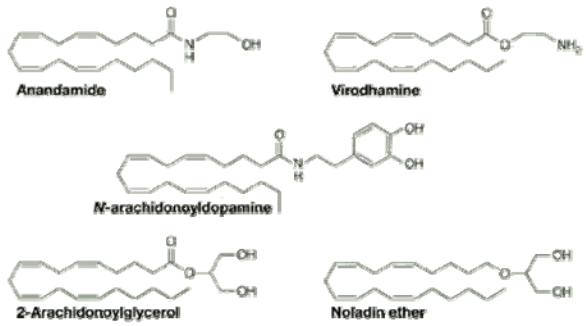


Figure 9. Chemical structure of endogenous compounds that bind to cannabinoid receptors (adapted from Piomelli, 2003).

The endocannabinoids share some common characteristics with other neurotransmitters. However, they differ from the classical neurotransmitter because of two main characteristics: they act as retrograde messengers (Chevaleyre *et al.* 2006) and do not accumulate in synaptic vesicles. These compounds are synthesised on demand and act in the proximity where they are released (Di Marzo V *et al.* 1994). Thus, in response to a concrete stimulus, the endocannabinoids are released from the postsynaptic neurons in the synaptic cleft and stimulate the cannabinoid receptors situated on the presynaptic neuron (Wilson and Nicoll 2002). Once that they are released, the endocanabinoids are rapidly inactivated and degradated by specific enzymes (Piomelli 2003) (figure 10).

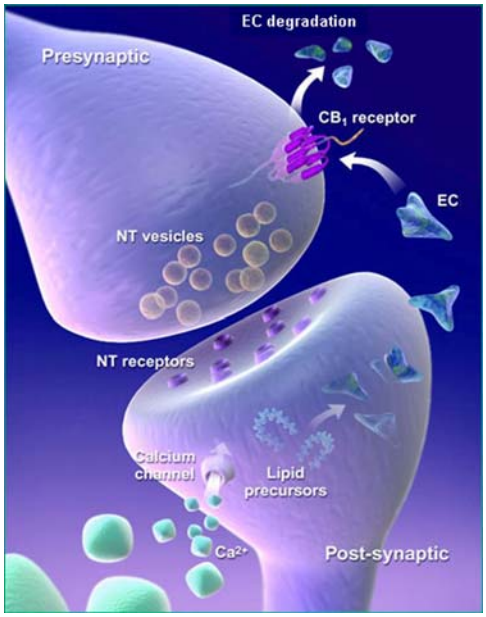


Figure 10. Retrograde signalling by endocannabinoids (Di Marzo V, 1998, 2005)

The two best studied endocannabinoids are anandamide (N-arachidonoylethanolamine) and 2-arachidonoylglycerol (2-AG) (figure 9). Anandamide is synthesised from the phosphatidylethanolamine present on the cell membrane by the activation of two enzymes: the N-acyltransferase and phospholipase D (Di Marzo V *et al.* 1994). After release, the anandamide is transported from the synaptic cleft inside the cell through passive diffusion or by a selective transporter which can be inhibited by *N*-(4-hydroxyphenyl)-arachidonamide (AM404) **(**Fegley D at al. 2004). However, the transporter has not been identified yet. Anandamide is hydroxylated by the fatty acid amide hydrolase (FAAH) (Cravatt *et al.* 1996). The 2-AG is the most abundant endogenous cannabinoid in the brain and its concentration is about 200 times higher than the anandamide (Stella *et al.* 1997). 2-AG is generated from diacylglycerol that is synthesised from phosphoinositides or from phosphatidic acid (Bisogno *et al.* 2005). The synthesis of 2-AG is

mediated mainly by the phospholipase C (Piomelli 2003). The 2-AG reuptake is taking place by similar mechanisms than for anandamide 2-AG degradation is mainly due to the action of the monoacylglycerol lipase MAGL (Dinh *et al.* 2002).

Other endogenous cannabinoids that have been identified are the 2-arachidonilglicerol ether, also called noladin ether, (Hanus *et al.* 2001), the virodhamine (Porter *et al.* 2002) and N-arachidonoyldopamine (Huang *et al.* 2001) (figure 9).

1.2.5 Effects induced by cannabinoids

The activation of the cannabinoid system through THC or other phytocannabinoids, synthetic cannabinoids or endocannabinoids causes numerous actions that have been extensively reported (Grotenhermen 2004). The cannabinoid system plays an important role in multiple aspects of the neural functions including learning and memory, emotion, addictive-like behaviour, feeding and metabolism, pain and neuroprotection. It is also involved in the modulation of different processes at the cardiovascular and immunological levels, among others (table 3).

Cannabinoids interact with multiple neurotransmitters and neuromodulators, among them ACh, DA, GABA, histamine, 5-HT, glutamate, norepinephrine, prostaglandins and opioid peptides (Dewey 1986; Grotenhermen 2004) (see table 4). A number of pharmacological effects can be explained at least in part on the basis of such interactions (Grotenhermen 2004).

Table 3 Physiological and physiopathological processes in which the cannabinoids participate

Physiological and physiopathological processes	Effects of cannabinoid agonists	Recent reviews
1. Learning and memory	Cognitive deficit	Riedel and Davis, 2005
2. Food intake	Increase in appetite	Di Marzo and Matias, 2005
3. Pain	Antinociception	Leveran and Rice, 2007
4. Motor control	Hypolocomotion, catalepsy, ataxia	Fernández-Ruiz and Gonzales, 2005
5. Anxiety	Anxyogenesis, anxyolisis	Viveros et al, 2005
6. Motivation	Euphoria, disphoria	Solinas et al, 2008
7. Corporal temperature	Hypothermia	Wenger and Moldrich, 2002
8. Inflammation	Antiinflamatory	Ashton, 2007
9. Neurodegeneration	Neuroprotection	van der Stelt and Di Marzo, 2005
10. Immune system	Immunosuppressant	Klein and Cabral, 2006
11. Cardiovascular system	Vasodilatation, tachycardia, hypotension	Sarzani, 2008
12. Bone metabolism	Stimulation of bone formation; inhibition	Bab and Zimmer, 2008
13. Cancer	Antitumoral	Velasco et al, 2007
14. Sleep	Sleep stimulation	Murillo-Rodriguez, 2008
15.Reproductive system	Hormonal secretion inhibition	Rossadoet al, 2008

Table 4 Neurotransmitter functions under cannabinoid control (modified from F.Grotenhermen 2004)

Neurotransmitter	Associated disorder
1. Excitatory amino acids	
Glutamate	Epilepsy, nerve-cell death in stroke
2. Inhibitory amino acids	
GABA	Spinal cord motor disorders, epilepsy, anxiety
Glycine	Startle syndromes
3. Monoamines	
Noradrenaline	Autonomic homeostasis, depression, hormones
Serotonin	Depression, anxiety, migraine
Dopamine	Drug addiction Parkinson's disease, schizophfrenia, vomiting, pituitary hormones
Acetylcholine	Neuromuscular disorders, autonomic homeostasis, dementia, Parkinson's disease, epilepsy, sleep-wake cycle
Neuropeptides	Pain, movement, neural development, anxiety

The involvement of the cannabinoid system in cognitive processes, food intake and pain will be described in the following sections.

1.2.5.1 Cannabinoids and cognitive processes

Learning and memory impairments are among the most commonly reported effects of cannabinoids both in animals (Hampson and Deadwyler 1998) and humans (Chait and Perry 1992). Preclinical studies have shown that the administration of cannabinoid agonists impairs the acquisition and alters working-memory in various tasks such as radial maze, object recognition task, active and passive avoidance and Morris water maze (Lichtman and Martin 1996; Molina-Holgado *et al.* 1995; Winsauer *et al.* 1999), in particular spatial memory (Lichtman and Martin 1996; Molina-Holgado *et al.* 1995) and short-term memory (Molina-Holgado *et al.* 1995). In rodents, cannabinoid agonists (Hoffman *et al.* 2007) and endocannabinoids decrease the long-term potentiation (LTP) in the hippocampus (Stella *et al.* 1997), which is related to the impairment of memory in various behavioural tasks. These cognitive effects of cannabinoid agonists are attenuated by the administration of the cannabinoid receptor antagonist, rimonabant (Collins *et al.* 1995; Mallet and Beninger 1998; Terranova *et al.* 1995; Terranova *et al.* 1996). On the other hand, rimonabant facilitates working memory in delayed-nonmatch-to-sample behavioral task (Hampson and Deadwyler 1998). In agreement with these pharmacological data, mice lacking CB_1 cannabinoid receptors showed an increase of LTP in the hippocampus (Bohme *et al.* 2000), an improvement in memory retention in the object recognition paradigm (Maccarrone *et al.* 2002; Reibaud *et al.* 1999) and an increased number of conditional changes in the active avoidance task (Martin *et al.* 2002). In addition, the endocannabinoid system has a specific role in facilitating extinction and/or forgetting processes (Marsicano *et al.* 2002; Varvel and Lichtman 2002). In this sense, CB_1 deficient mice showed strongly impaired short-term and long-term extinction in auditory fear-conditioning tests, with unaffected memory acquisition and consolidation.

Treatment of wild-type mice with rimonabant mimicked the phenotype of CB_1 deficient mice, revealing that CB_1 is required at the moment of memory extinction. Consistently, tone presentation during extinction trials resulted in elevated levels of endocannabinoids in the basolateral amygdala complex, a region known to control extinction of aversive memories (Marsicano *et al.* 2002). In the same line, Varvel and Lichtman (2002) have shown that the CB_1 receptor deficient mice exhibited a deficit in learning the new platform location during the task in reversal test of the Morris water maze. These mice continued to return to the previously learned location, despite being repeatedly shown the new location.

The specific mechanisms involved in the modulation of learning and memory processes induced by cannabinoids have not been still fully clarified. Thus, memory impairment produced by cannabinoids has been related to an inhibition of cholinergic activity in the CNS (Braida and Sala 2000). Hippocampus is a brain structure that plays a crucial role in learning and memory processes and contains high levels of CB_1 cannabinoid receptors. This brain area acquire, encode and consolidate new information in short-term memory which is then processed in the PFC and it is also involved in LTP (Egerton *et al.* 2006). Both *in vitro* (Gifford and Ashby, Jr. 1996) and *in vivo* (Gessa *et al.* 1997) studies have shown that the cannabinoid agonists induce an inhibition of ACh released in rat hippocampus. Otherwise, pharmacological (Gessa *et al.* 1997; Gifford and Ashby, Jr. 1996) and genetical (Kathmann *et al.* 2001) blockade of CB_1 receptor increased the ACh in hippocampus. In this brain area, CB_1 receptors are highly expressed and are mainly localized in GABAergic terminals of basket cells (Katona *et al.* 1999; Katona *et al.* 2001) to control GABA release (Katona *et al.* 2001). In addition, CB_1 receptors are localized, to a minor extent in glutamatergic terminals (Katona *et al.* 2006; Kawamura *et al.* 2006) where they play a critical role in neuroprotection (Monory *et al.* 2006) through the modulation of

glutamate release (Takahashi and Castillo 2006). In a recent study it has been demonstrated, using different lines of CB_1 receptor conditional knockout mice lacking CB_1 receptor in GABAergic or glutamatergic terminals, that THC-induced cognitive impairment is mainly mediated through the stimulation of CB_1 receptor in GABAergic terminals, while those in glutamatergic terminals did not participate in these cognitive responses (Puighermanal *et al.* 2009). These findings suggest that the stimulation of CB_1 receptor, mainly expressed in GABAergic interneurons would contribute to an unbalance between the excitatory and inhibitory inputs in the hippocampus leading to the cognitive impairment produced by cannabinoids (Puighermanal *et al.* 2009).

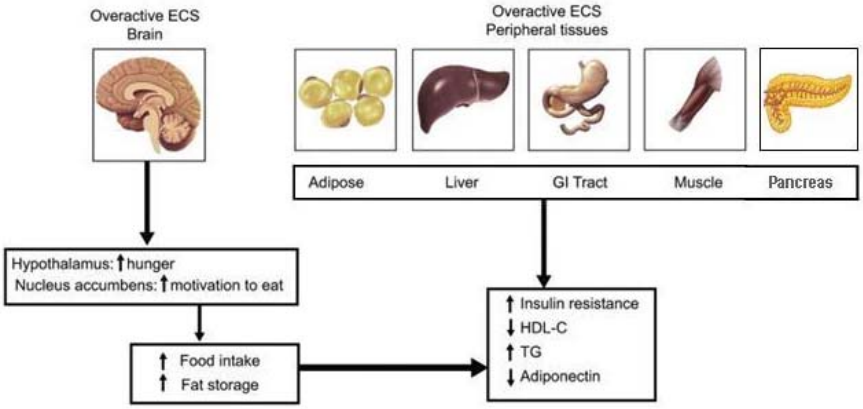
In humans it has been reported that marijuana acutely impairs performance on short-term memory tasks (Chait and Perry 1992; Miller and Branconnier 1983) and its chronic consumption affects long term-memory storage. In addition to reduced learning, heavy cannabis use is also associated with a decreased mental flexibility, increased perseveration and reduced ability to sustain attention (Lundqvist 2005). It has also been described that long-term heavy cannabis users show impairments in memory and attention that persist beyond the period of intoxication and get worse with increasing years of regular cannabis use (Solowij *et al.* 2002). Brain imaging studies of cannabis users have demonstrated altered function, blood flow, and metabolism in prefrontal and cerebellar regions (Solowij *et al.* 2002).

The most common used form of cannabis is the hashish that is always mixed with tobacco. In spite of this current association of cannabis and tobacco in humans, the behavioural and biochemical consequences of the interaction between these two drugs are poorly documented. Thus, future studies are necessary in order to better understand the underling mechanisms of cannabis and tobacco association in memory and learning processes.

1.2.5.2 Cannabinoids in food intake and energy balance

The orexigenic properties of *Cannabis sativa* derivatives have been known for centuries (Abel 1975). Indeed, the administration of either exogenous or endogenous cannabinoid agonists have been shown to increase food intake in humans and in animal models when the doses employed were low or moderate, while these compounds were anorexigenic at higher doses probably due to their sedative properties (Giuliani *et al.* 2000; Kirkham *et al.* 2002; Williams and Kirkham 2002). The effects of cannabinoids on food intake and metabolism are mainly mediated through the activation of the CB_1 cannabinoid receptor (Pagotto *et al.* 2006), although recent studies suggest also an involvement of CB_2 cannabinoid receptor (Agudo *et al.* 2010). Cannabinoid agonists are effective in the clinic to increase food intake in some pathological conditions related to weight loss, namely cancer and AIDS (Cota *et al.* 2003a; Kirkham *et al.* 2002). On the contrary, acute administration of CB_1 cannabinoid antagonists suppresses food intake and food-motivated behaviour (Foltin and Haney 2007; Salamone *et al.* 2007; Sink *et al.* 2008). Genetic or chronic pharmacological impairment of the endogenous cannabinoid system mainly results in a short-term hypophagia and long-lasting reduction in body weight. Thus, mice chronically treated with the selective CB_1 antagonist, rimonabant or lacking the CB_1 cannabinoid receptors are leaner, have lower motivation for food and lower plasma leptin levels, as well as a transitory lower caloric intake than their corresponding controls (Cota *et al.* 2003b; Ravinet *et al.* 2004; Ward and Dykstra 2005). CB_1 cannabinoid receptor activation modulates the effects of cannabinoids on food intake and metabolism (Pagotto *et al.* 2006) both at the CNS and in the peripheral tissues (Di, V 2008; Pagotto *et al.* 2006) (Figure 11).

Figure 11. Role of endocannabinoid system in food intake and energy balance (modified from Di Marzo, 2008)



At the central level, it has been well described that the endocannabinoid system plays a dual role in the regulation of food intake by homeostatic and non-homeostatic (or hedonic) energy regulation (Berthoud 2006). In the mesolimbic system, the activation of the CB_1 receptor increases the motivation for the incentive value of food (Di Marzo and Matias 2005), and CB_1 activation in the hypothalamus enhances appetite by regulating the responses of several orexigenic and anorectic mediators (Di Marzo V *et al.* 2004; Di Marzo and Matias 2005; Kirkham *et al.* 2002; Osei-Hyiaman *et al.* 2005). Thus, CB_1 receptors regulate the release of agouti-related protein, orexins and melanocyte-concentrating hormone or anorexic neuropeptides such as those produced from pro-opiomelanocortin and the cocaine and amphetamine-regulated transcript (CART) (Matias *et al.* 2008). On the other hand it was suggested that the orexigenic effects of endocannabinoids could be in part mediated by neuropeptide Y since stimulation or blockade of hypothalamic CB_1 receptors increases or decreases respectively the levels of this mediator (Gamber *et al.* 2005; Verty *et al.* 2005). Hypothalamic endocannabinoid levels are decreased after systemic leptin administration in rats, and

increased in rodent models where leptin signalling or its biosynthesis is defective (Di Marzo V *et al.* 2001). Leptin, secreted by adipocytes into the peripheral circulation, regulates not only food intake but also the central regulation of energy expenditure. It appears, therefore, that the endocannabinoid system participates in the orexigenic hypothalamic network regulated by leptin (Matias *et al.* 2008) increasing feeding behaviour (Jo *et al.* 2005a).

At the peripheral level, the activation of the endocannabinoid system reduces energy expenditure and this process has been shown to take place in the adipose tissue, liver, skeletal muscle, gastrointestinal tract and pancreas (Matias *et al.* 2008). In the adipose tissue, CB_1 receptor activation increases energy storage by several mechanisms. First, CB_1 activation stimulates the growth and differentiation of preadipocytes into fully mature adipocytes (Bellocchio *et al.* 2008; Matias *et al.* 2006) and increases energy accumulation within these cells promoting by these mechanisms the adipogenesis. The accumulation of energy comes from the storage of triglycerides in the adiposities as a result of the enhanced lipolysis and from exogenous fatty acids (Tucci *et al.* 2004). Moreover, CB_1 activation in adipocytes increases insulin signalling which promotes glucose uptake and decreases adiponectine (Bellocchio *et al.* 2008; Matias *et al.* 2006), both leading to an enhancement of energy storage. In addition, the endocannabinoids decrease the 5AMP-activated protein kinase (AMPK) activity in the adipose tissue (Cavuoto and Wittert 2009; Kola *et al.* 2005), resulting in an increase adiposity and lipogenesis.

In the liver, the activation of CB_1 receptor leads also to decrease energy expenditure by reducing the AMPK activity that alters fatty acid and glucose oxidation and increases lipogenesis (Matias *et al.* 2008).

Skeletal muscle is a significant site of lipid and glucose oxidation, accounting for as much as 30% of basal energy expenditure. In this tissue, the activation of CB_1 receptor leads to a decrease in oxygen consumption

(Cavuoto and Wittert 2009), fatty acid oxidation (Bellocchio *et al.* 2008) and glucose uptake (Liu *et al.* 2005). In addition, it has been suggested that endocannabinoids could reduce muscle contraction by a CB_1-dependent mechanism (Newman *et al.* 2007).

The effects of cannabinoids on insulin secretion by pancreatic beta cells remain unresolved (Cavuoto and Wittert 2009). There are conflicting data regarding the distribution of CB_1 and CB_2 in the pancreas, although both have been reported to be present (Starowicz *et al.* 2008). Activation of the CB_1 receptors has been shown to induce insulin and glucagon secretion, whereas CB_2 mediates a decrease in glucose-dependent insulin secretion (Bermudez-Silva *et al.* 2008).

On the other hand, it has been described that cannabinoids inhibit gastrointestinal motility in both rodents and humans (Esfandyari *et al.* 2007), through CB_1 receptor activation. Moreover, CB_1 activity in the gastrointestinal tract enhances food intake by regulating the release of gut peptides such as cholecystokinin and ghrelin (Gomez *et al.* 2002).

All these data demonstrate the crucial role of the endocannabinoid system in the regulation of food intake and metabolism and underline the relevance of this system as a new therapeutic target to fight against obesity and also to treat the cachectic states present in some pathologies. Thus, cannabinoid agonists, such as Nabilone and Dronabinol (Marinol) (Δ9-tetrahydrocannabinol) are available in the clinic as appetite stimulants, primarily for AIDS, chemotherapy and gastric bypass patients.

In spite of the current association of cannabis and tobacco consumption in humans, the behavioural and biochemical consequences of the interaction between these two drugs related to feeding behaviour and metabolism are poorly documented. Thus, future studies are necessary in order to elucidate the interaction between the endocannabinoid system and nicotine in food intake and metabolism.

1.2.5.3 Cannabinoids and pain

One of the ancestral uses of cannabis was to treat pain. Historical documents reveal the use of cannabis for surgical anaesthesia in ancient China and for relieving pain of diverse origin in ancient Israel, Greece, Rome, and India (Mechoulam and Hanus 2000). Cannabinoids exert their antinociceptive effects through complex mechanisms involving effects on brain (Fox *et al.* 2001; Hohmann *et al.* 1999; Martin *et al.* 1993; Meng *et al.* 1998) spinal cord (Chapman 1999; Drew *et al.* 2000; Lichtman and Martin 1991b; Lichtman and Martin 1991a; Naderi *et al.* 2005; Suplita *et al.* 2005) and peripheral sensory nerves (Amaya *et al.* 2006; Calignano *et al.* 1998; Fox *et al.* 2001; Johanek and Simone 2004; Jordt *et al.* 2004). This is consistent with the anatomical location of CB_1 receptors in areas relevant to pain in the brain, spinal dorsal horn, DRG, and peripheral afferent neurons (Hohmann *et al.* 1999; Sanudo-Pena *et al.* 1999) (Figure 12).

At the central level, the antinociceptive effects induce by cannabinoids are due mainly to CB_1 located in the spinal cord and supraspinal structures (Ledent *et al.* 1999; Meng *et al.* 1998). However, it has been recently shown that CB_2 receptors also participates in pain modulation in the spinal cord (Taylor 2009). Although the endocannabinoid system plays an outstanding role in the control of the ascending pathways involved in the transmission of nociceptive stimuli, another central mechanism for the antinociceptive effects induced by cannabinoids seems to be modulation of the descending inhibitory pathways. Several studies demonstrated the presence of a bidirectional control of pain transmission in the periaqueductal grey matter and rostral ventromedial medulla that can exert both inhibitory and facilitatory control (Fields 2004). This dual control results from the activity of two neuronal subpopulations. One class, termed “OFF cells”, shows a pause in firing that begins before the

withdrawal reflex and its activation promotes analgesic effects. The other class, "ON cells" shows a burst of activity that begins before the reflex and the activation of these cells facilitates pain transmission. Consistent with their role in pain modulation, rostral ventromedial medulla ON and OFF cell axons project directly and selectively to dorsal horn laminae that relay nociceptive signals (Fields *et al.* 1995). Microinjection of cannabinoid agonists into the periaqueductal grey substance (Martin and Lichtman 1998; Martin *et al.* 1998) and rostral ventromedial medulla (Martin *et al.* 1999) as well as the electro stimulation of these areas (Fields *et al.* 1991) resulted in analgesia by enhancing the activity of "OFF cells".

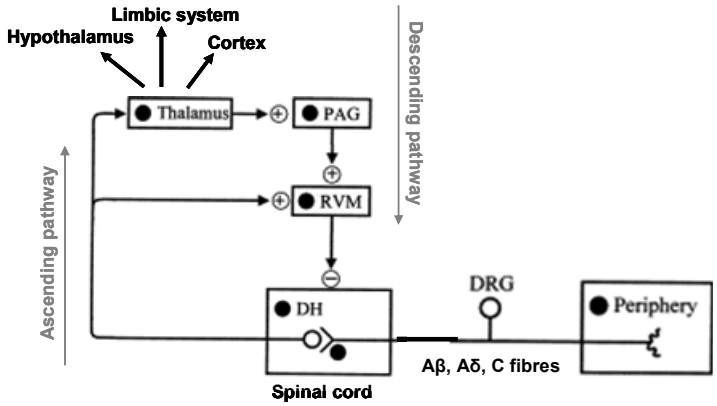


Figure 12. Sites at which cannabinoids act through CB_1 receptors to induce antinociception in rodents. DRG: dorsal root ganglion; DH: dorsal horn of spinal cord; RVM: rostral ventromedial medulla, PAG: periaqueductal grey (adapted from Pertwee, 2001)

The cannabinoids may stimulate the descending inhibitory pathway by activating neurons from both brain regions. This activation seems to be induced by the inhibition of GABA release in the axon terminal of presynaptic interneurons located in rostral ventromedial medulla and

periaqueductal grey matter, through a mechanism similar to the one described for the analgesic effects of opioids.

At the spinal level, the CB_1 receptors are found mainly in the dorsal horn of the spinal cord. Most of the primary afferent neurons that express CB_1 mRNA are Aβ fibers or large diameter fibers, involved in the sensitive, non nociceptive transmission (Hohmann and Herkenham 1998). However, CB_1 receptors are also expressed in nociceptive fibers with small diameter including C-fibers, able to inhibit the release of neurotransmitters involved in pain transmission (Drew *et al.* 2000; Kelly and Chapman 2001; Wilson and Nicoll 2001). CB_1 mRNA is also highly expressed in the DRG (Bridges *et al.* 2003; Hohmann 2002). At this level, stimulation of CB_1 receptors blocks the presynaptic Ca^{+2} dependent channels, decreasing by this mechanism the release of neurotransmitters (Millns *et al.* 2001). At the supraspinal level, cannabinoids are also able to modify the subjective interpretation of pain by modulating the neuronal activity mainly at the level of the limbic structures such as amygdala (Manning *et al.* 2001).

At the peripheral level, the activation of both CB_1 and CB_2 receptors seems to participate in the antinociceptive effects of cannabinoids. In this line, several studies have proposed the existence of a synergism between the responses mediated by CB_1 and CB_2 receptors in the periphery (Malan, Jr. *et al.* 2001).

The analgesic effects of cannabinoid compounds have been demonstrated in multiple behavioural studies in both animals and humans (Martin and Lichtman 1998; Pacher *et al.* 2006). The most employed acute nociceptive models used in animals that apply thermal stimuli to reveal cannabinoid antinociception are the hot plate (Buxbaum 1972; Hutcheson *et al.* 1998; Martin 1985) and tail flick paradigms (Buxbaum 1972). These two tests evaluate different behavioural responses. While the antinociceptive responses obtained in the hot plat test are modulated by the activation of

supraspinal pathways, the behavioural responses observed in the tail flick paradigm are mainly due to spinal mechanisms. Cannabinoids agonists were proved to be efficient in both tests (Gomez *et al.* 2002; Hohmann 2002; Lichtman and Martin 1991b; Martin and Lichtman 1998; Tham *et al.* 2005). Other animal models where cannabinoid agonists were proved to be efficient, are mechanical models that measure motor (Smith *et al.* 1994) or reflex (Gilbert 1981) responses, chemical models such as the writhing response induced by acetic acid or the administration of fenilbenzoquinone (Ulugol *et al.* 2006; Welch *et al.* 1995) and models of electric stimulation of paw (Weissman *et al.* 1982), sciatic nerve (Bicher and Mechoulam 1968) or dental pulp (Kaymakcalan *et al.* 1974). The cannabinoid agonists also produced antinociceptive effects in models of inflammatory pain such as the hyperalgesia induced by carrageenan (Mazzari *et al.* 1996), capsaicin (Li *et al.* 1999), formalin (Calignano *et al.* 1998; Moss and Johnson 1980) and Freud's adjuvant (Martin *et al.* 1999). Moreover, recent studies have shown that the cannabinoids were also efficient in models of neuropathic pain (Goya et al. 2003). Thus, an upregulation of spinal CB1 receptor was revealed after a chronic constriction of sciatic nerve that promotes the enhancement of the analgesics effects of Win 55,212-2 on neuropathic pain in rats (Lim et al. 2003). By contrast, a genetic study using CB1 knockout mice, has shown that CB1 cannabinoid receptors are not critically involved in the development of neuropathic pain nor in the anti-allodynic and anti-hyperalgesic effects of gabapentin in a model of neuropathic pain induced by partial sciatic nerve ligation (Castañé et al. 2006). CB2 cannabinoid receptors have also been involved in this pathological state. Indeed, the selective CB2 cannabinoid agonist AM1241 produced a dose-dependent inhibition of tactile and thermal hypersensitivity induced in rats by spinal nerve ligation (Ibrahim et al. 2003). The crucial role of CB2 receptor in the regulation of central immune responses during neuropathic pain was

recently demonstrated using mice lacking CB2 receptor, transgenic mice overexpressing this receptor and bone marrow chimera mice (Castañé et al. 2006; Racz et al. 2008b; Racz et al. 2008a). Thus, CB2 knockout mice and mice reconstituted with CB2 deficient bone marrow cells exposed to nerve injury developed similar neuropathic pain in the ipsilateral side as wild-type animals. However, they showed a contralateral mirror-image of pain accompanied by glial activation. In contrast, neuropathic pain was attenuated in transgenic mice overexpressing CB2 receptors (Racz et al. 2008b; Racz et al. 2008a). Therefore, CB2 cannabinoid agonists could represent a future new group of pharmacological agents for the treatment of neuropathic pain devoid of any psychoactive side effects (Racz et al. 2008b; Racz et al. 2008a)

In humans, cannabinoid agonists are already used to alleviate some manifestations of pain. Indeed, Sativex® (Δ9-tetrahydrocannabinol with cannabidiol) is prescribed for the symptomatic relief of neuropathic pain in adults with multiple sclerosis and as an adjunctive analgesic treatment for adult patients with advanced cancer (Pertwee 2009). One important challenge at the present moment would be to identify additional therapeutic targets for cannabinoid agonists considering the promising findings already reported.

2. Neuropathic pain

2.1 Definition and classification

Neuropathic pain is defined by the International Association for the Study of Pain (IASP) as pain initiated or caused by a primary lesion or dysfunction in the nervous system. The classification of neuropathic pain is a complex matter. A traditional approach classifies neuropathic pain according to the aetiology, as well as the presumed location of the nerve injury (peripheral or central). The etiology-based classification of neuropathic pain can be summarized as follows (adapted from (Baron 2006):

1. Focal or multifocal lesions of the peripheral nervous system: Entrapment syndromes, phantom limb pain, stump pain, post-traumatic neuralgia, postherpetic neuralgia, diabetic mononeuropathy, ischemic neuropathy and polyarteritis nodosa.

2. Generalized lesions of the peripheral nervous system (polyneuropathies): Diabetes mellitus, cancer associated neuropathy, alcohol, amyloid, plasmocytoma, HIV neuropathy, hypothyroidism, hereditary sensory neuropathies, vitamin B deficiency, toxic neuropathies (arsenic, thallium, chloramphenicol, metronidazole, nitrofurantoin, isoniazid, vinca alkaloids, taxoids), Fabry's disease and Bannwarth's syndrome (neuroborreliosis).

3. Lesions of the CNS: Spinal cord injury, brain infarction (especially in the thalamus and brainstem), spinal infarction, syringomyelia and multiple sclerosis.

4. Complex neuropathic disorders: Complex regional pain syndromes type I and II, reflex sympathetic dystrophy and causalgia.

2.2 Symptoms and signs in neuropathic pain:

Neuropathic pain is characterised by the existence of spontaneous pain and abnormal stimulus-evoked pain (hyperalgesia, allodynia, paresthesia and dysesthesia). When a stimulus that usually causes mild pain is perceived by patient as producing severe pain, this situation is called hyperalgesia. Depending on the nature of the stimulus, the resultant condition is known as heat, cold or mechanical hyperalgesia. However, in some cases painless stimuli (such as the rubbing of clothing) are felt as painful, and this situation is known as allodynia. Allodynia may be very distressing for some patients. Hyperalgesia and allodynia may also appear in other pathological situations such as inflammatory pain (e.g. sunburn). Besides, hyperalgesia and allodynia, there are other evoked sensory phenomena, such as paresthesia (abnormal sensation, different from pain, whether spontaneous or evoked, not unpleasant) or dysesthesia (abnormal sensation, different from pain whether spontaneous or evoked, unpleasant (Baños et al. 2003). Another characteristic of neuropathic pain is the appearance of Tinel's sign which is pathognomonic for nervous injury and very useful to determine the anatomical level of the lesion. It is a tingling sensation in the distal end of a limb when percussion is made over the site of a divided nerve. Table 5 defines several sensory signs and symptoms that can be found in painful neuropathies, and summarizes the appropriate tests to assess these symptoms.

Table 5. Definition and assessment of sensory symptoms or signs in neuropathic pain (modified from Baron, 2006).

Symptom/sign	Definition	Assessment	Expected pathological response
Negative signs and symptoms			
Hypoesthesia	Reduced sensation to non-painful stimuli	Touch skin with painter's brush, cotton swab or gauze	Reduced perception, numbness
Pallhypoesthesia	Reduced sensation to vibration	Apply tuning fork to bone or joint	Reduced perception threshold
Hypoalgesia	Reduced sensation to painful stimuli	Prick skin with single pin stimulus	Reduced perception, numbness
Thermohypoesthesia	Reduced sensation to cold or warm stimuli	Touch skin with objects of 10 °C (metal roller, glass of water, coolants like acetone) Touch skin with objects of 45 °C (metal roller, glass of water)	Reduced perception
Spontaneous sensations/pain			
Paraesthesia	Non-painful ongoing sensation (ant crawling)	Grade intensity (0–10) Area in cm^2	–
Paroxysmal pain	Shooting electrical attacks for seconds	Number per episode Grade intensity (0–10) Threshold for evocation	–
Superficial pain	Painful ongoing sensation, often of burning quality	Grade intensity (0–10) Area in cm^2	–
Evoked pain			
Mechanical dynamic allodynia	Normally non-painful light-pressure moving stimuli on skin evoke pain	Stroking skin with painter's brush, cotton swab or gauze	Sharp burning superficial pain in the primary affected zone, spreading into unaffected skin areas (secondary zone)
Mechanical static allodynia	Normally non-painful gentle static pressure stimuli on skin evoke pain	Manual gentle mechanical pressure to the skin	Dull pain in the area of affected (damaged or sensitized) primary afferent nerve endings (primary zone)
Mechanical punctate or pinprick hyperalgesia	Normally stinging-but-not-painful stimuli evoke pain	Manual pricking of the skin with a safety pin, sharp stick or stiff von Frey hair	Sharp superficial pain in the primary affected zone, spreading into unaffected skin areas (secondary zone)
Temporal summation	Repetitive application of identical single noxious stimuli is perceived as increasing pain sensation (wind-up-like pain)	Pricking the skin with safety pin at <3 s intervals for 30 s	Sharp superficial pain of increasing intensity
Cold allodynia	Normally non-painful cold stimuli evoke pain	Touch skin with objects of 20 °C (metal roller, glass of water, coolants like acetone) Control: touch skin with objects of skin temperature	Painful, often burning, temperature sensation in the area of affected (damaged or sensitized) primary afferent nerve endings (primary zone)
Heat allodynia	Normally non-painful heat stimuli evoke pain	Touch skin with objects of 40 °C (metal roller, glass of water) Control: touch skin with objects of skin temperature	Painful burning temperature sensation in the area of affected (damaged or sensitized) primary afferent nerve endings (primary zone)
Mechanical deep somatic allodynia	Normally non-painful pressure on deep somatic tissues evokes pain	Manual light pressure at joints or muscle	Deep pain in joints or muscles

2.3 Fisiopathologycal mechanisms of neuropathic pain

Most of the current hypothesis to explain the pathophysiology and mechanisms underlying neuropathic pain originated from experimental work in animal models. These animal studies delineated a series of partially independent peripheral and central pathophysiological

mechanisms to explain the development and manifestations of neuropathic pain (Baron 2006).

2.3.1 Peripheral sensitization on the nociceptors and sensory fibers

The nociceptors are located in the terminals of afferent neurons responsible for the pain stimuli detection and transmission. Like all primary afferent neurons, the nociceptors have a cellular body localised in the DRG, and two prolongations. The central prolongation ends into the dorsal horn of the spinal cord and the peripheral prolongation ends in the peripheral organs and constitutes the sensory fiber. Taking into account the myelinization, the diameter and the conduction speed, the cutaneous sensory fibers are classified in three categories: Aβ fibers (large myelinated afferents), Aδ fibers (small myelinated afferents) and C fibers (small unmyelinated afferents) (Table 6). In physiological conditions, all of these fibers can transmit innocuous information, whereas only Aδ and C fibers transmit nociceptive information. When a nociceptive stimulus acts on the skin, the Aδ nociceptors are responsible for the transmission of the immediate acute pain which is followed by a more diffused pain transmitted by the activation of nociceptors located on C fibers characterised by a slower conduction speed. During chronic pain, nociceptor sensitization is caused by inflammatory mediators, like amines, prostaglandins, leukotrienes and bradykinins that are released after injury decreasing the threshold of nociceptive stimuli and increasing the response to suprathreshold stimuli (Baños *et al.* 2003). In pathological conditions, mainly in the presence of tissue inflammation or peripheral nerve injury, neurochemicals alterations may occur in the Aβ neurons and these fibers can start to transmit nociceptive information.

Table 6. Clasification of sensory cutaneous fibers

Fiber type	Diameter(µm)	Myelinisation	Conduction speed (m/sec)
Aβ	> 10	thick	30-100
Aδ	02-jun	thin	dic-30
C	0,4-1,2	absent	0,5-2

After a peripheral nerve lesion, the nociceptors become abnormally sensitive and develop pathological spontaneous activity. These pathological changes are underpinned by molecular and cellular changes at the level of the primary afferent nociceptor that are triggered by the nerve lesion (Baron 2006) (Figure 13). These changes lead to ectopic (abnormal) and spontaneous discharges, abnormal nerve conduction, alterations of ionic channel expression, collateral spouting of primary afferent neurons, sprouting of sympathetic neurons and nociceptor sensitisation (Baños *et al.* 2003)

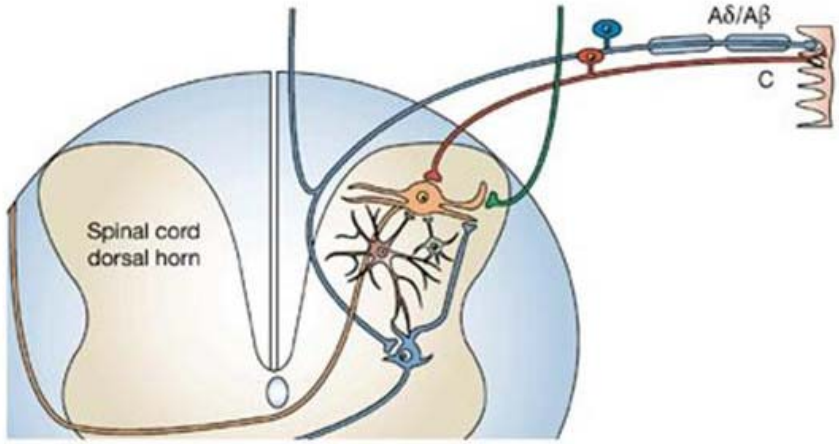


Figure 13: Primary afferent pathways and their connections in the spinal cord dorsal horn. Nociceptive C-fibers (red) terminate at spinothalamic projection neurons in upper laminae (orange neuron), whereas myelinated A-fibers (blue) project to deeper laminae (adapted from Baron, R, 2006).

The ectopic and spontaneous discharges are expressed as a large increase in spontaneous firing in the afferent neurons linked to the injury site,

which originates in the DRG and along the nerves (Wall *et al.* 1974; Wall and Devor 1983). At least two subpopulations of primary afferents develop ectopic activity in the presence of nerve injury: injured afferent neurons and their uninjured neighbours. Thus, both populations of afferents are hypothetically capable of initiating as well as maintaining the behavioural changes observed in the presence of nerve injury (Gold 2000). These abnormal discharges can be spontaneous due to instability of the membrane potential or caused by undetectable stimuli.

Ectopic and spontaneous activity following nerve injury is matched by increased expression of messenger RNA for voltage-gated sodium channels in the primary afferent neurons. Clustering of sodium channels at sites of ectopic impulse generation might be responsible for the lowering of the action-potential threshold and consequent hyperactivity (Lai *et al.* 2003). The genes that encode the voltage-gated sodium channels are expressed selectively in nociceptive primary afferent neurons (Wood *et al.* 2004). After peripheral nerve damage, sodium channel clusters accumulate not only at the site of the nerve lesion, but also within the intact DRG. Within the DRG, an alternation between a phasically activating voltage-dependent tetrodotoxin sensitive sodium conductance and a passive voltage-independent potassium leak generates characteristic membrane potential oscillations (Amir *et al.* 2002).

Damage to peripheral nerves also induces upregulation of various receptor proteins some of which are only marginally expressed under physiological conditions at the membrane of primary afferents. Thus, partial nerve injury and streptozotocin-induced diabetes produce a downregulation of vanilloid receptors type 1 (TRPV1) on many damaged afferent neurons and novel expression of TRPV1 on uninjured C-fibers and A-fibers (myelinated Aβ and Aδ) (Hong and Wiley 2006; Hudson *et al.* 2001). Recent studies also reveal an upregulation of TRPV1 in medium and large injured DRG cells (Ma *et al.* 2005). TRPV1 are located predominantly on

nociceptive afferent fibers and transmit noxious heat (>43°C) (Caterina *et al.* 2000). The observation that TRPV1-deficient mice do not develop heat hyperalgesia after tissue inflammation (Caterina *et al.* 2000; Davis *et al.* 2000) supports the idea that these changes might contribute to the development of peripheral sensitization and the associated heat hyperalgesia (Baron 2000). TRPV1 does not seem to be the only transduction mechanism for thermal sensitization after nerve injury since wildtype and TRPV1-null mice exhibited comparable persistent enhancement of mechanical and thermal nociception after partial sciatic nerve ligation (Caterina *et al.* 2000).

Investigations into temperature-sensitive excitatory ion channels also identified a cold and menthol-sensitive transient receptor potential (TRP) channel activated in the 8–28 °C range (Patapoutian *et al.* 2003) that is expressed in small-diameter DRG neurons (McKemy *et al.* 2002) and that results upregulated after peripheral injury (Wasner *et al.* 2004). This up-regulation seems to participate in the peripheral sensitization of cold-sensitive nociceptors located on C fibers which results in the sensory phenomenon of cold (Wasner *et al.* 2004) and mechanical hyperalgesia (Price *et al.* 2001). Experimental nerve injury also triggers the expression of functional α_1-adrenoceptors and α2-adrenoceptors on cutaneous afferent fibers which could also participate in the peripheral sensitization (Price *et al.* 1998). The concept of a pathological adrenergic coupling between sympathetic postganglionic fibers and afferent neurons forms the conceptual framework for the use of sympathetic antagonists in some pain processes, such as complex regional pain syndromes (Price *et al.* 1998).

Another peripheral mechanism that occurs during neuropathic pain and contributes to the sensitization is the collateral sprouting of primary afferent neurons. This means that the fibers of the primary afferent neurons spread in their vicinity and eventually establish new synapses. The induction of the sprouting is a consequence of nerve grow factor

action at the level of DRG, where the levels of mRNA are increased after nerve injury (Sebert and Shooter 1993). Sprouting of sympathetic neurons (noradrenergic perivascular sympathetic postganglionic axons) into DRG forms barkets around the large diameter neurons that do not transmit pain under physiological conditions. This sympathetic input could activate the neurons because the terminals of the sprouted neurons establish functional synapses-like structures with the cell bodies having as consequence the aberrant transmission of pain.

2.3.2 Central sensitization

Main addaptative changes also occur in the spinal cord dorsal horn and supraspinal structures during the development of neuropathic pain. Indeed, peripheral nerve injury leads to an increase in the general excitability of the spinal cord neurons. This hyperexcitability is manifested by increased neuronal activity in response to noxious stimuli, expansion of neuronal receptive fields and spread of spinal hyperexcitability to other segments (Baron 2006). This phenomenon participates in the so called central sensitization, which is mainly initiated and maintained by the activity of the pathologically sensitized C-fibers. These fibers sensitize spinal cord dorsal horn neurons by releasing glutamate, which acts on post synaptic N-methyl- D- aspartate (NMDA) receptors and the neuropeptide substance P which acts on neurokinin 1 (NK1) receptors (Baron 2006). Several intracellular cascades contribute to this central sensitization at the level of the spinal cord, in particular the MAPK (Ji and Woolf 2001). After central sensitization, normally innocuous tactile stimuli become capable of activating spinal cord pain-signalling neurons via Aδ and Aβ low-threshold mechanoreceptors (Tal and Bennett 1994).

Another adaptive modification leading to the central sensitization is the reorganization of spinal neurons. The spinal reorganisation is a response to peripheral nerve injury of the Aβ-fibers that sprout into lamina II of the dorsal horn, which is normally innervated by C-fibers. At this level, the Aβ-fibers establish functional synaptic contact with other second order neurons that are involved in pain transmission (Scholz and Woolf 2002) (Figure 14). As a consequence of these synapses, low threshold non noxious inputs from the Aβ-fibers can be interpreted as nociceptive in origin although they are not (Bridges *et al.* 2001). Furthermore, Aβ fibers suffer a phenotypic switch and begin to express nociceptors, substance P and calcitonin gene-related peptide that have an excitatory effect on postsynaptic neurons and potentiate the effects of substance P. All these nociceptors and neurokines are normally expressed by primary afferent C-fibers and Aδ-fibers, but not in Aβ fibers (Miki *et al.* 1998).

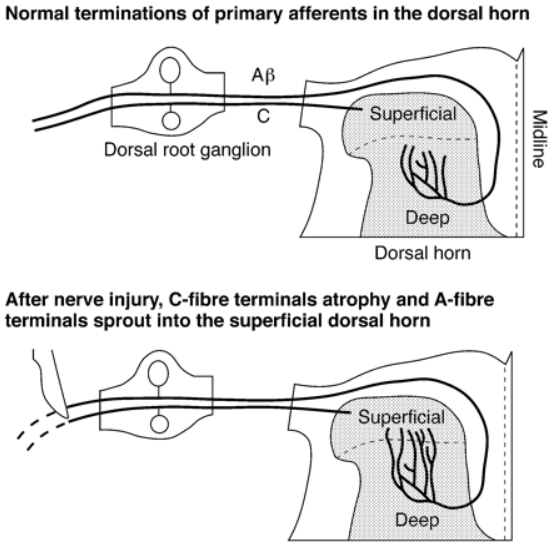


Figure 14: Schematic representation of reorganization of the spinal dorsal horn which is observed after peripheral nerve injury (adapted from Bridges D, 2001).

Changes in inhibitory pathways, such as reduction in the inhibitory control over dorsal horn neurons through different mechanisms, are also important in the central sensitization produced during neuropathic pain (Sugimoto *et al.* 1990; Woolf and Mannion 1999). Dorsal horn neurons receive a strong inhibitory input from GABA-releasing interneurons. In rodents, peripheral nerve injury promotes a selective apoptotic loss of GABA-releasing inhibitory neurons in the superficial dorsal horn of the spinal cord (Moore *et al.* 2002), a mechanism that further increases central sensitization. Dorsal horn neurons receive a powerful descending modulating control, from supraspinal brainstem centers, that has inhibitory as well as facilitatory effects (Vanegas and Schaible 2004). It was hypothesized that a loss of function in descending inhibitory serotonergic and noradrenergic pathways contributes to central sensitization during neuropathic pain. In animals, mechanical allodynia after peripheral nerve injury depends on tonic activation of descending pathways that facilitate pain transmission, indicating that structures in the mesencephalic reticular formation, possibly the nucleus cuneiformis and the periaqueductal gray are involved in central sensitization during neuropathic pain (Ossipov *et al.* 2000). However, another alternative mechanism of intraspinal desinhibition following peripheral nerve injury has been recently proposed. This mechanism involves a trans-synaptic reduction in the expression of the potassium-chloride exporter KCC2 in lamina I neurons, which disrupts anion homeostasis in these neurons. The resulting shift in the transmembrane anion gradient changes inhibitory anionic synaptic currents to be excitatory. The effect is that GABA release from normally inhibitory interneurons now paradoxically exerts an excitatory action on lamina I neurons, which contributes also to increase central sensitization (Coull *et al.* 2003).

Central sensitization may also be enhanced in the spinal cord by glial cells, mainly astrocytes and microglia. Astrocytes and microglia are

activated under conditions associated with enhanced pain (Wieseler-Frank *et al.* 2005). Thus, peripheral nerve injury activates spinal cord glia and these activated cells enhance pain by releasing neuroexcitatory glial proinflammatory cytokines (TNF, IL-1 and IL-6) and glutamate (Wieseler-Frank *et al.* 2005) (Figure 15). The complete neuroinflammatory process leading to the progression of the neuropathic pain requires the coactivation of both microglia and astrocytes (Colburn *et al.* 1999). Cytokines that are released by the activated microglia can be responsible for the subsequent activation of astrocytes, which permits the consolidation of the neuropathic pain state (John *et al.* 2004; Racz *et al.* 2008b). These mechanisms open new perspectives regarding the development of novel analgesic compounds for the treatment of neuropathic pain.

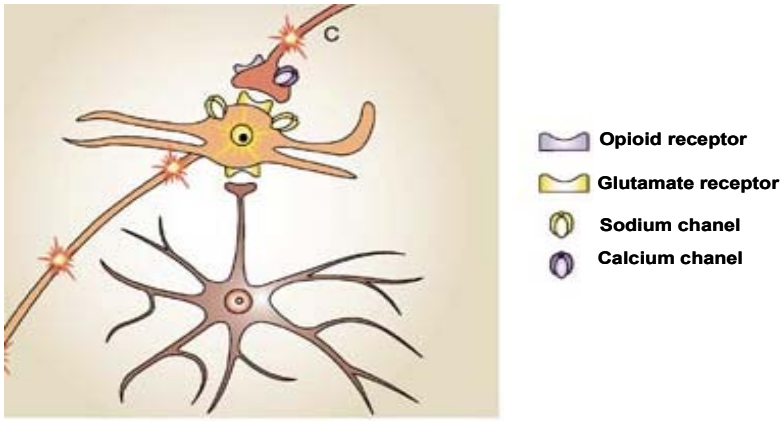


Figure 15: Peripheral nerve injury activates spinal cord non-neural glial cells (brown cell), which further enhances excitability in neurons by releasing cytokines and increasing glutamate.(adapted from Baron, R, 2006)

Most animal experiments investigating the mechanisms involved in central sensitization have been concentrated on the dorsal horn of the spinal cord. However, sensitized neurons are also found in the thalamus and primary somatosensory cortex after peripheral nerve injury in rodents

(Guilbaud *et al.* 1992). Furthermore, magneto-encephalography, positron emission tomography and functional MRI studies demonstrate fundamental changes in the somatosensory cortical representation and excitability in patients with phantom limb pain, complex regional pain syndromes and central pain syndromes, (Flor *et al.* 1995; Maihofner *et al.* 2005; Pleger *et al.* 2004; Willoch *et al.* 2004) as well as in experimental pain models (Baron *et al.* 1999; Baron *et al.* 2000). Interestingly, these changes correlate with the intensity of the perceived pain and disappear after successful treatment of the pain symptoms (Maihofner *et al.* 2004; Pleger *et al.* 2005).

2.4 Treatment of neuropathic pain

The current management of neuropathic pain includes non-pharmacological and pharmacological therapies. The surgical treatment and the conservative interventions can be included in the first category.

2.4.1 Non pharmacological treatment of neuropathic pain

There are several non pharmacological treatments available to attenuate the neuropathic pain symptoms. Among these categories it is worth mentioning physiotherapy, trascutaneous electric nerve stimulation, manual therapies, percutaneous electrical nerve stimulation, acupuncture or electropuncture, magnetotherapy interferential therapy, low-level laser therapy, superficial heat, ultrasonography, psychological-cognitive behavioural techniques and chiropractic. However, there are situations in which the only effective solution to treat neuropathic pain is through surgery procedures. These situations mainly occur in the case of traumatic peripheral nerve injury, lumbar hernia, or in the case of tumours like neuromas that can also generate neuropathic pain. Surgical procedures

include surgical release of entrapped nerve in traumatic peripheral injury, removal of prolapsed nucleus pulposus material of an intervertebral disc in lumbar hernia or surgical excision in the case of tumors.

The interventional techniques for neuropathic pain management can usually be considered when standard pharmacological treatments fail and psychological screening shows emotional stability (Moulin *et al.* 2007). Evidence of efficacy for these techniques is generally less than for pharmacological or surgical interventions. Intravenous lidocaine infusions are generally safe and can provide significant pain relief for two to three weeks at a time. Other interventional techniques are costly and labour-intensive. Among these techniques, continuous spinal infusion of an opioid or clonidine via an implantable pump may be beneficial (Krames 2002). Longitudinal studies of spinal cord stimulation have consistently shown significant pain relief in 50% to 60% of patients with extremity neuropathic pain (Carter 2004).

2.4.2 Pharmacological treatment of neuropathic pain

Large systematic reviews of neuropathic pain treatment have shown that only 60%-70% patients achieved moderate or better levels of pain relief after pharmacological treatment (Collins *et al.* 2000; Sindrup and Jensen 1999). Intolerable side-effects often limit the ability to achieve adequate pain control with a single agent, leading either to discontinuation of specific agents or to progressive treatment strategies to optimize pain control for individual patients (Namaka *et al.* 2004). Even within the same disease, responses to neuropathic pain treatment may vary from patient to patient. Most patients receive multiple agents with divergent mechanisms of action that collectively work to diminish the peripheral and central manifestations of pain. In the last fifty years, the treatment of neuropathic pain has included antidepressants, antiepileptics, anticonvulsants,

antiarrhytmics, topical local anaesthetics, capsaicin and not without controversy, opioids (Baños *et al.* 2003).

Antidepressants

The antidepressants are drugs used in the treatment of chronic pain due to their analgesic and antidepressant properties and in many occasions represent the first line treatment for neuropathic pain. Indeed, tricyclic antidepressants have repeatedly been shown to reduce neuropathic pain (Sindrup and Jensen 1999). Their analgesic actions may be attributable to noradrenaline and 5-HT reuptake blockade (presumably enhancing descending inhibition), NMDA-receptor antagonism and sodium-channel blockade (Stahl 1998), or to the central potentiation of the endogenous opioid system (Schreiber *et al.* 1999). Several authors have reviewed the available clinical evidence of the efficacy of tricyclic antidepressants in controlled clinical trials (Sindrup and Jensen 1999). The main conclusion drew from these studies was that the tricyclics may alleviate 60-70% of patients with neuropathic pain in a wide rage of conditions, such as painful neuropathy, post therapeutic neuralgia, central post-stroke pain and direct nerve-injury pain (Baños *et al.* 2003). Otherwise, selective 5-HT reuptake inhibitors (Sindrup and Jensen 1999) and mixed 5-HT–noradrenaline reuptake inhibitors (venlafaxine and duloxetine) (Goldstein *et al.* 2005) do not appear to be as effective as tricyclic antidepressants like amitriptyline or nortriptylin (Finnerup *et al.* 2005). Side-effects of antidepressants treatment are frequent and include increased heart rate, drowsiness, dry mouth, constipation, urinary retention, blurred vision, dizziness, confusion, and sexual dysfunction.

Anticonvulsivants

Anticonvulsant drugs represent an important option in the treatment of neuropathic pain and they have shown to be effective almost four decades

ago (Baños *et al.* 2003). Several anticonvulsivants such as carbamazepine, phenytoine and more recently gabapentin, pregabalin and lamotrigine are used to treat neuropathic pain. Based on clinical trials, carbamazepine and phenytoin present modest efficacy in diabetic peripheral neuropathy (Finnerup *et al.* 2005). Both have significant adverse effects, making them generally poor candidates for first-line therapy. Carbamazepine, however, is still considered first-line therapy for trigeminal neuralgia, a unique neuropathic pain condition (Finnerup *et al.* 2005). Oxcarbazepine, a newer anticonvulsant structurally related to carbamazepine, may also be useful although only one randomized controlled clinical trial in diabetic peripheral neuropathy has been published (Dogra *et al.* 2005). Gabapentin is a voltage-gated calcium channel antagonist that has repeatedly demonstrated analgesic efficacy and improvements in mood and sleep in several clinical trials in neuropathic pain (Gilron 2007). Gabapentin effectiveness was comparable to amitryptiline in some clinical studies, but with fewer side-effects (Morello *et al.* 1999). Dizziness and somnolence are the most frequent adverse effects of gabapentin, although they are generally well tolerated. A gabapentin analogue that is currently widely used in the treatment of neuropathic pain is pregabalin. Pregabaline has a similar mechanism of action as gabapentin, but has higher calcium-channel affinity, better bioavailability and showed effectiveness in clinical trials in diabetic peripheral neuropathy and postherpetic neuralgia studies (Gilron 2007).

Opioids

The effectiveness of opioids extends from nociceptive to neuropathic pain states (Smith 2008). Although neuropathic pain does not respond reliably to opioids, randomized clinical trials have shown effect of opioids in different neuropathies (Baños *et al.* 2003). Opioid compounds have important side-effects that can limit their use in some patients. Most of

them, such as nausea, vomiting, constipation, confusion and sedation are frequent and unavoidable consequences of their use. However, respiratory depression, the most feared effect of opioid, may be avoided with careful titration of opioid dosage and advice for a close monitoring of patients. Other important side-effects, such the development of tolerance and dependence can also be avoided when using an appropriate medical praxis (McCleane and Smith 2007). Nevertheless, the potential development of tolerance and dependence may complicate the use of these drugs in patients with non-cancer chronic pain and may represent sometimes an additional limitation for the clinical use of opioids (Benyamin *et al.* 2008).

Drugs acting on NMDA receptors

NMDA antagonists given as intravenous infusions have been reported to relieve neuropathic pains of different origin (Sang 2000). Oral NMDA antagonists, such as riluzole and memantine have been studied mainly in small trials in neuropathic pain, with either no or minor pain relieving effect (Finnerup *et al.* 2005). Unfortunately, available agents have limited efficacy and produce intolerable side-effects. Ketamine, an intravenous anaesthetic with NMDA-antagonist activity, has been found to be effective although with important psychomimetic side-effects that are dose limiting (Hocking and Cousins 2003).

Drugs acting on noradrenergic pathways

Presynaptically alpha-2 adrenergic receptors are present on small primary afferent neurons and their activation results in hyperpolarization and diminished release of neurotransmitters involved in relaying pain signals (Wolff *et al.* 2007). Alpha-2 adrenergic agonists also activate spinal cholinergic neurons, which may potentiate their analgesic effects (Roh *et al.* 2008a). Clonidine is an alpha2-agonist that blocks the release of P substance at the presinaptic level and the activation of nociceptive

neurons. mainly in the spinal cord (Roh *et al.* 2008a). Clonidine showed benefit in animal models of neuropathic pain (Roh *et al.* 2008a) and also in a subset of patients with painful diabetic neuropathy (Byas-Smith *et al.* 1995) when administered intrathecal and transdermal, respectively.

Cannabinoids and the neuropathic pain treatment

The cannabinoids are analgesic agents with strong evidence of efficacy in animal models of neuropathic pain and increasing evidence of efficacy in humans. Thus, the systemic administration of the cannabinoid agonists WIN55,212,2, CP-55,940 and HU-210 reversed the mechanical hyperalgesia in a rat neuropathic pain model of sciatic ligation model (Fox *et al.* 2001). WIN55,212,2 has also been shown to reduce thermal and mechanical hyperalgesia as well as mechanical allodynia in two different neuropathic pain models, the spinal nerve ligation and the chronic constriction injury (Bridges *et al.* 2001a; Herzberg *et al.* 1997). Studies using knockout animals have demonstrated that overexpression of CB_2 cannabinoid receptor in mice attenuated the behavioural manifestations of neuropathic pain, whereas these neuropathic pain manifestations were increased in knockout mice deficient of CB_2 receptors (Racz *et al.* 2008b; Racz *et al.* 2008a). In accordance, several CB_2 agonists were efficient to attenuate the manifestations of neuropathic pain in the rat spinal nerve ligation model (Thakur *et al.* 2009).

In clinical trials, dronabinol has been shown to produce modest analgesia in a randomized trial of central pain in multiple sclerosis patients (Svendsen *et al.* 2004). Sativex® , a 50/50 mixture of tetrahydrocannabinol and cannabidiol in the form of an oral mucosal spray provided significant benefit in another trial of central pain in multiple sclerosis (Rog *et al.* 2005).

Other drugs used for neuropathic pain treatment

Lidocaine patches (5%) are attractive options for the neuropathic pain treatment because they may cause minimal systemic side-effects. The lidocaine patch has been shown to relieve localized pain in postherpetic neuralgia with no incidence of significant side-effects (Finnerup *et al.* 2005). In addition, lidocain patches were found efficient in a prospective clinical trial in other types of neuropathic pain such as postmastectomy pain, intercostals neuralgia and painful diabetic peripheral neuropathy (Vadalouca *et al.* 2006).

Capsaicin, an ingredient of hot peppers, is an agonist of the TRPV1 where acts to produce its known burning and analgesic effects. The analgesic effects of capsaicin are a consequence of repeated activation of the VR1 receptors that finally desensitize them (Baños *et al.* 2003) The analgesia produced by capsaicin is preceded by an intense burning sensation that corresponds to the activation of VR1. This dual effect is poorly tolerated by some patients and, hence, new drugs that do not stimulate the VR1 and that still have the blocking action are needed. The results of randomized control trial that compared topical capsaicin with placebo in patients with painful diabetic peripheral neuropathy, postherpetic neuralgia, and post-mastectomy pain have been inconsistent (Finnerup *et al.* 2005; Mason *et al.* 2004). Interpretation of efficacy is problematic in these studies because the burning sensation associated with capsaicin use may have compromised blinding in the trials in which superiority to placebo was found.

Baclofen, a GABA β-receptor agonist, is a muscle relaxant that has shown effectiveness in trigeminal neuralgia (Fromm *et al.* 1984). It has also been shown that intrathecal baclofen suppresses central pain in patients with spinal lesions (Herman *et al.* 1992).

Mexiletine is a local anaesthetic and antiarrythmic agent acting through the blockade of sodium channels. In patients with painful diabetic

peripheral neuropathy and other types of neuropathic, mexiletine has shown either modest benefits or no differences compared to placebo (Finnerup *et al.* 2005; Tremont-Lukats *et al.* 2005). When evidence of efficacy was found in these trials, it was at higher dosages, which are often poorly tolerated because of side-effects.

2.5 New targets for the neuropathic pain treatment

2.5.1 Adenosine 2A receptors and neuropathic pain

2.5.1.1 Adenosine and the adenosine receptors The presence of adenosine receptors has been demonstrated in almost every tissue or organ examined (Fredholm *et al.* 2000; Fredholm *et al.* 2001a). Adenosine, a purine nucleoside, is produced in response to metabolic stress and cell damage, ischemia, hypoxia, inflammation and trauma (Hasko and Cronstein 2004). The main pathway leading to high extracellular adenosine levels during metabolic stress is the release of precursor adenine nucleotides, mostly ATP, from the cell followed by extracellular catabolism to adenosine by a cascade of ectonucleotidases (Zimmermann 2000). Another significant source of extracellular adenosine is intracellular adenosine, which is released through nucleoside transporters when intracellular adenosine levels rise (Hasko and Pacher 2008). This occurs mostly as a result of degradation of intracellular ATP in ischemic conditions. Adenosine produces a wide range of physiological responses by binding to and activating four cell surface adenosine receptors, designated as A_1, A_{2A}, A_{2B} and A_3. The adenosine receptors contain seven transmembrane domains and couple to intracellular GTP-binding proteins (G proteins). Adenosine can activate A_1, A_{2A}, and A_3 receptors with high potency whereas the potency of adenosine at A_{2B} receptors is lower (Fredholm *et al.* 2001b). As physiological adenosine concentrations rarely

exceed 1 μM, physiological levels of adenosine can activate A_1, A_{2A}, and A_3 receptors, whereas A_{2B} receptor activation requires pathophysiological conditions. In general, A_1 and A_3 receptors are coupled to Gi/o proteins and their stimulation decreases the intracellular cAMP levels. A_{2A} and A_{2B} receptors are coupled to Gs proteins and stimulate adenylyl cyclase and cAMP accumulation (Figure 16).

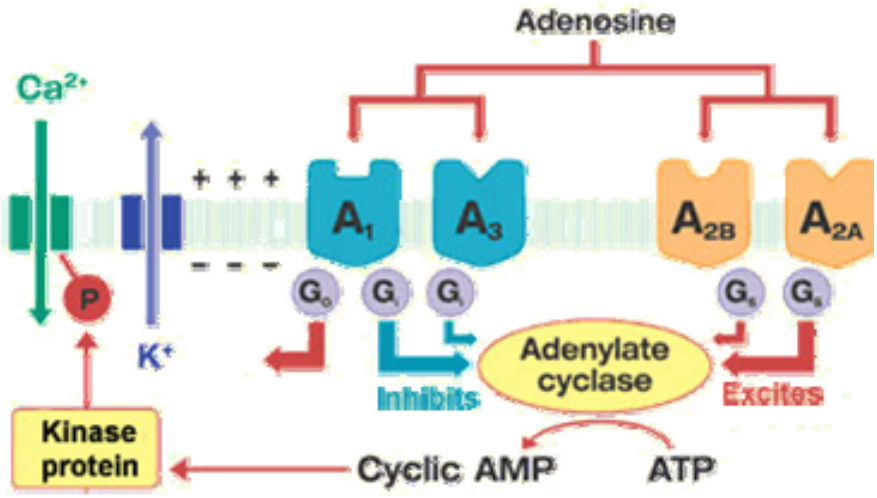


Figure 16: Adenosine receptors. The A_1 and A_3 coupled to Gi protein inhibit the adenylate cyclase. The A_{2A} and A_{2B} receptors coupled to Gs protein stimulate the adenylate cyclase

The distribution of the A_1 and A_{2A} receptors has been well characterized because the appropriate pharmacological tools, including radioligands and knockout animals, are available. In the case of the A_{2B} and A_3 receptors, less data are available due to the absence of these experimental tools.

The overall distribution of adenosine receptors in the brain is similar in rodents, humans, or other primates (Dixon *et al.* 1996; Moreau and Huber 1999). In rodents, there is a high density of A_1 adenosine receptors in the SNC in areas such as cortex, cerebellum and dorsal horn of spinal cord (Fredholm *et al.* 2001a). The A_{2A} adenosine receptors are highly

expressed in the striatum, nucleus accumbens and olfactory bulb and is also present in lower concentrations in thalamus and hippocampus (Sebastiao and Ribeiro 1996).

Both A_1 and A_{2A} adenoine receptors are mostly located presynaptically in the brain, whereas A_1 receptors have also a significant post-synaptic localization. The striatum is clearly the exception, where A_{2A} receptors are most densely located post-synaptically. Apart from this predominant neuronal localization, both A_1 and A_{2A} receptors are also located in astrocytes and microglia, A_1 receptors are present in oligodendrocytes and A_{2A} receptors in blood vessels (Cunha 2005).

The purinergic receptors (A_1 A_{2A} A_{2B} A_3) are also found in the peripheral tissues as well including the heart, lungs, kidney and liver among others (Table 7).

Table 7. Summary of adenosine receptors distribution (modified from Fredholm et al, 2001)

Receptor A1	Receptor A2A	Receptor A2B	Receptor A3
High expression	**High expression**	**High expression**	**High expression**
Brain (cortex, cerebellum, hippocampus). Dorsal horn of spinal cord. Eye, adrenal gland, atria	leukocytes (both lymphocytes and granulocytes), blood platelets. Striatopallidal GABAergic neurons (in caudate-putamen, nucleus accumbens, tuberculum bulb olfactorium), olfactory	Cecum, colon, bladder	Testis, mast cells (rat)
Intermediate levels	**Intermediate levels**	**Intermediate levels**	**Intermediate levels**
Other brain regions. Skeletal muscle, liver, kidney, adipose tissue, salivary glands, esophagus, colon, antrum, testis	Thalamus, hippocampus Heart, lung, blood vessels	Lung, blood vessels, eye, median eminence mast cells	Cerebellum, lung hippocampus spleen, pineal (sheep)
Low levels	**Low levels**	**Low levels**	**Low levels**
Lung, pancreas	Other brain regions	Brain, adipose tissue, adrenal gland, kidney liver, pituitary gland ovary,	Thyroid, spleen, most of brain, liver adrenal gland, heart kidney, intestine, testis (human)

Given this wide distribution, the A_{2A} adenosine receptors are involved in various physiological responses and pathological conditions. Thus, these receptors participate in the regulation of motor functions, anxiety, aggressiveness, stress, motivation, reward processes, memory, aging, pain, sleep and wakefulness (Moreau and Huber 1999).

2.5.1.2 A_{2A} receptors and pain

Adenosine plays an important role in the modulation of pain and the receptors initially thought to be predominantly involved were the A_1 adenosine receptors. However, a special attention was conferred in the last years to the role of A_{2A} adenosine receptors in pain modulation. As described in the previous paragraphs, A_{2A} adenosine receptor is mainly localized in the brain, in the dorsal and ventral striatum, which have no major role in the pain circuitry. However, nociceptive neurones reach both the dorsal and ventral striatum (Newman *et al.* 1996) and, like many areas of the limbic system, modulates pain processing (Millan 1999). In addition, the presence of A_{2A} has also been demonstrated in the somatosensory cortex (Johansson *et al.* 1997; Kelly *et al.* 2004), which provides a potential role of these receptors in the central integration of pain. Early studies suggested that A_{2A} receptors were present in the dorsal spinal cord of the rat (Choca *et al.* 1987), but not in the mice (Bailey *et al.* 2002). In agreement, recent immunohistochemistry studies coupled with functional electrophysiology have shown responses to the A_{2A} selective agonist CGS 21680 in the rat spinal cord (Brooke *et al.* 2004) and these receptors have been proposed to be located on presynaptic inhibitory terminals of descending fibers from higher centers (Brooke *et al.* 2004). Thus, the possibility of spinal effects of A_{2A} receptor activation in the modulation of pain cannot be excluded. The A_{2A} receptor gene is also expressed in the DRG (Kaelin-Lang *et al.* 1998) and a retrograde transport of the receptor to the peripheral terminals has been proposed in sensory

nerve fibers suggesting that another site of A_{2A} receptor modulation of pain could to be at peripheral nerve terminals (Sawynok 1998).

In addition to these data, an important location of the A_{2A} receptor for pain modulation is at the microglia and astrocyte level. Astrocytes are the major population of glial cells in the CNS and they undergo a process of proliferation, changes in gene expression and morphology (hypertrophy of cell bodies, thickening and elongation of astrocytic processes) in response to noxious stimuli (Liberto *et al.* 2004). This process, which is termed astrogliosis, is associated with enhanced release of growth factors and neurotrophins (Liberto *et al.* 2004). Astrocytes activation is also associated with enhanced production of inflammatory cytokines, increased expression of major histocompatibility complex II and augmented release of free radicals (Dong and Benveniste 2001). On the other hand, microglia responds rapidly and relatively uniformly to several kinds of injury with characteristic morphological changes, proliferation, upregulation of cell-surface molecules and production of soluble mediators. It has been reported that the blockade of A_{2A} adenosine receptor has neuroprotective effects in several pathological situations by inhibiting the proliferation of both astrocytes and microglia (Hasko *et al.* 2005) (Figure 17)

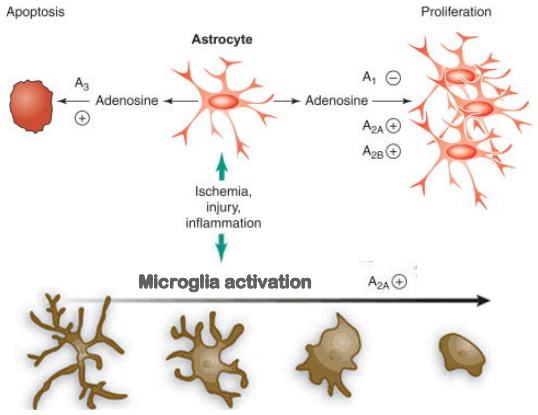


Figure 17. Regulation of astrocyte and microglial cells proliferation and apoptosis by adenosine receptors.

Although sometimes contradictory, both pharmacological and genetical studies have also confirmed the involvement of A_{2A} receptors in pain modulation. Thus, the A_{2A} receptor agonists, CGS 21680 and DPMA showed antinociceptive effects in the writhing test, a visceral pain model (Bastia *et al.* 2002; Pechlivanova and Georgiev 2002). CGS 21680 also induced antinociception in thermal tests after intrathecal administration (Suh *et al.* 1997) and in inflammatory (Poon and Sawynok 1998) and neuropathic pain models (Lee and Yaksh 1996). However, CGS 21680 has been shown both antinociceptive (Borghi *et al.* 2002) and pronociceptive effects in the formalin test in rats (Doak and Sawynok 1995). The same antinociceptive and pronociceptive effects were observed with the A_{2A} receptor agonist APEC in mice (Karlsten *et al.* 1992). A_{2A} receptor agonists are also pronociceptive in a mechanical paw pressure test in the rat (Taiwo and Levine 1990). Studies with A_{2A} receptor antagonists have been rather more consistent. SCH 58261 an A_{2A} antagonist, has antinociceptive effects in the writhing (Bastia *et al.* 2002), tail flick and hot plate test (Godfrey *et al.* 2006). In contrast, the A_{2A} receptor antagonist, DMPX, did not produce antinociception in the writhing test in mice (Pechlivanova and Georgiev 2002), but did reverse formalin-induced flinching responses in the rat (Doak and Sawynok 1995). Genetical studies showed that A_{2A} receptor knockout mice (Ledent *et al.* 1997) presented hypoalgesia in the tail immersion and hot-plate test (Bailey *et al.* 2002; Godfrey *et al.* 2006), but these thermal nociceptive responses were not altered at lower temperature (Bailey *et al.* 2002). In addition, the nociceptive latencies were not modified in these knockout mice in the tail pressure test (Bailey *et al.* 2002). Moreover, A_{2A} receptor knockout mice had reduced biting and flinching responses to intraplantar formalin injection. In spite of this wide evidence suggesting a possible role of A_{2A} adenosine receptor in pain modulation, little information has

been provided about the specific involvement of these receptors in neuropathic pain.

2.5.2 Sigma-1 receptors and pain

2.5.2.1 Clasification, distribution and functions Sigma (σ) receptors were first defined as a subclass of opioid receptors (Skuza and Wedzony 2004) and later confounded with the high affinity phencyclidine (PCP) binding sites (Skuza and Wedzony 2004). However, today sigma receptors are considered as unique binding sites, distinct from opioid and PCP receptors and related to independent brain function (Skuza and Wedzony 2004). Biochemical and pharmacological studies suggest that there are several subtypes of sigma receptors, but only two have been well characterized at the present moment: sigma-1 and sigma-2 receptor (Bowen 2000; Hellewell and Bowen 1990; Quirion *et al.* 1992). In addition, only the sigma-1 receptor has been recently cloned from various sources, including guinea-pig liver (Hanner *et al.* 1996), human placental choriocarcinoma cells (Kekuda *et al.* 1996), human brain (Prasad *et al.* 1998), rat brain (Mei and Pasternak 2001; Seth *et al.* 1998), and mouse brain (Pan *et al.* 1998). It was suggested that sigma-1 receptor includes a single putative transmembrane domain based on hydropathy analysis of the amino acid sequence of this receptor (Mei and Pasternak 2001). However, recent studies suggest that the sigma-1 receptor has two transmembrane segments (Aydar *et al.* 2002) (Figure 18). Despite of considerable advances in understanding the sigma receptors over the past years, an endogenous ligand has not yet been found (Guitart *et al.* 2004). The amino acid sequence of the sigma receptor is not consistent with that of a classical G protein- coupled receptor, which makes especially difficult to predict the effects of agonists or antagonists of this receptor, at

least when considering the traditional way of direct linkage to stimulation or inhibition of intracellular signalling pathways.

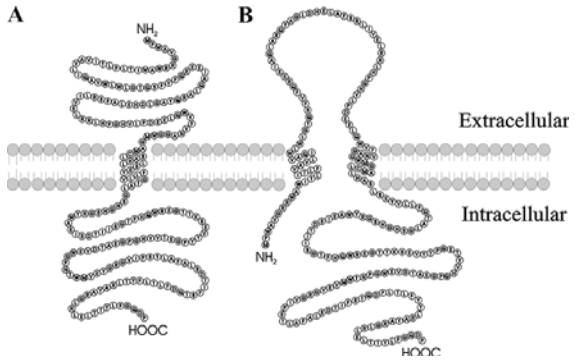


Figure 18 The two structural models that have been proposed for the sigma-1 receptor: with a single putative transmembrane domain (A) and two putative transmembrane domains (B) (adapted form Guitart, X et al, 2004)

The hydrophobic region has been suggested to be important for the binding of (+)- pentazocine (Yamamoto *et al.* 1999), although it has not been yet elucidated whether this binding site is shared by neurosteroids. Nevertheless, biochemical (Beart *et al.* 1989; Connick *et al.* 1992; Itzhak 1989) and pharmacological studies (Gonzalez-Alvear and Werling 1995; Monnet *et al.* 1992; Pascaud *et al.* 1993) have identified a substantial number of molecules with activity as agonists or antagonists of sigma-1 receptors such as (+)-pentazocine (Chaki *et al.* 1994), OPC-14523 (Oshiro *et al.* 2000) and sertraline (Schmidt *et al.* 1989) as a putative agonists, and haloperidol, (Chaki *et al.* 1994) and SR31742A (Poncelet *et al.* 1993), as antagonists (Guitart and Farre 1998). Classical peptides and neurotransmitters have been shown to be ineffective in displacing selective sigma ligands from sigma receptors. Neurotransmitters that do not interact with the sigma receptor include 5-HT, norepinephrine, DA, and histamine (Haven-Hudkins and Fleissner 1992; Weber *et al.* 1986) as well as several amino acids, such as glutamate, glycine, aspartate, and

cysteine (Craviso and Musacchio 1983; Klein and Musacchio 1989) and peptides, such as the β-endorphin, dynorphins, enkephalins and substance P (Haven-Hudkins and Fleissner 1992; Samovilova *et al.* 1988).

The distribution of this receptor in the adult rat CNS has been described using immunohistochemistry with a specific antibody against the sigma-1 receptor (Alonso *et al.* 2000). High levels of sigma-1 immunostaining were found in neurons of specific brain regions, including the olfactory bulb, several hypothalamic nuclei, the septum, the central gray matter, certain motor nuclei of the hindbrain, and the dorsal horn of the spinal cord. Cells with intense immunostaining were also observed in the hippocampus particularly in the dentate gyrus. Only a small number of weakly stained cells was observed in the dorso-lateral striatum, whereas moderately immunostained cells were observed in the nucleus accumbens. The sigma- 1 receptor is also widely distributed in peripheral organs. Thus, binding assays and autoradiography studies showed that sigma-1 receptors are present in the mucosal and submucosal regions of the digestive tract and with less labeling in the muscular regions (Samovilova and Vinogradov 1992). Sigma-1 receptors were also found in the liver (Dumont and Lemaire 1991; Hellewell *et al.* 1994; Maurice *et al.* 1996), kidney (Hellewell *et al.* 1994), heart (Ela *et al.* 1996; Jansen *et al.* 1992) and sexual organs (Jansen *et al.* 1992).

Subcellular localization studies have shown that the sigma-1 receptor is primarily associated in the brain with neuronal perikarya and dendrites, and is localized in the plasma membrane, the mitochondrial membrane and the endoplasmic reticulum (McLean and Weber 1988; Hayashi and Su 2001; Morin-Surun *et al.* 1999). Given this unusual distribution, it has been proposed that the sigma-1 receptor is translocated after activation from the endoplasmic reticulum to the plasma membrane or to the nuclear membrane (Hayashi and Su 2001; Morin-Surun *et al.* 1999).

A large number of pharmacological, biochemical, and lately genetical studies (Langa *et al.* 2003) has shown that sigma-1 receptors are involved in several physiological and pathological conditions. In the CNS, sigma-1 receptors participate in the modulation of mood disorders, amnesic and cognitive deficits, reward circuits, movement disorders, and nociception (Guitart *et al.* 2004). In peripheral organs, sigma receptors could also be implicated in gastrointestinal movements under stress, duodenal bicarbonate secretion, contraction of vas deferens, regulation of plasma levels of corticosterone, prolactin, adrenocorticotropin and contractility of cardiac cells (Guitart *et al.* 2004).

2.5.2.2 Sigma-1 receptors and pain

A possible role for sigma receptors in antinociception was initially suggested from studies showing a relationship between sigma receptors and opioid analgesia (Chien and Pasternak 1993; Chien and Pasternak 1994). Thus, an antiopioid role has been suggested for sigma-1 receptor since sigma-1 receptor antagonists potentiate opioid analgesia (Chien and Pasternak 1993; Chien and Pasternak 1994). Thus, sigma-1 antagonists, such as haloperidol, have no effect on withdrawal latencies in the tail-flick assay when given alone, but significantly increase morphine analgesic responses. Haloperidol also increased the antinociceptive responses of the selective κ-receptor agonists in the tail flick test, whereas the sigma-1 agonist (+)-pentazocine produces opposite effects (Guitart *et al.* 2004). Treatment with sigma-1 receptor antisense oligodeoxynucleotides enhanced the antinociception produced by agonists of μ-, δ- and κ-opioid receptors in the tail flick test (Mei and Pasternak 2002). These data suggest that sigma-1 receptors modulate opioid-induced antinociception in acute models of pain.

Activation of NMDA receptors is involved in different types of chronic pain (Coderre and Melzack 1992; Petrenko *et al.* 2003). Interestingly, sigma-1 receptor agonists have been shown to potentiate NMDA-induced activation of neuronal firing (Debonnel and de 1996). In agreement, the expression of formalin-induced pain(Cendan *et al.* 2005) was reduced in sigma-1 receptor knockout mice through their known ability to modulate NMDA-mediated responses (Debonnel and de 1996). Furthermore, intrathecal administration of sigma-1 antagonists dose-dependently reduced formalin and nerve injury-induced pain (Kim *et al.* 2006; Roh *et al.* 2008b). Moreover, mice lacking sigma-1 receptors showed decreased levels of thermal and mechanical allodynia after partial sciatic nerve ligation induced neuropathic pain (Puente *et al.* 2009). Therefore, the sigma-1 receptor is a constituent of the mechanisms modulating pain sensitization and could represent a new potential target for drugs designed to alleviate neuropathic pain.

2.6. Experimental models for neuropathic pain evaluation

Animal models of neuropathic pain are essential to improve the research in order to better understand the mechanisms underlying the pathophysiology of this disease and to design novel therapeutic strategies to obtain new compounds for clinical use (Bridges *et al.* 2001) The experimental animal models of neuropathic pain developed in the last three decades include both CNS and peripheral nerves injuries. The last category includes a higher variety of models due to the better accessibility, and includes neuropathic pain induced by injury (nerve injury or constriction), physical methods (laser or cryogenics), metabolic disorder (diabetes), neurotoxicity (chemotherapic agents like paclitaxel and cisplatin), immunological induction (Freud adjuvant [FA], tumour necrosis factor [TNF] and nerve growth factor [NGF]). Table 8 summarises the main neuropathic pain experimental models.

Table 8. The main neuropathic pain experimental models

Model	Reference
Central nervios system	
Posterior rhizotomy	Basbaum and Wall, 1991
Dysesthesic syndrome	Levitt, 1991
Ischaemic spinal cord injury photochemically induced by laser irradiation	Hao et al, 1991
Neurotoxicity induced by intraspinal injections of quisqualic acid	Yezierski and Park, 1993
Peripheral nerves	
Mechanic injury	
Nerve section	Wall et al, 1979
Chronic constriction injury of sciatic nerve	Bennet and Xie, 1988
Sciatic partial nerve ligati	Seltzer et al, 1990
Spinal nerve ligation	Kim and Chung, 1992
Neuropathy induced by fixed-diameter polyethylene cuffs applied to the sciatic nerve	Mosconi and Kruger, 1996
Phisical injury	
Peripheral cryogenic nerve lesion	DeLeo et al, 1991
Laser induced ischemia of sciatic nerve	Kupers et al, 1998
Metabolic neuropathy	
Streptozotocin induced diabetic neuropathy	Ahlgren and Levine, 1993
Chemotherapic drugs induced neuropathy	
Vincristine	Authier et al 1999
Paclitaxel	Polomano et al 2001
Cisplatin	Authier et al 2003
Neuroinflammation induced neuropathy	
TNF administration	Wagner and Myers 1996
NGF adminstration	Ruiz et al, 2004
FA administration	Eliav et al, 1999

Most of the currently used neuropathic pain models share alterations in hind-paw cutaneous sensory thresholds following partial injury of a peripheral (usually sciatic) nerve as a common feature. Demonstration of hyperalgesia to noxious thermal stimuli and allodynia to cold and mechanical stimuli are currently used as outcome measures. The three most commonly used peripheral models are the chronic constriction injury

(CCI) of sciatic nerve (Bennett and Xie 1988), the partial sciatic nerve ligation model (PSNL) (Malmberg and Basbaum 1998; Seltzer *et al.* 1990) and the spinal nerve ligation model (SNL) (Kim and Chung 1992) (Figure 19).

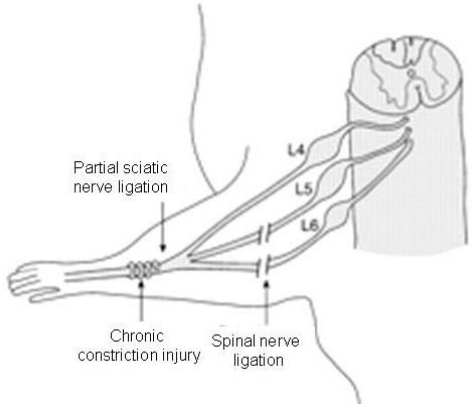


Figure 19. Schematic drawing of the partial sciatic nerve ligation (tight ligation of 33–50% of the sciatic nerve trunk), CCI (loose ligations of the sciatic nerve trunk), and SNL (tight ligation and transection of the L5 and L6 spinal nerves) animal models of neuropathic pain (adapted from Bridges et al, 2001)

The CCI model consists of the loose ligation of the sciatic nerve at mid-thigh level with chromic gut sutures (Bennett and Xie 1988). An inflammatory reaction develops in response to the catgut and consequentially a loss of most A-fibers and some C-fibers occurs, although few cell bodies are lost (Tandrup *et al.* 2000). This injury is associated with spontaneous pain-related behaviour, allodynia and hyperalgesia. A significant inflammatory component is associated to the development of the painful neuropathy since the CCI rats exposed to this procedure showed decreases thermal hyperalgesia after anti-inflammatory treatment (Wagner *et al.* 1998). In addition, there is a large degree of operator variability in this model, depending on differences in the tightness of the ligatures (Bridges *et al.* 2001). The PSNL model also consists of injury to the sciatic nerve at mid-thigh level. In this model, a

tight ligation is created around 33–50% of the sciatic nerve, leaving the rest of the nerve 'uninjured' (Malmberg and Basbaum 1998; Seltzer *et al.* 1990). This is associated with the development of spontaneous pain-like behaviour, allodynia and hyperalgesia. Although this model is regarded as having less inflammatory component than the CCI model, there is still high variability depending on the number of ligated neurones per animal. In addition, it is not easy to relate the PSNL injury to a specific DRG or level of the spinal cord since usually a random mixture of L4 and L5 spinal nerve afferents are injured (Bridges *et al.* 2001). The SNL model consists of injury to the L5 and L6 spinal nerves, that contribute to the sciatic nerve (Kim and Chung 1992). This injury is also associated with the development of spontaneous pain-like behaviour as well as long lasting allodynia and hyperalgesia. A tight ligation of only the L5 spinal nerve resulted in comparative symptoms to the L5 and L6 ligation group and hence some experimenters now use this procedure as a modified SNL model (Bridges *et al.* 2001). SNL model allow to examine cellular responses to the injury at the DRG level since the L5 and L6 DRGs are affected, whereas the L4 DRG is not (Li *et al.* 2000).

These existing animal models allow the study of the manifestations of neuropahic pain, the neurobiological mechanism involved and the efficacy of possible pharmacological treatments. However, the different compounds tested in the currently available models are administered in a non contingent manner, which limits the evaluation of their efficacy and possible side-effects. Therefore, it would be of great interest to develop new animal models allowing the contingent administration of the analgesic compounds by the animal suffering pain. This would represent an important advance for the evaluation of the new therapeutic strategies since the results obtained in these models would certainly have a better extrapolation to the human clinical situation. In addition, these models would also permit the measurement of a large range of behavioural

responses that would be difficult to determine in a non contingent model, such as the abuse liability of the new compounds that could be easily evaluated using the appropriate controls in a contingent model of self-administration of the analgesic compounds. Therefore, these new models would provide more valuable information to predict the clinical benefit/risk ratio of new analgesic compounds to treat chronic pain.

OBJECTIVES

Objective 1

To investigate the possible interactions between the cannabinoid and cholinergic systems in memory and learning processes by using genetic and pharmacological approaches in two different behavioural models, the active avoidance and the object recognition test.

Article #1

SA Bura, A Castañé, C Ledent, O Valverde and R Maldonado (2007). "Genetic and pharmacological approaches to evaluate the interaction between the cannabinoid and cholinergic systems in cognitive processes". *British Journal of Pharmacology,* 150(6):758-65.

Objective 2

To evaluate the effects of chronic nicotine administration and withdrawal in food intake, metabolic parameters and anxiety-like behaviour in CB_1 knockout mice and wild-type littermates using a new highly sensitive food and drink monitoring system developed in collaboration with our laboratory.

Article # 2

S.Andreea Bura, Aurelijus Burokas, Elena Martín-García, Rafael Maldonado (2010). "Effects of chronic nicotine on food intake and anxiety-like behaviour in CB1 knockout mice". *European Neuropsychopharmacology,* 20(6):369-378.

Objective 3

To assses the possible involvement of adenosine A_{2A} receptors in the development of neuropathic pain and the expression of microglia and astrocytes in the spinal cord after sciatic nerve injury.

Article # 3

S. Andreea Bura, Xavier Nadal , Catherine Ledent , Rafael Maldonado and Olga Valverde " (2008). A_{2A} adenosine receptor regulates glia proliferation and pain after peripheral nerve injury". *Pain,* 140(1):95-103.

Objective 4

To set-up a new operant model of drug self-administration in mice exposed to neuropathic pain that can be used to evaluate the therapeutic potential and possible side-effects of novel compounds for neuropathic pain.

Article # 4

S.Andreea Bura, Thomas Guegan, Daniel Zamanillo, José Miguel Vela and Rafael Maldonado. "A new operant model in mice to evaluate the therapeutic potential of novel compounds for neuropathic pain". Manuscript in preparation to be sent to *Pain*.

RESULTS

ARTICLE #1

Genetic and pharmacological approaches to evaluate the interaction between the cannabinoid and cholinergic system in cognitive processes

Bura SA, Castañe A, Ledent C, Valverde O, Maldonado R

Br J Pharmacology (2007); 150(6):758-65.

Objectives:

To investigate the possible interactions between the cannabinoid and cholinergic systems in memory and learning processes by using genetic and pharmacological approaches in two different behavioural models, the active avoidance and the object recognition test.

Main results:

- Nicotine (0.5 mg/kg) did not modify the performance of CB_1 knockout mice and wild-type mice in the active avoidance model, whereas scopolamine (0.5 mg/kg) impaired the performance in both genotypes.
- Physostigmine (0.1 mg/kg) increased the active avoidance performance in wild-type, but not in CB_1 knockout mice.
- Rimonabant given in a wide range of doses did not modify the performance in the active avoidance test, given alone or co-administered with nicotine.
- Nicotine enhanced the performance in the object recognition task but this response was attenuated by rimonabant co-administration.

Conclusion:

The present findings revealed that the cognitive effects of nicotine and physostigmine are attenuated in the absence of CB_1 receptor activity.

Scopolamine effects are independent from CB_1 receptors, whereas nicotine and physostigmine effects are mediated by these receptors.

Bura SA, Castañé A, Ledent C, Valverde O, Maldonado R. Genetic and pharmacological approaches to evaluate the interaction between the cannabinoid and cholinergic systems in cognitive processes. Br J Pharmacol. 2007; 150(6): 758-65.

ARTICLE #2

Effects of chronic nicotine on food intake and anxiety-like behaviour in CB_1 knockout mice

S.Andreea Bura, Aurelijus Burokas, Elena Martín-García and Rafael Maldonado

Eur Neuropsychopharmacol. (2010); 20(6):369-378

Objectives:

To evaluate the consequences of non contingent chronic nicotine administration and its withdrawal in food intake and preference, metabolic parameters and emotional behaviour in CB_1 knockout mice and wild-type littermates.

Main results:

- The deleterious effects of the high fat diet on metabolic parameters as well the effects of nicotine on anxiogenic-like responses and body weight were prevented in CB_1 knockout mice.
- Mutant mice showed lower preference for high palatable drink in the absence of nicotine treatment
- Nicotine reduced body weight in wild-type mice but not in CB_1 knockout mice
- Anxiogenic-like effects of nicotine were found in wild-type animals, but not in CB_1 knockout mice.

Conclusion:

These results provide a new evidence of the important role played by the endocannabinoid system in the pharmacological responses of nicotine that will be useful to better understand the interaction between nicotine and cannabinoid compounds.

Bura SA, Burokas A, Martín-García E, Maldonado R. Effects of chronic nicotine on food intake and anxiety-like behaviour in CB1 knockout mice. Eur Neuropsychopharmacol. 2010; 20(6): 369-78.

ARTICLE #3

A_{2A} adenosine receptor regulates glia proliferation and pain after peripheral nerve injury

S. Andreea Bura, Xavier Nadal , Catherine Ledent , Rafael Maldonado and Olga Valverde

Pain, (2008); 140(1):95-103.

Objectives:

To evaluate the possible involvement of A_{2A}Rs in the development of neuropathic pain and the expression of microglia and astrocytes in the spinal cord after sciatic nerve injury.

Main results:

- In wild-type animals, sciatic nerve injury led to a neuropathic pain syndrome characterized by the presence of mechanical and thermal allodynia, as well as thermal hyperalgesia.
- A significant decrease of the mechanical allodynia and a suppression of thermal hyperalgesia and allodynia were observed in A_{2A}R deficient mice.
- The expression of microglia and astrocytes was enhanced in wild-type mice exposed to sciatic nerve injury and this response was attenuated in knockout animals.

Conclusion:

Our results demonstrate the involvement of A_{2A}Rs in the control of neuropathic pain and propose this receptor as an interesting target for the development of new drugs for the management of this clinical syndrome.

Bura SA, Nadal X, Ledent C, Maldonado R, Valverde O. A2A adenosine receptor regulates glia proliferation and pain after peripheral nerve injury. Pain. 2008; 140(1): 95-103.

ARTICLE #4

A new operant model in mice to evaluate the therapeutic potential of novel compounds for neuropathic pain

S.Andreea Bura, Thomas Guegan, Daniel Zamanillo, José Miguel Vela and Rafael Maldonado

Manuscript in preparation to be sent to *Pain*

Objectives:

To validate a new operant model of drug self-administration in mice exposed to neuropathic pain that can be used to evaluate the therapeutic potential of novel compounds for neuropathic pain.

Main results:

- Neuropathic pain was significantly reduced in animals exposed to partial sciatic nerve ligation self-administering the sigma antagonist S1RA at both doses tested (3 and 6 mg/kg/infusion).
- Both sham-operated and partial sciatic nerve ligated mice acquired a stable operant responding behaviour to obtain S1RA at the dose of 3 mg/kg/infusion. However, only partial sciatic nerve ligated mice acquired the operant responding to obtain the highest dose of S1RA (6 mg/kg/infusion).
- Mice exposed to partial sciatic nerve ligation showed an anhedonic state revealed by a decreased consumption of palatable drink that was significantly attenuated by S1RA chronic administration (25 mg/kg) twice daily during 10 days.

Conclusion:

The present findings reveal the analgesic efficacy of the new sigma receptor antagonist, S1RA, in neuropathic pain. This effect was associated to an improvement of the emotional negative consequences of chronic pain. S1RA administered at the highest dose was devoid of abuse liability. The operant responses evaluated in this animal model can have a high predictive value to estimate the clinical benefit/risk ratio of new analgesic compounds to treat chronic pain, such as S1RA.

A new operant model in mice to evaluate the therapeutic potential of novel compounds for neuropathic pain

S.Andreea Bura[1], Thomas Guegan[1], Daniel Zamanillo[2], José Miguel Vela[2] and Rafael Maldonado[1]

[1]Laboratori de Neurofarmacologia, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, C/Doctor Aiguader 80, 08003 Barcelona, Spain.

[2]Laboratorios Dr. Esteve S.A. Av. Mare de Déu de Montserrat, 221 08041 Barcelona, Spain

[#] To whom correspondence should be addressed: Dr. Rafael Maldonado, Laboratori de Neurofarmacologia, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, PRBB Barcelona, Spain

Dr. Aiguader 88, 08003 Barcelona, Spain;

rafael.maldonado@upf.edu

Telephone: +34 933160824, fax: +34 933160901

Abstract

The treatment of neuropathic pain is unsatisfactory at the present moment due to the side effects and/or insufficient efficacy of the currently available drugs. The aim of this study was to validate a new operant model of drug self-administration in mice exposed to neuropathic pain that can be used to evaluate the therapeutic potential of novel compounds for neuropathic pain. First, chronic pain was developed in mice by a partial ligation of the sciatic nerve. Once that chronic pain had reached a steady-state, mice were trained to maintain an operant behaviour to self-administer a new analgesic compound, the sigma receptor antagonist S1RA (3 and 6 mg/kg/infusion), which has been previously reported to alleviate neuropathic pain manifestations. The possible abuse liability of the analgesic compound was identified by evaluating the self-administration behaviour in sham-operated mice. The anhedonic state related to chronic pain and the influence of S1RA treatment on this emotional response was also evaluated by measuring the preference for palatable drink (2 % sucrose solution) using a new highly sensitive behavioural device (Phecomp food and drink monitoring system). Both sham-operated and partial sciatic nerve ligated mice acquired a stable operant responding to obtain S1RA at 3 mg/kg/infusion, althought only partial sciatic nerve ligated mice acquired the operant responding to obtain the highest dose of S1RA (6 mg/kk/infusion). After 10 days of training on the drug self-administration paradigm, neuropathic pain was significantly reduced in animals exposed to partial sciatic nerve ligation receiving S1RA at both doses (3 and 6 mg/kg/infusion). In addition, mice exposed to partial sciatic nerve ligation showed an anhedonic state revealed by a decreased consumption of palatable drink that was significantly attenuated by S1RA (25 mg/kg). Our results reveal the analgesic efficacy of the new sigma receptor antagonist, S1RA, in neuropathic pain. This effect was associated to an improvement of the

emotional negative consequences of chronic pain. S1RA administered at the highest dose was devoid of reinforcing effects. The operant responses evaluated in this animal model can have a high predictive value to estimate the clinical benefit/risk ratio of new analgesic compounds to treat chronic pain, such as S1RA.

Key words: sigma receptor, S1RA, self-administration, anhedonia, allodynia, hyperalgesia

1. Introduction

Neuropathic pain is defined by the International Association for the Study of Pain (IASP) as pain initiated or caused by a primary lesion or dysfunction in the nervous system that is often associated with hyperalgesia, allodynia, spontaneous pain and emotional alteration. Several compounds are currently used to treat neuropathic pain [2;31]. However, these compounds have a limited efficacy and present side effects that can limit their use. One of the most important side effects is the potential development of abuse liability and hypolocomotion that represent serious limitation for the clinical use of some drugs. The relationships between treatment of chronic pain and addiction are largely recognise [1;14]. There are certain regions of the brain, such as the nucleus accumbens and the anterior cingulated gyrus that are involved in both pain and addiction. It has been suggested that there may be a shared neural system for mediaing both aversive and rewarding stimuli [4]. Moreover, both chronic pain and addiction involve sensitization and synaptic plasticity which alter the responses of the nerve circuits to sensory inputs, including the painful stimuli [16]. At present, the most reliable technique to evaluate the relationships between chronic pain and addiction in preclinical research is the operant drug self-administration paradigm. Studies in rats, showed that chronic pain altered drug self-administration [10;17] and in animals exposed to neuropathic pain, heroin and methadone were more effective in maintaining self-administration when administered at analgesic doses, whereas lower doses of these two opioids were similarly self-administered in both nerve-injury and control rats [21]. In spite of these data, there is no animal model with high predictive value to estimate the clinical benefit/risk ratio of new analgesic compounds to treat chronic pain. Thus, nowadays severe pain treatment remains an open issue to deal with and there is an urgent need for more effective drugs and new animal models with high predictive value

to evaluate the analgesic and side effects of new compounds. A possible animal model developed in mice could open new perspectives given the possibility to identify specific genes involved in the relationships between pain and addiction thanks to the new lines of genetically modified mice now available.

In the last years, a special attention has received the sigma 1 receptor (σ1R) and its implication in pain modulation. σ1Rs are expressed in key areas for pain control such as the superficial layers of the dorsal horn, periaqueductal gray matter, locus coeruleus and rostroventral medulla [11]. Both, genetical studies employing σ1R knockout mice and pharmacological blockade of these receptors revealed decrease behavioural manifestation of neuropathic pain in a partial sciatic nerve ligation (PSNL) model. Furthermore, σ1Rs exert a modulatory role on the NMDA receptors, a key receptor involved in central sensitization present in chronic pain. These data indicate that σ1R play an important function in the development and maintenance of neuropathic pain and in the processes underling this pathology.

The aim of this study was to validate a new operant model of drug self-administration in mice exposed to neuropathic pain that can be used to evaluate the therapeutic potential of novel compounds for neuropathic pain. Chronic pain was developed in mice by a PSNL. Once that chronic pain had reached a steady-state, mice were trained to maintain an operant behaviour to self-administer a new analgesic compound, the sigma receptor antagonist S1RA (3 and 6 mg/kg/infusion), which has been previously reported to alleviate neuropathic pain manifestations. The possible abuse potential of the analgesic compound was identified by evaluating the self-administration behaviour in sham-operated mice. The anhedonic state related to chronic pain and the influence of S1RA treatment in this emotional response was also evaluated by measuring the preference for palatable drink (2 % sucrose solution) using a new highly sensitive behavioural device.

2. Materials and methods

2.1 Animals

Experiments were performed in C57BL/6 male mice (Charles River, France) weighting 22–24 g at the beginning of the experiments. Mice were housed individually in a temperature (21 ± 1°C) and humidity-controlled (55 ± 10%) room. Mice were tested during the dark phase of a 12 h light/dark reverse cycle (light off at 08:00 AM, light on at 8:00 PM). Food and water were available *ad libitum* except during the training for the food maintained operant behaviour. In this sequence of the study animals had a restricted diet. Mice were isolated in individual cages, and habituated to their new environment and handled for 1 week, before starting the experimental procedure. The observer was blind to treatment in all the experiments. Animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the local ethical committee (CEEA-IMAS-UPF).

2.2 Drugs

The sigma 1 receptor antagonist, S1RA, (Laboratorios Dr. Esteve, Barcelona, Spain) was dissolved in physiological sterile saline solution (0.9%) and administered intravenously (i.v.) in the self-administration paradigm and intraperitonealy (i.p.) in the behavioural paradigm used to evaluate anhedonia.

2.3 Operant model to evaluate the therapeutic potential in neuropathic pain

For the self-administration paradigm, mice were first trained to acquire an operant behaviour to obtain food. In a second step, mice were operated for the partial sciatic nerve injury and then intravenous catheters were implanted in order to train the animals to acquire an operant responding maintained by drug self-administration. The nociceptive behavioural tests were performed as indicated in Figure 1.

2.3.1 Acquisition of an operant responding to obtain food

After 7 days of habituation, mice were food deprived during 3 days until they reached 85% of their initial weight. The same food deprivation regime was maintained during the whole period of evaluation of food-operant behaviour. Water was available *ad libitum* during the whole experiment. Three days after starting food deprivation, mice were trained in operant chambers to nose-poke for food pellets as previously reported [3;27]. The experimental chambers contain two manipulanda (holes of 1.2 cm diameter), one was selected as active hole for delivering the reinforcer and the other as inactive hole. Nose-poking on the active hole resulted in a reinforcer (food pellet), while nose-poking on the inactive hole had no consequences. A stimulus light, located above the active hole, was paired contingently with the delivery of the reinforcer. Mice were trained during 10 days under a FR1 schedule of reinforcement, 1 nose-poke results 1 food pellet delivery. A 10 sec time-out period was established after each reinforcement. During this 10 sec period the cue light was off and no reward was provided. Responses on the inactive hole and all the responses during the 10 sec time-out period were also recorded. The session was finished after 100 reinforces were delivered or after 1 hour whichever occurred first. After the last session of food-self-administration, food and water were available *ad libitum*.

2.3.2 Partial sciatic nerve ligation

Next day after the last session of food self-administration, animals were habituated for the neuropathic pain experiments, 2 hours to each different experimental test, (Von-Frey and plantar test). The following day, basal values were measured and then the partial ligation of the sciatic nerve at mid-thigh level was used to induce neuropathic pain, as previously described [7;18]. Briefly, mice were anaesthetized with isofluorane (induction, 5%; surgery, 2%) and the common sciatic nerve was exposed at the level of

the mid-thigh of the right hind paw. At ~1 cm proximally to the nerve trifurcation, a tight ligature was created around 33–50% of the sciatic nerve using 9–0 18-inch non-absorbable virgin silk suture (Alcon® surgical, Texas, USA), leaving the rest of the nerve 'undamaged'. The muscle was then stitched, and the incision was closed with wound clips. Control animals (sham-operated mice), underwent the same surgical procedure except that the sciatic nerve was not ligated.

2.3.3 Nociceptive behavioural tests

Hyperalgesia to noxious thermal stimulus and allodynia to cold and mechanical stimuli were used as outcome measures of neuropathic pain. The behavioural manifestations of neuropathic pain were evaluated the day before and 3 days after the PSNL, as well as the day before and 11 days after drug self-administration, by using the following behavioural models.

2.3.3.1 Plantar test

Thermal hyperalgesia was assessed in the plantar test (Ugo Basile, Varese, Italy), by measuring paw withdrawal latency in response to radiant heat as previously reported [7;15]. A cut-off time of 20 s was used to prevent tissue damage in the absence of a response. The mean paw withdrawal latencies for the ipsilateral and contralateral hind paws were determined from the average of three separate trials, taken at 5 min intervals to prevent thermal sensitization and behavioural disturbances.

2.3.3.2 Von Frey paradigm

Mechanical allodynia was quantified by measuring the hind paw withdrawal response to von Frey filament stimulation, as previously reported [7;8]. The filament of 0.4 g was first used. 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 the Basbaum's laboratory (UCSF, San Francisco, USA). Clear paw withdrawal, shaking or licking was considered as a nociceptive-like response. Both ipsilateral and contralateral hind paws were tested.

2.3.3.3 Cold plate test

Thermal allodynia to a cold stimulus was assessed by using the hot/cold-plate analgesia meter (Colombus, OH, USA), as previously described [5;7]. The number of elevations of each hind paw was recorded in the mice exposed to the cold plate (5 ± 0.5 C) during 5 min. A score was calculated for each animal as the difference of number of elevations between ipsilateral and contralateral paw.

2.3.4 Acquisition of drug self-administration

Three days after sciatic nerve surgery and following the evaluation of the behavioural manifestation of neuropathic pain, mice were implanted with indwelling intravenous silastic catheter, as previously reported [27]. Briefly, a 6-cm length of silastic tubing (0.3 mm inner diameter, 0.6 mm outer diameter) (Silastic®, Dow Corning, Houdeng-Goegnies, Belgium) was fitted to a 22-gauge steel cannula (Semat, Herts, UK) that was bent at a right angle and then embedded in a cement disk (Dentalon Plus, Heraeus Kulzer, Wehrheim, Germany) with an underlying nylon mesh. The catheter tubing was inserted 1.3 cm into the right jugular vein and anchored with suture. The remaining tubing ran subcutaneously to the cannula, which exits at the midscapular region. All incisions were sutured and coated with antibiotic ointment (Bactroban, GlaxoSmithKline, Madrid, Spain). Food and water were available *ad libitum* during this experimental phase. Drug self-administration sessions were conducted for 10 consecutives days as describe above for food-maintained responding, except that responses were maintained by drug delivery in a volume of 23.5 µl over 2 sec. Stable acquisition of self-administration behaviour was achieved when mice followed all the next

criteria for at least three consecutive sessions: (1) less than 20% of deviation from the mean of the total number of responses in active hole (80% of stability), (2) 85% of discrimination between holes, (3) a minimum of four infusions per session. After 10 days of drug self-administration, mice were tested on a progressive ratio (PR) schedule for the dose they were trained on. In this paradigm, the requirement to earn an injection escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000. The breaking point, defined as the last ratio completed before self-administration behaviour extinguished in a 2-h session, was determined for each animal once. Only the values of mice that reached the acquisition criteria were considered. After PR session neuropathic pain manifestations were measured and the patency of i.v. catheters was evaluated by infusion of 0.1 ml of tiobarbital (Figure 1).

2.4 Anhedonia model

Mice were individualized and habituated in a room with a reversed light/dark cycle with food and water *ad libitum*. The anhedonic state related with chronic pain and the effects of S1RA treatment on the emotional response were evaluated using a new food and drink monitoring system that we have recently developed in the laboratory in collaboration with Harvard Instruments (http://www. panlab.com/ panlabWeb/ Hardware/ php/ display Hard.php? campo=Metabolism&nameHard =PHECOMP) that allows to evaluate with an extremely high sensitivity (less than 0.02 g for both food and drink) the preference for a palatable food or/and drink. In this study, the anhedonic state was evaluated by measuring the preference for sucrose solution 2% as a palatable drink. After one week of habituation to the reversed light/dark cycle, mice were subjected to four 4 h session every second day during 1 week in the monitoring boxes in order to be familiarised with the new environment and drink taste. After each session, mice were replaced in their home cage. After this habituation period, baseline values of drink

intake were measured. Thus, mice were deprived for 2 h before starting the 4 h session in the monitoring box. One day after the baseline measurement, mice were exposed to partial sciatic nerve ligation or sham-operation and were then subjected every second day to the 2 h deprivation followed by the 4 h session in the monitoring box during 16 days. The chronic treatment with S1RA started 7 days after PSNL. Mice were injected twice daily with S1RA (25 mg/kg i.p) or saline during 10 days. Animals received the first day injection 30 min before starting the 4 h session in the monitoring box and the second one immediately before the light period of the cycle started (Figure 7).

2.9 Statistical analysis

Data obtained in the plantar test, cold plate test and von Frey filament paradigm were compared each experimental day by using two-way ANOVA (surgery and treatment as between factors of variation), followed by Newman Keuls *post hoc* comparisons when required. The same statistical analysis was used for the data obtained the last day of drug self-administration, the breaking-point values obtained following the PR schedule and the mean of 10 days of sucrose preference on the anhedonia model. Data obtained in food self-administration paradigm were analysed using three-way ANOVA with repeated measures (surgery and treatment as between-subjects factors and day as within-subjects factor of variation). Pearson's χ^2 test was used to compare the percentage of sham-operated and PSNL mice that acquired criteria at the different doses tested. The differences between means were considered statistically significant when the p value was below 0.05. SPSS statistical package was used.

3. Results

3.1 Food self-administration

Three-way ANOVA, calculated for number of nose-pokes for the active and inactive hole, showed significant main effects of day ($F_{(9, 459)} = 23.599$, $p < 0.001$; and $F_{(9,468)} = 23.599$, $p < 0.001$ respectively), no effect of surgery ($F_{(1, 51)} = 2.643$, N.S.; and $F_{(1, 52)} = 2.403$, N.S. respectively) or treatment ($F_{(1, 51)} = 3.022$, N.S.; and $F_{(1, 52)} = 0.666$, N.S. respectively) and no interaction among these factors ($F_{(9, 459)} = 0.378$, N.S.; and $F_{(9,468)} = 1.814$, N.S. respectively). The factors surgery and treatment were introduced in order to demonstrate that all groups were homogenous at the beginning of the drug self-administration training (data not shown)

3.2 Self-administration of sigma receptor 1 antagonist is increased in animals with PSNL

On day 10 of drug self-administration, both sham-operated and PSNL mice showed an increased in the number of operant responses to obtain S1RA at the dose of 3 mg/kg/infusion when compared with vehicle control groups. However, the number of operant responses to obtain S1RA at the dose of 6 mg/kg/infusion was significantly higher in PSNL mice when compared to sham-operated mice trained to obtain the same treatment. Indeed, two-way ANOVA (treatment and surgery as between-subjects factors of variation) revealed a significant effect of treatment ($F_{(1,57)} = 5.358$, $p < 0.01$) and surgery ($F_{(1,57)} = 4.036$, $p < 0.05$) but no interaction between these two factors ($F_{(1,57)} = 1.812$, N.S.). *Post hoc* Newman Keuls analysis underlined that the number of responses of animals self-administering S1RA in a dose of 3 mg/kg/infusion was significantly higher in both, sham ($p < 0.01$) and PSNL ($p < 0.5$) animals when compared with their corresponding saline controls. However, the number of operant responses to obtain S1RA at the dose of 6 mg/kg/infusion was significantly higher in PSNL mice when

compared to sham-operated mice trained to obtain the same treatment ($p < 0.05$, Newman Keuls *post hoc* analysis) (Figure 2A).

No differences were observed in responses in the inactive hole. Thus, two-way ANOVA revealed no significant effect of treatment ($F_{(1,57)} = 2{,}735$, N.S.), or surgery ($F_{(1,57)} = 0.657$, N.S.), nor interaction between these two factors ($F_{(1,57)} = 0.096$, N.S.) (Figure 2B).

No differences were revealed in data obtained in PR. Thus, two-way showed no effect of treatment ($F_{(5,10)} = 0.270$, N.S.) or surgery ($F_{(5,10)} = 3.033$, N.S.), and no interaction between these two factors ($F_{(5,10)} = 2.890$, N.S.).

Pearson's χ^2 test was used to compare the percentage of sham-operated and PSNL mice that reached the acquisition criteria at the different doses tested. χ^2 showed that there were no differences in the percentage of mice that reached the acquisition criteria in sham-operated group when compared with saline ($\chi^2 = 0.778$, N.S. for the dose of 3 mg/kg/infusion and $\chi^2 = 2.800$, N.S. for the dose of 6 mg/kg/infusion). The same analysis revealed significant differences in the percentage of mice that reached the acquisition criteria in the PSNL animals self-administering the S1RA, at both doses tested, when compared with saline group ($\chi^2 = 11.344$, $p < 0.001$ for the dose of 3 mg/kg/infusion and $\chi^2 = 7.500$, $p < 0.01$ for the dose of 6 mg/kg/infusion).

3.3 Sigma receptor 1 antagonist decreased the neuropathic pain manifestation after intravenous self-administration.

3.3.1 Mechanical allodynia was significantly decreased after S1RA self-administration

Sciatic nerve injury led to a profound decrease of the threshold for evoking withdrawal of the hind ipsilateral paw to a mechanical stimulus and this response was significantly attenuated in animals after self-administering S1RA at both doses tested (Figure 3). Baseline values were similar in all the animal groups, as revealed by two-way ANOVA calculated for ipsilateral paw (Table 1). Nerve injury led to a significant decrease of the threshold for evoking hind paw withdrawal to mechanical stimulation on the injured side, as revealed by two-way ANOVA (Table 1). The threshold for evoking withdrawal at the ipsilateral paw to a mechanical stimulus was significant on day 3 ($p < 0.001$, for all groups), day 6 ($p < 0.001$, for all groups) and day 17 ($p < 0.001$ for saline group, $p < 0.01$ for 3 mg/kg/infusion group and $p < 0.05$ for 6 mg/kg/infusion group) after surgery (*post hoc* Newmann Keuls). However, a significant decrease of mechanical allodynia was observed on day 17 after PSNL in mice self-administering S1RA in both doses, ($p < 0.001$ for 6 mg/kg/infusion and $p < 0.05$ for 3mg/kg/infusion) when compared to the saline group (*post hoc* Newmann Keuls). Withdrawal latencies of the contralateral paw were not modified in any experimental group during the whole experiment (Table 1).

3.3.2 Significantly reduced of hyperalgesia after S1RA self-administration

Sciatic nerve ligature decreased ipsilateral paw withdrawal latency to thermal stimulus and this response was significantly attenuated in animals after self-administering S1RA at both doses tested (Figure 4). Baseline values calculated for ipsilateral paw were similar in all the animal groups as revealed by two-way ANOVA (Table 1). A marked

and long-lasting decrease of the paw withdrawal latencies was observed in the ipsilateral paw of all mice exposed to sciatic nerve injury as showed by two-way ANOVA (Table 1). Paw withdrawal latencies for ipsilateral side were significant on day 3 ($p < 0.001$) and day 6 ($p < 0.001$) after surgery (*post hoc* Newmann Keuls). On day 17, *post hoc* analysis, revealed a decrease in paw withdrawal latency to thermal stimulus only in mice self-administering saline ($p < 0.01$) when compared with sham-operated animals, and this response was abolished in mice receiving the S1RA (N.S.). Moreover, a decrease of this response was found in mice self-administering S1RA at both doses ($p < 0.05$) when compared with saline group (*post hoc* Newman Keuls).

Withdrawal latencies of the contralateral paw were not modified in any of the experimental groups (two-way ANOVA with treatment and surgery as between factors of variation) (Table 1).

3.3.3 Thermal allodynia was significantly reduced in mice self-administering S1RA

Sciatic nerve ligature enhanced the score values (see materials and methods) during the cold thermal stimulation as revealed by two-way ANOVA (table 1). A significant difference of the score values was displayed in animals with PSNL on day 3 ($p < 0.05$ in mice receiving saline and 3 mg/kg/infusion of S1RA, $p < 0.001$ in mice receiving 6 mg/kg/infusion of S1RA) and day 6 ($p < 0.001$ in mice receiving S1RA 3 mg/kg/infusion, $p < 0.01$ in mice receiving saline and $p < 0.05$ in mice receiving S1RA 6 mg/kg/infusion) when compared with sham-operated animals (*post hoc* Newmann Keuls). On day 17 a significant increase in score values of mice exposed to sciatic nerve injury receiving saline ($p < 0.01$) was observed when compared with sham-operated animals (*post hoc* Newmann Keuls). This response was reduced in mice self-administering S1RA at both doses (3 mg/kg/infusion and also 6 mg/kg/infusion, $p < 0.05$) when compared with mice receiving saline (*post hoc* Newman Keuls). Baseline

score values were similar in all these groups as showed by two-way ANOVA (Table 1) (Figure 5).

3.4 The S1RA significantly improved the anhedonic state related with chronic pain

Neuropathic pain induced an anhedonic like state in animals treated with saline that were previously exposed to PSNL. This anhedonia was evaluated by measuring the sucrose preference in a free choice paradigm. Non - contingent administration of the σR1 antagonist improved this emotional deficit. Indeed, two-way ANOVA (treatment and surgery) of the mean of sucrose preference calculated for the all treatment period (10 days), revealed a significant effect of surgery ($F_{(1,44)} = 6.025$, $p < 0.05$) and treatment ($F_{(1,44)} = 7.813$, $p < 0.01$), and significant interaction between these two factors ($F_{(1,44)} = 6.025$, $p = 0.056$). Subsequent *post hoc* Newmann Keuls indicated that animals exposed to nerve injury receiving saline displayed a lower preference for sucrose ($p < 0.01$) when compared with sham-operated animals. This emotional deficit disappeared in mice receiving S1RA exposed to neuropathic pain. Indeed, both, mice previously exposed to nerve injury and sham-operated that received S1RA at a dose of 25 mg/kg (twice daily during 10 days), showed a higher preference for sucrose ($p < 0.05$ for both, sham and mice exposed to PSNL) when compared with their corresponding saline group (Newman Keuls *post hoc*) (Figure 6)

4.Discussion

In this study, we succeed to validate a new operant model of drug self-administration in mice exposed to neuropathic pain that will permit to evaluate the analgesic and some of the most relevant side effects of these compounds, such as the possible abuse liability and the potential effects upon the locomotor activity. In order to set up this model, a new analgesic compound, the σR1 antagonist, S1RA, was used. Furthermore, the possible effect of this compound on the emotional consequences of neuropathic pain was also evaluated.

In clinical practice, antiepileptic and antidepressants drugs are considered the first-choice treatment of neuropathic pain. At present, the gabapentinoids gabapentine and pregabalin, and the serotonin norepinephrine reuptake inhibitors are dominating the field [12;13]. One of the most important side effects that can limit the use of pharmacological compounds to treat neuropathic pain is the potential abuse liability. In addition, hypolocomotion represents a serious limitation for the clinical use of some of these new drugs. Both potential side effects of the novel therapeutic agents can be easily evaluated in an operant model of contingent self-administration of analgesic compounds. The relationships between the treatment of chronic pain and addiction is largely recognise and these relationships are difficult to be explored in the currently available behavioural models[1;14]. Indeed, the presence of pain and the motivation to alleviate it seems to minimize the possible development of addictive behaviours with an appropriate opioid treatment protocol [20]. In this sense, the rate of abuse of opioid analgesics in pain patients is relatively moderate [9]. However, other data have shown an increased prevalence of drug abuse in patients receiving opioids for chronic pain [19]. Several animal studies also suggest that chronic pain attenuates the rewarding properties of opioids. Thus, inflammatory [30] and neuropathic pain [23] reduced

morphine-induced conditioned place preference in rodents. Certain areas of the brain, such as the nucleus accumbens and the anterior cingulated gyrus are involved in both pain and addictive processes [4]. The relationships between chronic pain and addiction could be explored in the operant drug self-administration paradigm that we have set-up in our study by comparing the response of mice exposed to neuropathic pain and those of sham-operated animals

In the last years, a special attention has received the σ1R for its implication in pain modulation. Indeed, σ1Rs are expressed in key areas for pain control, such as the superficial layers of the dorsal horn, periaqueductal gray matter, locus coeruleus and rostroventral medulla[11]. Studies using both σ1R knockout mice and pharmacological blockade of these receptors revealed that these interventions decrease the behavioural manifestations of neuropathic pain in a partial sciatic nerve ligation model [11]. Furthermore, σ1Rs exert a modulatory role on the NMDA receptors, a key receptor involved in the central sensitization developed during neuropathic pain [11]. These data indicate that σ1Rs play an important role in the neurobiological mechanisms leading to the development and maintenance of neuropathic pain.

In this study, mice exposed to neuropathic pain were then trained to acquire an operant behaviour to self-administer intravenously the analgesic compound in order to set-up the new operant model of contingent self-administration of analgesic compounds. First, the animals were trained to acquire a food-maintained operant behaviour. Chronic pain was then developed in mice by partial sciatic nerve ligation. Once that chronic pain had reached a steady-state, mice were trained to maintain an operant behaviour to self-administer a new analgesic compound, the σ1R antagonist S1RA, which has been previously reported to alleviate neuropathic pain manifestations. The possible abuse potential of this new analgesic compound was identified by evaluating the self-

administration behaviour in sham-operated mice. Two doses of this sigma antagonist, 3 and 6 mg/kg/infusion, were used in the intravenously self-administration paradigm. The sciatic nerve ligation led to a neuropathic pain syndrome characterised by mechanical and thermal allodynia, and thermal hyperalgesia that were present from the first day of measurement and were maintained during the whole experimental sequence. We showed that the σR1 antagonist, S1RA, decreased the behavioural manifestations of neuropathic pain after intravenous self-administration at both doses tested. Indeed, after 10 days of S1RA self-administration, animals with partial sciatic nerve ligation showed a reduction of thermal and mechanical allodynia and thermal hyperalgesia. These results are in accordance with the previous studies revealing the role of σR1 in the manifestations of neuropathic pain [25;26]. In these previous studies, thermal hyperalgesia was not modified after the decrease of σR1 activity. However, neuropathic pain model used in the pharmacological study was the chronic constriction injury that is different from the one used in the present work. One important difference between these two models is that the manifestations of neuropathic pain after the partial sciatic nerve ligation are dependent on the activity of the sympathetic nervous system since the simpathectomy reverts these pain manifestations [18], in contrast to the chronic constriction injury model. The difference between the results obtained in the present study and σR1 knockout mice could also be due to the possibility of adaptive compensatory changes in the genetic model. In addition, the mouse is self-administering itself the σR1 antagonist in the present study, whereas in the previous studies the drug was administered in a non-contingent way. Studies using a yoking procedure have demonstrated that the neurobiological and neurochemical consequences of drug intake can differ depending on whether the animal self-administers the drug or if the drug is passively administered [20]. In this sense, drug self-administration, unlike non-

contingent infusions, merge many additional features of the drug different from its analgesic properties, including reinforcing effects and the consequent motivation to seek for the drug. Our operant model allows the animal exposed to the chronic neuropathic pain to seek for drug delivery in order to alleviate the pain, but also permits to evaluate the possible reinforcing effects of the drug by comparing the operant responses between sham-operated and animals exposed to the partial sciatic nerve ligation.

Before acquiring the intravenous drug self-administration, mice were previously trained in the same operant paradigm to seek for food. Therefore, the response of the animals during the first days of drug self-administration animals is influenced by the previous acquisition of the food maintained operant behaviour. Consequently, differences in self-administration of the different doses of S1RA reached the significance on the last day of training. Interestingly, mice that developed neuropathic pain maintained an operant behaviour to self-administer the σR1 antagonist compound at 3 and 6 mg/kg/infusion. The self-medication with this analgesic alleviates neuropathic pain, as revealed by the significant attenuation of both hyperalgesia and allodynia on the last day of training to self-administer S1RA at both doses tested. In contrast, control mice exposed to sham surgery did not maintain such an operant behaviour to self-administer the sigma ligand at the dose of 6 mg/kg/infusion, whereas a moderate self-administration was observed at the dose of 3 mg/kg/infusion. These results clearly reveal that mice exposed to chronic neuropathic pain maintained an operant behaviour to self-administer an elevated dose of the sigma ligand in order to alleviate pain. However, a lower dose of the same sigma ligand was also self-administered by mice not exposed to neuropathic pain, revealing the reinforcing effects of this compound. The progressive ratio schedule of reinforcement, design as a measure of motivation was performed in the last day of intravenous drug self-administration and did not reveal significant differences between

the different animal groups. However, interesting results were found when the percentages of acquisition were compared. Thus, no differences in percentage of acquisition were found within the sham-operated group, whereas within the sciatic nerve ligated group, this percentage was higher in animals that self-administered both doses of S1RA when compared with mice self-administering saline. These results revealed that sham-operated animals display the same incentive to seek for the sigma antagonist compound than saline, whereas this incentive to seek the antagonist was enhanced in mice exposed to neuropathic pain.

The results obtained in our experiment were not due to a modification in the locomotor activity or any physical alteration that would prevent the mice from nose-poking or from maintaining a sufficient activity to obtain drug infusion. Indeed, no differences in the number of inactive hole responses were seen between the different animal groups exposed or not to S1RA.

Chronic pain is often associated to several emotional consequences that impair the quality of life of these patients and difficult the therapeutic approach. Indeed, depressive-like symptoms are often present in patients suffering chronic pain, [6], which aggravates the consequences and manifestations of pain [22]. Because depression and chronic pain are often associated, an appropriate treatment of both emotional components of the chronic pain and painful symptoms may improve the beneficial effects in these patients [6]. In a second experiment, we evaluated the consequence of non-contingent S1RA administration on the anhedonic state induced by the sciatic nerve ligation. This emotional feature was assessed by measuring the preference for sucrose solution in the Phecomp food and drink monitoring system. It has been previously described that the consumption and the preference for highly palatable sweet solutions are decreased during an anhedonic state [24;28;29]. In accordance, we found that

anhedonia induced by the exposure to neuropathic pain diminishes the sucrose intake. In addition, non-contingent administration of S1RA improved this emotional response. Indeed, animals exposed to partial sciatic nerve ligation receiving S1RA displayed the same preference for sucrose solution as sham-operated mice. These data show that S1RA is able to improve both physical and emotional manifestations of neuropathic pain pointing the high potential interest of this new compound for the treatment this pathology.

Our results revealed the analgesic efficacy of a new σR1 antagonist, S1RA, in the treatment of neuropathic pain. This analgesic effect was observed together with an improvement of the emotional consequences associated to the presence of chronic pain. S1RA administered at the highest dose was devoid of reinforcing effects. The operant responses evaluated in this animal model have a high predictive value to estimate the clinical benefit/risk ratio of new analgesic compounds to treat chronic pain, such as S1RA.

Acknowledgements

This work was supported by the CENIT2006 program from the Spanish Ministry of Industry (Project GENIUS PHARMA), the Spanish Ministry of Science and Innovation (#SAF2007-64062), the Catalan Government (SGR2009-00131), the ICREA Foundation (ICREA Academia-2008) and the DG Research of the European Commission (GENADDICT, #LSHM-CT-2004-05166; and PHECOMP, #LSHM-CT-2007-037669).

[1] Ballantyne JC, LaForge KS: Opioid dependence and addiction during opioid treatment of chronic pain. Pain 2007;129:235-255.
[2] Banos JE, Sanchez G, Berrendero F, Maldonado R: Neuropathic pain: some clues for future drug treatments. Mini Rev Med Chem 2003;3:719-727.
[3] Barbano MF, Castane A, Martin-Garcia E, Maldonado R: Delta-9-tetrahydrocannabinol enhances food reinforcement in a mouse operant conflict test. Psychopharmacology (Berl) 2009;205:475-487.
[4] Becerra L, Breiter HC, Wise R, Gonzalez RG, Borsook D: Reward circuitry activation by noxious thermal stimuli. Neuron 2001;32:927-946.
[5] 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.
[6] Campbell JN, Basbaum AI, Dray A, Dubner R, Dworkin RH, Sang NC: Emerging strategies for the treatment of neuropathic pain. Seattle, IASP Press, 2006.
[7] Castane A, Celerier E, Martin M, Ledent C, Parmentier M, Maldonado R, Valverde O: Development and expression of neuropathic pain in CB1 knockout mice. Neuropharmacology 2006;50:111-122.
[8] 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.
[9] Cicero TJ, Inciardi JA, Munoz A: Trends in abuse of Oxycontin and other opioid analgesics in the United States: 2002-2004. J Pain 2005;6:662-672.
[10] 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;91:33-45.
[11] Diaz JL, Zamanillo D, Corbera J, Baeyens JM, Maldonado R, Pericas MA, Vela JM, Torrens A: Selective sigma-1 (sigma1) receptor antagonists: emerging target for the treatment of neuropathic pain. Cent Nerv Syst Agents Med Chem 2009;9:172-183.
[12] Dworkin RH, O'Connor AB, Backonja M, Farrar JT, Finnerup NB, Jensen TS, Kalso EA, Loeser JD, Miaskowski C, Nurmikko TJ, Portenoy RK, Rice AS, Stacey BR, Treede RD, Turk DC, Wallace MS: Pharmacologic management of neuropathic pain: evidence-based recommendations. Pain 2007;132:237-251.
[13] Finnerup NB, Otto M, McQuay HJ, Jensen TS, Sindrup SH: Algorithm for neuropathic pain treatment: an evidence based proposal. Pain 2005;118:289-305.
[14] Flugsrud-Breckenridge MR, Gevirtz C, Paul D, Gould HJ, III: Medications of abuse in pain management. Curr Opin Anaesthesiol 2007;20:319-324.
[15] 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.
[16] Ji RR, Kohno T, Moore KA, Woolf CJ: Central sensitization and LTP: do pain and memory share similar mechanisms? Trends Neurosci 2003;26:696-705.

[17] Lyness WH, Smith FL, Heavner JE, Iacono CU, Garvin RD: Morphine self-administration in the rat during adjuvant-induced arthritis. Life Sci 1989;45:2217-2224.
[18] Malmberg AB, Basbaum AI: Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. Pain 1998;76:215-222.
[19] Manchikanti L: National drug control policy and prescription drug abuse: facts and fallacies. Pain Physician 2007;10:399-424.
[20] Martin TJ, Ewan E: Chronic pain alters drug self-administration: implications for addiction and pain mechanisms. Exp Clin Psychopharmacol 2008;16:357-366.
[21] Martin TJ, Kim SA, Buechler NL, Porreca F, Eisenach JC: Opioid self-administration in the nerve-injured rat: relevance of antiallodynic effects to drug consumption and effects of intrathecal analgesics. Anesthesiology 2007;106:312-322.
[22] Ong KS, Keng SB: The biological, social, and psychological relationship between depression and chronic pain. Cranio 2003;21:286-294.
[23] Ozaki S, Narita M, Narita M, Iino M, Sugita J, Matsumura Y, Suzuki T: 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. J Neurochem 2002;82:1192-1198.
[24] Papp M, Willner P, Muscat R: An animal model of anhedonia: attenuation of sucrose consumption and place preference conditioning by chronic unpredictable mild stress. Psychopharmacology (Berl) 1991;104:255-259.
[25] Puente BD, Nadal X, Portillo-Salido E, Sanchez-Arroyos R, Ovalle S, Palacios G, Muro A, Romero L, Entrena JM, Baeyens JM, Lopez-Garcia JA, Maldonado R, Zamanillo D, Vela JM: Sigma-1 receptors regulate activity-induced spinal sensitization and neuropathic pain after peripheral nerve injury. Pain 2009.
[26] 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;109:879-889.
[27] Soria G, Mendizabal V, Tourino C, Robledo P, Ledent C, Parmentier M, Maldonado R, Valverde O: Lack of CB1 cannabinoid receptor impairs cocaine self-administration. Neuropsychopharmacology 2005;30:1670-1680.
[28] Strekalova T, Spanagel R, Bartsch D, Henn FA, Gass P: Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration. Neuropsychopharmacology 2004;29:2007-2017.
[29] Strekalova T, Steinbusch HW: Measuring behavior in mice with chronic stress depression paradigm. Prog Neuropsychopharmacol Biol Psychiatry 2010;34:348-361.
[30] Suzuki T, Kishimoto Y, Ozaki S, Narita M: Mechanism of opioid dependence and interaction between opioid receptors. Eur J Pain 2001;5 Suppl A:63-5.:63-65.
[31] Woolf CJ, Mannion RJ: Neuropathic pain: aetiology, symptoms, mechanisms, and management. Lancet 1999;353:1959-1964.

FIGURE LEGENDS

Figure 1. Experimental schedule for the self-medication model

Figure 2. Self-administration on day 10. Data are expressed as mean ± SEM of nose – pokes in active (A) and inactive (B) holes n = 8 -15 mice per experimental group.

★ $P < 0.05$, ★★ $P < 0.01$ mice receiving S1RA vs saline group (*post hoc* Newman Keuls). ☆ $P < 0.05$ mice exposed to sciatic nerve injury vs. sham–operated animals (*post hoc* Newman Keuls).

Figure 3. Development of mechanical allodynia in the ipsilateral paw in S1RA treated mice and their saline group control after sciatic nerve injury. Mechanical allodynia was evaluated by using the von Frey model. The behavioural responses were determined under basal conditions and on day 3, 6, and 17 after sciatic nerve surgery. Data are expressed as mean ± SEM in mice exposed to sciatic nerve injury (black) and sham-operated mice (white) receiving saline (rhombus) S1RA in a dose of 3 mg/kg/infusion (squares) and S1RA in a dose of 6 mg/kg/infusion (triangles); n = 8-15 animals per experimental group ★ $P < 0.05$, ★★ $P < 0.01$, ★★★ $P < 0.001$ PSNL vs. sham-operated (*post hoc* Newman Keuls). ☆ $P < 0.05$, ☆☆☆ $P < 0.001$ (S1RA treated mice vs. saline) (*post hoc* Newman Keuls).

Figure 4. Development of thermal hyperalgesia in the ipsilateral paw in S1RA treated mice and their saline group control after sciatic nerve injury. Thermal hyperalgesia was evaluated by using the plantar test. The behavioural responses were determined under basal conditions and on day 3, 6, and 17 after sciatic nerve surgery. Data are expressed as mean ± SEM in mice exposed to sciatic nerve injury (black) and sham - operated mice (white) receiving saline (rhombus), S1RA in a dose of 3 mg/kg/infusion (squares) and S1RA in a dose of 6 mg/kg/infusion (triangles); n = 8-15 animals per experimental

group ★★ P < 0.01, ★★★ P < 0.001 PSNL vs. sham-operated (*post hoc* Newman Keuls). ☆ P < 0.05, S1RA treated mice vs. saline (*post hoc* Newman Keuls).

Figure 5. Development of thermal allodynia in the ipsilateral paw in S1RA treated mice and their saline group control after sciatic nerve injury. Thermal allodynia was evaluated in the cold-plate test (score). The behavioural responses were determined under basal conditions and on day 3, 6, and 17 after sciatic nerve surgery. Data are expressed as mean ± SEM in mice exposed to sciatic nerve injury (black) and sham - operated mice (white) receiving saline (rhombus), S1RA in a dose of 3 mg/kg/infusion (squares) and S1RA in a dose of 6 mg/kg/infusion (triangles); n = 8-15 animals per experimental group ★ P < 0.05, ★★ P < 0.01, ★★★ P < 0.001 PSNL vs. sham-operated (*post hoc* Newman Keuls). ☆ P < 0.05, S1RA treated mice vs. saline (*post hoc* Newman Keuls).

Figure 6. Experimental schedule for the anhedonia model

Figure 7 Sucrose preference**.** Data are expressed as mean ± SEM of sucrose preference calculated for the treatment period (10 days). ☆P < 0.05 (sham operated vs sciatic nerve injury in saline group) (*post hoc* Newman Keuls). ★ P < 0.05 saline vs. S1RA, 25 mg/kg, sciatic nerve injury group (Newman Keuls *post hoc* analysis).

TABLE 1. The effect of intravenous self=administration of S1RA on the mantainance of the neuropathic pain mainifestations

Parameter	Baseline		Day 3		Day 6		Day 17	
	F-value (1, 57)	P <	F-value (1, 57)	P <	F-value (1, 57)	P<	F-value (1, 57)	P <
von Frey test								
Ipsilateral paw								
Surgery	0.135	N.S.	72.507	0.001	72.507	0.001	53.620	0.001
treatment	0.396	N.S.	2.077	N.S.	0.190	N.S.	2.401	N:S:
Interaccion	0.906	N.S.	1.728	N.S.	0.170	N.S.	5.854	0.01
Controlateral paw								
Surgery	1.419	N.S.	0.166	N.S.	0.483	N.S.	2.875	N.S.
treatment	1.383	N.S.	3.005	N.S.	0.992	N.S.	1.014	N.S.
Interaccion	1.953	N.S.	0.325	N.S.	1.119	N.S.	0.730	N.S.
Plantar test								
Ipsilateral paw								
Surgery	1.820	N.S.	85.156	0.001	73.223	0.001	0.633	N.S.
treatment	0.517	N.S.	0.878	N.S.	2.393	N.S.	3.117	N.S.
Interaccion	0.437	N.S.	0.957	N.S.	1.591	N.S.	4.328	0.05
Contralateral paw								
Surgery	0.669	N.S.	1.411	N.S.	1.846	N.S.	0.057	N.S.
treatment	0.805	N.S.	0.825	N.S.	2.434	N.S.	1.795	N.S.
Interaccion	0.659	N.S.	0.683	N.S.	0.322	N.S.	0.300	N.S.
Cold plate test (score)								
Surgery	0.048	N.S.	26.968	0.001	26.395	0.001	7.473	0.01
treatment	2.451	N.S.	0.651	N.S.	1.267	N.S	0.104	N.S.
Interaccion	0.294	N.S.	0.839	N.S.	2.005	N.S.	6.373	0.01

Figure 1

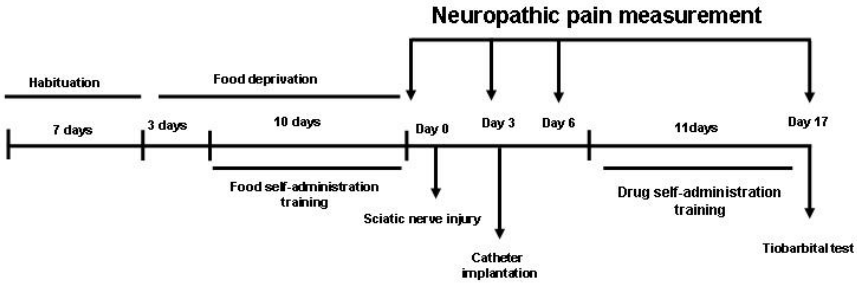


Figure 2A

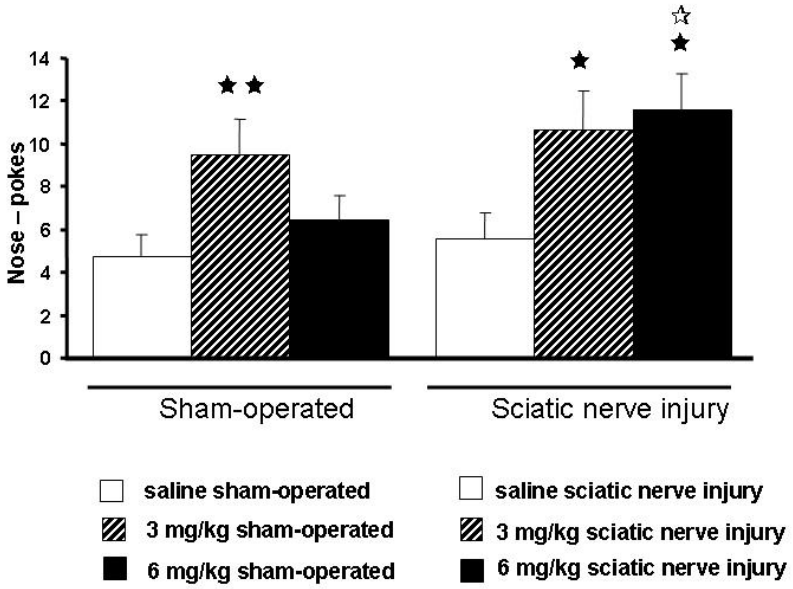


Figure 2B

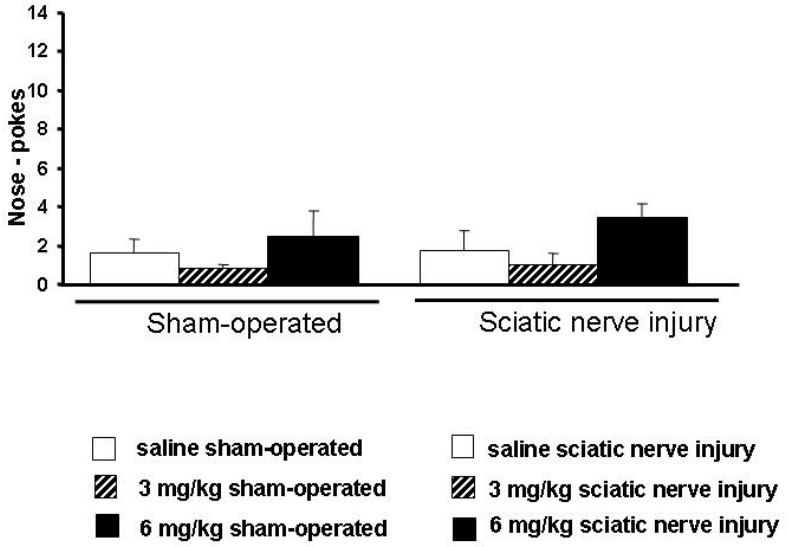


Figure 3

Ipsilateral paw

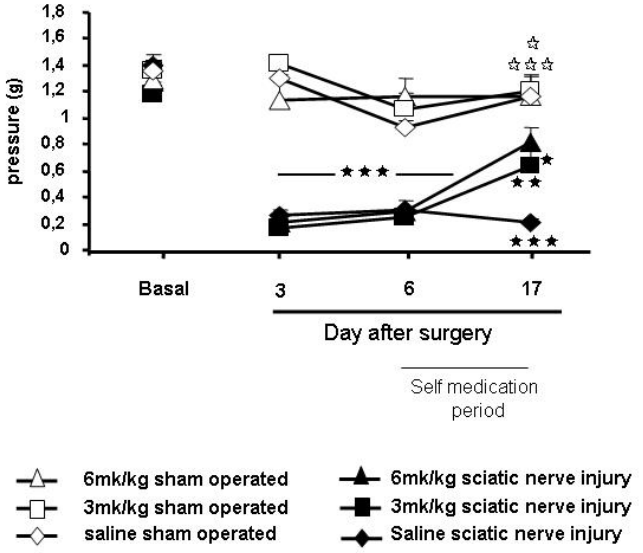


Figure 4

Ipsilateral paw

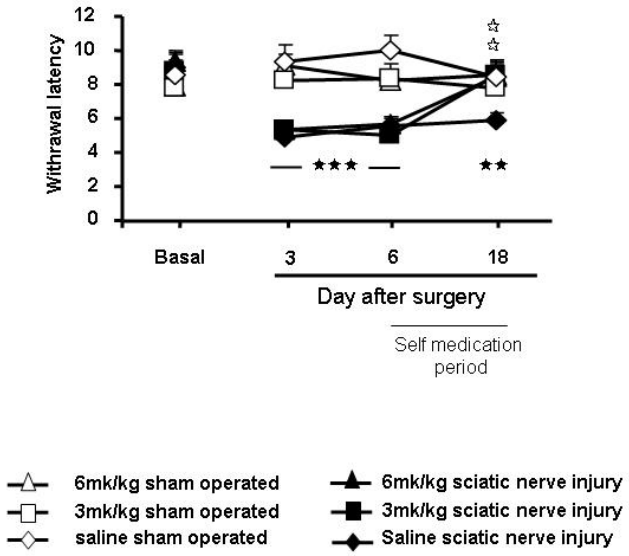


Figure 5

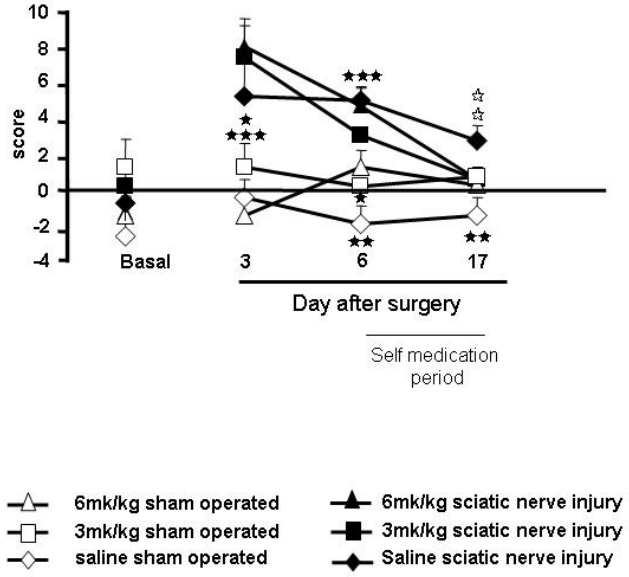


Figure 6

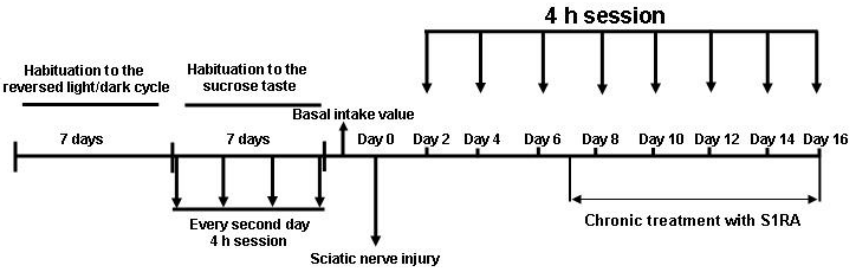
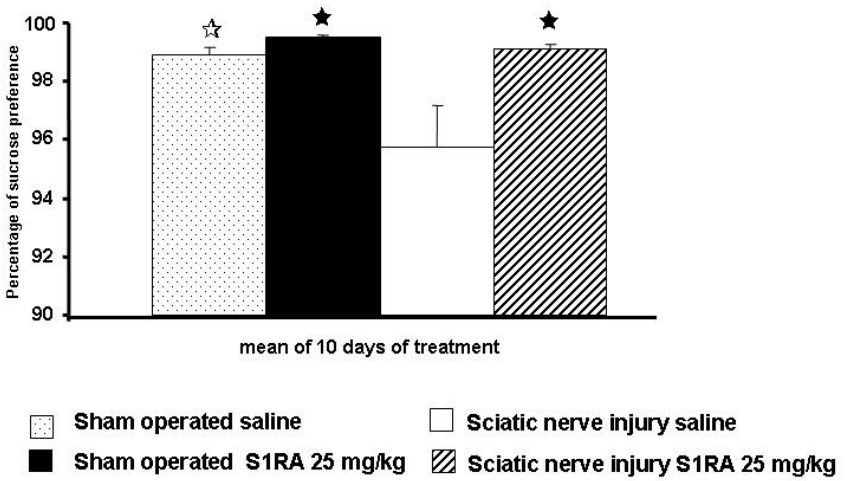


Figure 7



DISCUSSION

Involvement of the endocannabinoid system in nicotine responses

Nicotine and cannabinoids are among the most widely consumed drugs of abuse in humans and they are frequently use in combination. Both types of drugs share multiple common pharmacological properties including antinociception, hypothermia, impairment of locomotion, rewarding properties and physical dependence (Cook *et al.* 1998; Hildebrand *et al.* 1999; Hutcheson *et al.* 1998; Valjent and Maldonado 2000). In addition they have other biological actions in which show opposite effects, like the effects on memory and metabolism. Nicotine, the primary addictive substance in tobacco, exerts its effects through the activation of the nAChR, while THC the main psychoactive compound of *cannabis sativa*, acts through the cannabinoid receptors: CB_1 receptor mainly located in the CNS (Kano *et al.* 2009; Tsou *et al.* 1998) and CB_2 receptor abundant in the immune cells (Kano *et al.* 2009; Munro *et al.* 1993). The behavioural and biochemical consequences of the interaction between the cannabinoid and cholinergic systems are still poorly documented in spite of the current association of cannabis and tobacco consumption in humans. Indeed, few studies have reported this interaction in experimental models. In mice, nicotine has been shown to facilitate hypothermia, antinociception, hypolocomotion and anxiolytic-like responses induced by THC (Valjent *et al.* 2002), whereas THC decreased somatic and motivational manifestations of nicotine withdrawal (Balerio *et al.* 2004). On the other hand, rimonabant abolishes nicotine-induced anxiolytic-like effects and increased the anxiogenic-like responses of nicotine (Balerio *et al.* 2006). However, there are no studies focused on the interaction between the endocannabinoid and cholinergic system in memory and metabolism. For these reasons, the first objective of this thesis was to study in depth the interaction of these two systems in memory and metabolism.

Interactions between the endocannabinoid and cholinergic systems in cognitive processes (article 1)

Pharmacological and genetic approaches were used to investigate the possible interactions between the cannabinoid and cholinergic systems in cognitive processes in different behavioural paradigms. For this purpose, the effects induced by nicotine, physostigmine and scopolamine were studied in CB_1 knockout mice and wild-type littermates in the active avoidance paradigm. In addition, the effects of the pretreatment with rimonabant were evaluated on the pharmacological responses induced by nicotine in the active avoidance and the object recognition tasks in wild-type mice.

We showed that the effects of nicotine and physosotigmine were attenuated in the absence of CB_1 receptor activity, but the pharmacological responses of scopolamine were unchanged in the absence of this receptor. Thus, nicotine (0.5 mg/kg) did not modify the performance of CB_1 knockout mice and wild-type littermates in the active avoidance paradigm. Nevertheless, nicotine enhanced the performance of wild-type mice in the two trial object recognition task, but this cognitive response did not reach the significance when nicotine was combined with rimonabant, although the recognition index values were still high compared to saline. The different responses induced by nicotine in the two memory models could be due to the distinct neurobiological substrate and cognitive responses evoked in these behavioural tests. Thus, the active avoidance paradigm is a complex model in which other behavioural responses different from the cognitive processes, such as anxiety, play an important role in the trial performance. Several structures different from the hippocampus, that are involved in cognitive and emotional responses such as the prefrontal cortex and amygdala, participate also in the

responses obtained in this behavioural paradigm (Holland and Bouton 1999; LeDoux 2000). In contrast, the object recognition test is considered a rather pure working memory task (Ennaceur and Delacour 1988) in which the hippocampus plays a key role. The cholinergic innervation of hippocampus by neurons in the medial septal area has been reported to be critical for optimal memory performance in this model (Levin and Rezvani 2000). Moreover, we obtained in this study different results in the active avoidance paradigm with regards to the procedure used to block CB_1 receptor activity. Thus, a clear increase in the performance of CB_1 knockout mice was observed in this test. However, the CB_1 antagonist rimonabant administered at a large range of doses (from 0.3 to 10 $mg \cdot kg^{-1}$), did not modify the performance in the active avoidance test when given alone or co-administered with nicotine. In agreement with these results, rimonabant did not produce any cognitive effect in the object recognition test. In most of the previous studies where rimonabant improved the cognitive performance, this antagonist was administered after the original encounter with the cognitive paradigm (Terranova *et al.* 1996), which was not the case of the experimental conditions used in our study. Indeed, rimonabant was administered before the evaluation of the cognitive response in the active avoidance paradigm and the two trial recognition test. Another explanation for the difference between the results obtained with rimonabant and CB_1 knockout mice could be the possibility of adaptive compensation in the genetic model. Besides, the lack of effect of rimonabant in these behavioural paradigms could be due to the particular biodistribution of rimonabant, which presents a preferential biodisponibility at the peripheral tissues rather than at the CNS level (Despres *et al.* 2005).

The effects of the cholinergic antagonist scopolamine and the acetylcholinesterase inhibitor physostigmine on learning and memory were also evaluated in CB_1 knockout mice. Numerous pharmacological

studies have demonstrated that scopolamine impairs learning in different behavioural tasks (Fibiger *et al.* 1991; Gallagher and Colombo 1995; Zarrindast *et al.* 2002) and this impairment is directly related to a decrease in central cholinergic activity. In agreement, this study reveals an impairment in the active avoidance performance after scopolamine administration in both wild-type and CB_1 knockout mice, demonstrating that the amnesic effects of scopolamine are not mediated through the CB_1 receptor. On the other hand, physostigmine increased the active avoidance performance in wild-type mice in agreement with previous studies (Sansone *et al.* 1993), but did not modify the performance in CB_1 knockout mice. An enhanced ACh release (Kathmann *et al.* 2001) and improved long-term potentiation in the hippocampus (Bohme *et al.* 2000) have been reported in mice lacking CB_1 receptor, which are in part responsible for their improved memory function. Therefore, the cognitive responses mediated by the enhancement of ACh activity induced by physostigmine could be impaired in the mutant mice that already show an enhanced ACh release. Thus, it seems that a precise concentration of this neurotransmitter seems to be required at the synaptic level to improve memory and learning processes.

Our results demonstrate that the effects of nicotine and physosotigmine are attenuated in the absence of CB_1 receptor activity. However, scopolamine effects are independent from CB_1 receptor activity. The cognitive responses induced by rimonabant in the active avoidance paradigm were different to those observed in CB_1 knockout mice.

Interaction between nicotine and the endocannabinoid system in food intake and metabolism (article 2)

We investigated the consequences of chronic nicotine administration and withdrawal on food intake and metabolic parameters in CB_1 knockout mice as well as in their wild-type littermates in order to study the interaction between nicotine and the endocannabinoid system in these pharmacological responses. Anxiety-like behaviour was also evaluated in both genotypes before, during and after nicotine administration. Animals were chronically treated with nicotine at a dose that has been reported to develop physical dependence manifestations (Berrendero et al. 2005) with spontaneous withdrawal (Damaj *et al.* 2003). The effects of nicotine on the motivation for specific foods with different caloric and palatable value were evaluated before, during and after nicotine chronic treatment in CB_1 knockout animals and wild-type littermates using a new food and drink monitoring system with extremely high sensitivity that was developed in the laboratory in collaboration with the company Panlab SA (http//www .panlab.com /panlabWeb /Hardware/php/ displayHard.php? campo= Metabolism &nameHard=PHECOMP). The levels of cholesterol, insulin and glucose were also evaluated in these animals. Animals were exposed to a free-choice feeding paradigm consisting in offering two kinds of food (standard chow and high fat diet) and two kinds of beverage (water and saccharine solution) in order to assess the influence of CB_1 deletion and/or nicotine administration on feeding intake motivation. Control groups were exposed only to standard chow under similar experimental conditions. Wild-type and CB_1 knockout mice consumed almost exclusively high fat food (93.3 % and 90.9 % respectively) and saccharine solution (94.2 % and 83.5 % respectively) under basal conditions. Therefore, this free choice regimen resulted in a high fat feeding with a progressive gain of

body weight. No differences in metabolic parameters were observed between genotypes in animals fed with standard diet. After two weeks of free choice high fat diet, wild-type animals showed an enhancement in the levels of cholesterol, insulin and glucose compared with CB_1 knockout mice. These protective effects of CB_1 receptor deletion over metabolic unbalance have also been reported in previous studies using CB_1 knockout mice fed with high fat diet (Ravinet *et al.* 2003; Ravinet *et al.* 2004). In these previous studies, mutant mice did not develop insulin resistance on a high-fat diet and had a higher leptin sensitivity than wild-type mice, while maintaining similar levels of energy intake (Ravinet *et al.* 2004). In addition, previous studies have reported that the CB_1 antagonist rimonabant reduced leptin, insulin, glucose, triglycerides and low-density lipoprotein cholesterol levels in a model of diet-induced obese mice (Poirier *et al.* 2005; Ravinet *et al.* 2003). A very recent study using combined genetic and pharmacological approaches in mice showed that the control of glutamatergic transmission by CB_1 receptors is responsible, at least in part, for the well-known orexigenic role of the endocannabinoid system (Bellocchio *et al.* 2010).

In our study, chronic nicotine administration reduced body weight and decreased glucose levels in wild-type mice. Different results have been previously reported about the effects of nicotine on metabolic parameters. Several studies showed that nicotine increased cholesterol and glucose levels in mice fed with normal food (Lamota *et al.* 2008). In contrast, nicotine improved various metabolic parameters in animal models of obesity or diabetes. Thus, nicotine reduced hyperglycemia and the incidence of diabetes symptoms in mice exposed to streptozotocin (Mabley *et al.* 2002). Chronic nicotine administration also reduced blood glucose levels in obese rats by repressing gluconeogenesis and hepatic glycogen content, which results in a decreased hepatic glucose release (Liu *et al.* 2003). In humans, smoking was associated with a lower risk of

metabolic syndrome in a large cross-sectional study (Bernaards *et al.* 2005). All these data together suggest that nicotine impairs metabolic parameters in physiological conditions, but decreases glucose levels in overweight situations in spite of the well-known deleterious effects of nicotine on the overall cardiovascular risk (Bullen 2008). Interestingly, the decreased body weight and glucose levels induced by nicotine in wild-type mice exposed to high fat diet were not observed in CB_1 knockout animals. One of the mechanisms underling the decrease in body weight induced by nicotine is the activation of lipolysis in the adipocytes (Andersson and Arner 2001) and the repression of appetite by reducing in the hypothalamus orexigenic mediators (Chen *et al.* 2007). CB_1 mutant mice have altered expression of hypothalamic neuropeptides and impaired adipocyte function (Cota 2008), which may explain the lack of nicotine effect on body weight in these mutants. The fact that CB_1 knockout mice do not present metabolic unbalance after the hypercaloric diet and do not show body weight reduction after nicotine treatment, could explain the absence of nicotine effect on glucose levels in these mutants. The absence of nicotine effect in CB_1 knockout mice could also be explained by a floor effect considering the reduced glucose levels found under basal conditions in these mutant mice. These differences in glucose levels disappeared after withdrawal of nicotine treatment.

No differences between genotypes were revealed under basal conditions in the preference for high fat diet, although mutant mice showed lower preference for saccharine solution than wild-type animals. This result suggests that CB_1 receptor has a stronger effect on feeding motivational signals than on energy signals that are both crucial for the regulation of food intake. Our data are in agreement with previous literature showing that the disruption of CB_1 gene or the administration of rimonabant reduced the reinforcing effects of sweets, but not fat foods (Thornton-Jones *et al.* 2005; Ward and Dykstra 2005). In the same line, THC

enhanced food reinforcement predominantly for high palatable pellets with a moderate effect on high caloric pellets in a mouse operant conflict test (Barbano *et al.* 2009). Interestingly, the differences in saccharine preference between genotypes were revealed in basal conditions and after nicotine withdrawal, but disappeared during the nicotine treatment. This was due to the tendency of nicotine to reverse the decrease in the saccharine preference shown by CB_1 knockout mice, which suggests an improvement in the motivation of the mutants for this high palatable drink. In agreement, nicotine has been shown to improve motivational responses in both humans and animals with signs of anhedonia (Cook *et al.* 2007; Spring *et al.* 2008; Tizabi *et al.* 1999), and several manifestations of anhedonia have been widely reported in CB_1 knockout mice (Martin *et al.* 2002).

The endocannabinoid system has been involved in the effects produced by nicotine on anxiety like-responses (Balerio *et al.* 2004; Balerio *et al.* 2006). The possible interaction between chronic nicotine administration and the endocannabinoid system in anxiety-like behaviour was also evaluated in our study. CB_1 deficient mice showed an increase in anxiety-like behaviour under basal conditions when compared with wild-type animals, in agreement with previous pharmacological and genetic studies (Balerio *et al.* 2004; Balerio *et al.* 2006; Martin *et al.* 2002; Viveros *et al.* 2006). Several mechanisms have been involved in these anxiety-like responses. First, the endocannabinoid system plays a key role in the modulation of the hypothalamus-pituitary-adrenal axis (Rodriguez de Fonseca *et al.* 1991) and the deletion of CB_1 receptor increases the expression of corticotrophin releasing hormone and adrenocorticotrophin levels (Di Marzo and Matias 2005). Another important substrate for the behavioural phenotype of CB_1 knockout mice is the alteration reported on the functional activity of the serotonergic system (Aso *et al.* 2009). These changes in hypothalamus-pituitary-adrenal axis function and serotonergic

activity would promote stress and would decrease food intake (Cota 2008; Di Marzo and Matias 2005; Inui 1999). Therefore, the changes on these neurochemical systems could be involved in the anxiety-like phenotype of CB_1 mutant mice in basal conditions and could also contribute to the decrease food intake. Interestingly, nicotine increased anxiety-like behaviour only in wild-type mice, but did not modify this behavioural response in knockout animals. Nicotine exerts a stimulatory effect on corticotrophin releasing hormone and adrenocorticotrophin secretion that seems to be involved in its effects on anxiety-like behaviour (Mano-Otagiri *et al.* 2009; Weidenfeld *et al.* 1989). Thus, the stimulatory effects of nicotine on the hypothalamus-pituitary-adrenal axis could be absent in CB_1 mutant mice because these animals already present enhanced levels of corticotrophin-releasing factor and adrenocorticotrophin, which could explain the suppression of nicotine effects on anxiety.

The present findings confirm that the deleterious effects of the high fat diet on glucose, insulin and cholesterol levels were prevented in CB_1 knockout mice. Interestingly, nicotine reduced body weight and glucose levels, and induced anxiogenic-like effects in wild-type, but not in CB_1 knockout animals. The mutant mice also showed lower preference for high palatable drink in the absence of nicotine treatment. These results provide a new evidence of the important role played by the endocannabinoid system in the pharmacological responses of nicotine that will be useful to better understand the interactions between nicotine and cannabinoid compounds.

New targets for the treatment of neuropathic pain

The endocannabinoid system plays a crucial role in the control of the nociceptive responses. Indeed, several studies have shown that the activation of the cannabinoid system inhibits the transmission of

nociceptive stimuli acting at peripheral, spinal and supraspinal levels, modulates the integration of these nociceptive signals in several brain areas and stimulates the activity of the inhibitory descending pathway (Hohmann 2002). Indeed, one of the earliest and widely known uses of cannabis was to treat pain. The antinociceptive properties of cannabinoid compounds have been demonstrated in both acute and chronic pain animal models. Thus, cannabinoid agonists were proved to be efficient in both the hot plate and tail flick test (Gomez *et al.* 2002; Hohmann 2002; Lichtman and Martin 1991; Martin and Lichtman 1998; Tham *et al.* 2005), in mechanical models that measure motor (Smith *et al.* 1994) or reflex (Gilbert 1981) responses, chemical models such as the writhing response induced by acetic acid or administration of fenilbenzoquinone and (Ulugol *et al.* 2006; Welch *et al.* 1995) and also models of electric stimulation of paw (Weissman *et al.* 1982), sciatic nerve (Bicher and Mechoulam 1968) or dental pulp (Kaymakcalan *et al.* 1974).

In addition, cannabis has been shown to produce antinociceptive effects in several experimental models of chronic pain. Thus cannabinoid agonists reduced inflammatory pain such as the hyperalgesia induced by carrageenan (Mazzari *et al.* 1996), capsaicin (Li *et al.* 1999), formalin (Calignano *et al.* 1998; Moss and Johnson 1980) and Freud's adjuvant (Martin *et al.* 1999). Moreover, one of the most interesting potential application of the cannabinoid agonists would be the treatment of neuropathic pain. Indeed, several recent studies have shown that the cannabinoids were also efficient in models of neuropathic pain (Goya *et al.* 2003). An upregulation of spinal CB_1 receptor was observed following chronic constriction of sciatic nerve in rats, which enhanced the analgesic effects of Win 55,212-2 in this neuropathic pain model (Lim *et al.* 2003). By contrast, a genetic study using CB_1 knockout mice has shown that CB_1 cannabinoid receptors are not critically involved in the development of neuropathic pain nor in the anti-allodynic and anti-hyperalgesic effects of

gabapentin in a model of neuropathic pain induced by partial sciatic nerve ligation (Castañé *et al.* 2006). CB_2 cannabinoid receptors have also been involved in this pathological state. Indeed, the selective CB_2 cannabinoid agonist AM1241 produced a dose-dependent inhibition of tactile and thermal hypersensitivity induced in rats by spinal nerve ligation (Ibrahim *et al.* 2003). The crucial role of CB_2 receptor in the regulation of central immune responses during neuropathic pain was recently demonstrated using mice lacking CB_2 receptor, transgenic mice overexpressing this receptor and bone marrow chimera mice (Castañé *et al.* 2006; Racz *et al.* 2008b; Racz *et al.* 2008a). Thus, CB_2 knockout mice and mice reconstituted with CB_2 deficient bone marrow cells exposed to nerve injury developed similar neuropathic pain in the ipsilateral side as wild-type animals. However, they showed a contralateral mirror-image of pain accompanied by glial activation. In contrast, neuropathic pain was attenuated in transgenic mice overexpressing CB_2 receptors (Racz *et al.* 2008b; Racz *et al.* 2008a). Therefore, CB_2 cannabinoid receptors are crucial for the development of neuropathic pain and the CB_2 agonists could represent a future new group of pharmacological agents for the treatment of neuropathic pain devoid of any psychoactive side effects (Racz *et al.* 2008b; Racz *et al.* 2008a).

In spite of these promising results the treatment of neuropathic pain is often unsatisfactory due to the side effects and/or insufficient efficacy of the currently available drugs. For these reasons, the development of new drugs and new animal models able to predict the clinical benefit/risk ratio of novel analgesic compounds to treat neuropathic pain represents a major research priority in order to improve the management of this chronic disease. Therefore, the second main objective of this thesis was to identify new targets for the treatment of neuropathic pain and to develop new animal models that could help to anticipate the possible abuse liability of new analgesic compounds.

A_{2A} adenosine receptor as a new target for the treatment of neuropathic pain (article 3)

The purinergic system maintains a close anatomical (Herkenham *et al.* 1991; Svenningsson *et al.* 1999) and functional (Fredholm and Svenningsson 2003; Piomelli 2003) relationship with the endocannabinoid system, particulary with the CB_1 receptors, and it plays an important role in the control of pain. The purine nucleoside adenosine is an ubiquitous endogenous neurotransmitter (Dunwiddie and Masino 2001) which acts on four G-protein coupled receptors, named A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R (Fredholm *et al.* 2001). The endogenous ligand of these receptors, adenosine, can enhance or decrease nociception depending on the receptor subtype activated (Sawynok 1998). Genetic and pharmacological studies have shown that the stimulation of $A_{2A}R$ is pronociceptive while the activation of A_1R is thought to have opposite effects (Ferre *et al.* 2007). Indeed, mice lacking $A_{2A}R$ exhibit an increased nociceptive threshold after direct stimulation of peripheral sensory nerves or an inflammatory reaction (Godfrey *et al.* 2006; Ledent *et al.* 1999). Pharmacological studies, using $A_{2A}R$ ligands also demonstrated their role in the control of inflammatory pain (Godfrey *et al.* 2006; Hussey *et al.* 2007). However, few data are available on the involvement of the $A_{2A}R$ in neuropathic pain. To study this issue, we have used knockout mice deficient in $A_{2A}R$ (Ledent *et al.* 1999) and we have evaluated the consequences of this deletion in the development and expression of neuropathic pain after sciatic nerve injury. Hyperalgesia, mechanical and thermal allodynia were used as an outcome behavioural manifestation of neuropathic pain. We have also investigated in these mutant mice the expression of microglia and astrocytes that has been demonstrated to modulate neuronal changes occurring during neuropathic pain. A significant decrease in mechanical

allodynia and the abolishment of thermal hyperalgesia and thermal allodynia were revealed in $A_{2A}R$ knockout mice after the sciatic nerve injury during the whole experimental sequence, which indicates that this receptor participates both in the expression and development of neuropathic pain. A significant attenuation in the microglia and astrocyte expression was also revealed at different time points in the $A_{2A}R$ mutant animals after sciatic nerve injury. Thus, a significant increase in microglia expression was observed 3 days after sciatic nerve injury in the ipsilateral side in both genotypes. However on this day, the astrocyte activation appears only in wild-type mice. An important activation of both microglia and astrocyte expression was revealed 7 and 17 days after nerve injury in wild-type animals but not in mutant mice.

Nerve injury has been reported to promote activation of glial cells in the spinal cord, and activated glia may contribute to the initiation and maintenance of neuropathic pain (Clarke *et al.* 2008; Sawynok and Liu 2003; Watkins *et al.* 2001). Microglia responds quickly to peripheral nerve injury and releases several cytokines that promote neuron sensitization in the spinal cord (DeLeo and Yezierski 2001). In addition, different studies have demonstrated that spinal microglial activation precedes astrocyte activation (Colburn *et al.* 1999b; Tanga *et al.* 2004) and this astrocyte proliferation is more closely related to the maintenance of pain behaviour in different neuropathic pain models (Colburn *et al.* 1999a; Tanga *et al.* 2004). Both, microglia and astrocytes express $A_{2A}R$ (Hasko *et al.* 2005) and the secretor activity of microglia cells appears to be stimulated by $A_{2A}R$ (DeLeo and Yezierski 2001; Heese *et al.* 1997). $A_{2A}R$ stimulation enhances the proliferation and activation of astrocytes that occur as a consequence of a nerve injury (Brambilla *et al.* 2003; Hindley *et al.* 1994). An increase in the extracellular levels of adenosine has been revealed after nerve injury (Sawynok and Liu 2003). Under these pathological conditions, adenosine is released from a variety of cells types

such as neurons, neutrophils, mast cells, or fibroblasts and it comes also from the ATP, as a result of its dephosphorylation by ecto-50-nucleotidase (Sawynok and Liu 2003). One hypothesis to explain the absence of neuropathic pain manifestations in $A_{2A}R$ knockout mice could be that the adenosine released after nerve injury would act on the $A_{2A}Rs$ expressed by microglia and astrocytes generating the activation of these cells, which are responsible for the neuroinflamatory process occurring during neuropathic pain. A recent study published after our article showed that neuropathic pain was decreased in a chronic constriction injury model in rats after a single intrathecal injection of ATL313, an adenosine $A_{2A}R$ agonist. The attenuation of these behavioural manifestations was also associated to a decrease of microglia and astrocyte activation (Loram *et al.* 2009). These authors suggest that the glial activation reported in our study was attenuated in knockout mice as a consequence of the reduction in peripheral $A_{2A}R$ stimulation that is known to be pronociceptive (Taiwo and Levine, 1990; Doak and Sawynok, 1995; Khasar et al., 1995). In contrast, the authors propose that the glial activation was attenuated in their study by direct action within the spinal cord or, more likely, by the diffusion of cerebrospinal fluid derived IL-10 that has been reported to suppress glial activation. Indeed, intrathecal $A_{2A}R$ agonist administration induces the accumulation of IL-10 in cerebrospinal fluid, suggesting that this would contribute to both suppression of spinally mediated neuropathic pain and glial activation (Loram *et al.* 2009)

Contradictory results were also found in studies using $A_{2A}R$ agonists and antagonists. Indeed, peripheral administration of both $A_{2A}R$ agonist (ATL313) and antagonist (ZM241385) was able to improve pain manifestation in mice exposed to spinal cord injury. In contrast, the $A_{2A}R$ antagonist intrathecaly administered reverted the effects of ATL313, but did not improved neuropathic pain (Li *et al.* 2006). However, given the limited selectivity of these compounds, the pharmacological experiments

do not rule out the possibility that some of the effects of ATL313 or ZM241385 may be mediated by activation or inhibition of other adenosine receptors subtypes different from $A_{2A}R$ (Li *et al.* 2006). It seems that the protective effects of activation/blockade of $A_{2A}R$ depend on the animal model used and the route of administration. Moreover, the analgesic effects of the activation or blockade of $A_{2A}R$ depends also on the level of adenosine. Thus, positive effects of $A_{2A}R$ blockade likely occurs in a setting where local endogenous adenosine levels are high, while the protective effect of A_{2A} agonists likely occurs at a time preceding the release of large amounts of endogenous adenosine from the injured tissue. It may be possible to exploit the differences in the kinetics of these $A_{2A}R$-mediated responses to optimize spinal cord protection by sequential application of agonists and antagonists (Li *et al.* 2006).

The involvement of $A_{2A}R$ receptors in the control of pain is not limited to the periphery or the spinal cord. In the brain, the $A_{2A}Rs$ are found predominantly in the dorsal and ventral striatum, that are not directly involved in pain regulation (Moreau and Huber 1999). However, $A_{2A}Rs$ are also expressed with lower density in other brain regions, such as amygdala, thalamus and hypothalamus (Moreau and Huber 1999) that play a relevant role in the transmission and integration of nociceptive stimuli. Moreover, $A_{2A}R$ activation in the brain enhances the release of glutamate from glial cells (Moreau and Huber 1999; Nishizaki *et al.* 2002). Excessive accumulation of glutamate can lead to synaptic deregulation, which could also participate in the central sensitization produced during neuropathic pain. Therefore, the potential role of $A_{2A}Rs$ in the central integration of pain cannot be ignored.

In summary, our results provide new evidence to support the crucial role of $A_{2A}Rs$ in neuropathic pain modulation and reinforce the notion that $A_{2A}Rs$ could be an interesting target for the development of new drugs for the management of the clinical manifestations of this pathology.

Sigma receptors as a new target for neuropathic pain treatment; Evaluation of the effect of the selective sigma-1 receptor antagonist S1RA in neuropathic pain using a new operant model (article 4)

The existing animal models of neuropathic pain allow the study of the manifestations of this pathology and the efficacy of new potential pharmacological treatment. However the different compounds are always administered in these currently available models in a non contingent manner, which could limit the evaluation of their efficacy. Therefore, it would be of great interest to develop new animal models in which the animal could administer itself the analgesic compounds in a contingent manner. The potential results obtained in these contingent models would have a better extrapolation to the clinical human situation. In addition, these models would also permit the evaluation of a wide range of behavioural responses, which would provide important information to predict the clinical benefit/risk ratio of new analgesic compounds to treat chronic pain. Therefore, the next objective of the thesis was to set up a new operant model of contingent self administration of analgesic compounds in mice to evaluate the therapeutic potential of novel compounds for neuropathic pain.

One of the most important side effects that can limit the use of novel pharmacological compounds to treat neuropathic pain is the potential abuse liability. In addition, hypolocomotion represents a serious limitation for the clinical use of some these new drugs. Both potential side effects of the novel therapeutic agents can be easily evaluated in an operant model of contingent self-administration of analgesic compounds. The relationships between the treatment of chronic pain and addiction is largely recognise and these relationships are difficult to be explored in the currently available behavioural models (Ballantyne and LaForge 2007;

Flugsrud-Breckenridge *et al.* 2007). Indeed, the presence of pain and the motivation to alleviate it seems to minimize the possible development of addictive behaviours with an appropriate opioid treatment protocol (Martin and Ewan 2008). In this sense, the rate of abuse of opioid analgesics in pain patients is relatively moderate (Cicero *et al.* 2005). However, other data have shown an increased prevalence of drug abuse in patients receiving opioids for chronic pain (Manchikanti 2007). Several animal studies also suggest that chronic pain attenuates the rewarding properties of opioids. Thus, inflammatory (Suzuki *et al.* 2001) and neuropathic pain (Ozaki *et al.* 2002; Ozaki *et al.* 2003; Ozaki *et al.* 2004) reduced morphine-induced conditioned place preference in rodents. Certain areas of the brain, such as the nucleus accumbens and the anterior cingulated gyrus are involved in both pain and addictive processes (Becerra *et al.* 2001). The relationships between chronic pain and addiction could be explored in the operant drug self-administration paradigm that we have set-up in our study. Indeed, this operant model allows the evaluation of the analgesic (mice exposed to neuropathic pain) and the reinforcing effects (sham-operated animals) of a pharmacological compound in the same paradigm.

In the last years, a special attention has received the σ1R for its implication in pain modulation. Indeed, σ1Rs are expressed in key areas for pain control, such as the superficial layers of the dorsal horn, periaqueductal gray matter, locus coeruleus and rostroventral medulla (Diaz *et al.* 2009). Studies using both σ1R knockout mice and pharmacological blockade of these receptors revealed that these interventions decrease the behavioural manifestations of neuropathic pain in a partial sciatic nerve ligation model (Diaz *et al.* 2009). Furthermore, σ1Rs exert a modulatory role on the NMDA receptors, a key receptor involved in the central sensitization developed during neuropathic pain (Diaz *et al.* 2009). These data indicate that σ1Rs play an important role in

the neurobiological mechanisms leading to the development and maintenance of neuropathic pain.

In this study, mice exposed to neuropathic pain were then trained to acquire an operant behaviour to self-administer intravenously the analgesic compound in order to set-up the new operant model of contingent self-administration of analgesic compounds. First, the animals were trained to acquire a food-maintained operant behaviour. Chronic pain was then developed in mice by partial sciatic nerve ligation. Once that chronic pain had reached a steady-state, mice were trained to maintain an operant behaviour to self-administer a new analgesic compound, the σ1R antagonist S1RA, which has been previously reported to alleviate neuropathic pain manifestations. The possible abuse potential of this new analgesic compound was identified by evaluating the self-administration behaviour in sham-operated mice. Two doses of this sigma antagonist, 3 and 6 mg/kg/infusion, were used in the intravenously self-administration paradigm. The sciatic nerve ligation led to a neuropathic pain syndrome characterised by mechanical and thermal allodynia, and thermal hyperalgesia that were present from the first day of measurement and were maintained during the whole experimental sequence. We showed that the σR1 antagonist, S1RA, was able to decrease the behavioural manifestations of neuropathic pain after intravenous self-administration at both doses tested. Indeed, after 10 days of S1RA self-administration, animals with partial sciatic nerve ligation showed a significant reduction of thermal and mechanical allodynia and thermal hyperalgesia when compared with the saline group. These results are in accordance with the previous studies revealing the role of σR1 in the manifestations of neuropathic pain (Puente *et al.* 2009; Roh *et al.* 2008). In these previous studies, thermal hyperalgesia was not modified after the decrease of σR1 activity. However, neuropathic pain model used in the pharmacological study was the chronic constriction injury that is different from the one

used in the present work. One important difference between these two models is that the manifestations of neuropathic pain after the partial sciatic nerve ligation are dependent on the activity of the sympathetic nervous system since the simpathectomy reverts these pain manifestations (Malmberg and Basbaum 1998), in contrast to the chronic constriction injury model. The difference between the results obtained in the present study and σR1 knockout mice could also be due to the possibility of adaptive compensatory changes in the genetic model. In addition, the mouse is self-administering itself the σR1 antagonist in the present study, whereas in the previous studies the drug was administered in a non-contingent way. Studies using a yoking procedure have demonstrated that the neurobiological and neurochemical consequences of drug intake can differ depending on whether the animal self-administers the drug or if the drug is passively administered (Martin and Ewan 2008). In this sense, drug self-administration, unlike non-contingent infusions, merge many additional features of the drug different from its analgesic properties, including reinforcing effects and the consequent motivation to seek for the drug. Our operant model allows the animal exposed to the chronic neuropathic pain to seek for drug delivery in order to alleviate the pain, but also permits to evaluate the possible reinforcing effects of the drug by comparing the operant responses between sham-operated and animals exposed to the partial sciatic nerve ligation.

Before acquiring the intravenous drug self-administration, mice were previously trained in the same operant paradigm to seek for food. Therefore, the response of the animals during the first days of drug self-administration animals is influenced by the previous acquisition of the food maintained operant behaviour. Consequently, differences in self-administration to the different doses of this analgesic compound reached the significance on the last day of training. Interestingly, mice that developed neuropathic pain maintained an operant behaviour to self-

administer the σR1 antagonist compound at 3 and 6 mg/kg/infusion. The self-medication with this analgesic alleviates neuropathic pain as revealed by the significant attenuation of both hyperalgesia and allodynia on the last day of training to self-administer S1RA at both doses tested. In contrast, control mice exposed to sham surgery did not maintain such an operant behaviour to self-administer the sigma ligand at the dose of 6 mg/kg/infusion, whereas a moderate self-administration was observed at the dose of 3 mg/kg/infusion. These results clearly reveal that mice exposed to chronic neuropathic pain maintained an operant behaviour to self-administer an elevated dose of the sigma ligand in order to alleviate the pain. However, a lower dose of the same sigma ligand was also self-administered by mice not exposed to neuropathic pain, revealing the reinforcing effects of this compound. The progressive ratio schedule of reinforcement, design as a measure of motivation and performed in the last day of intravenous drug self-administration revealed no significant differences between the different animal groups. However, interesting results were found when the percentages of acquisition were compared. Thus, no differences in percentage of acquisition were found within the sham operated group, whereas within the sciatic nerve ligated group, this percentage was higher in animals that self-.administered both doses of S1RA when compared with mice self-administering saline. These results revealed that sham animals display the same incentive to seek for the sigma antagonist compound than saline, whereas this incentive to seek the antagonist was enhanced in mice exposed to neuropathic pain.

The results obtained in our experiment were not due to a modification in the locomotor activity or any physical alteration that would prevent the mice from nose-poking or from maintaining a sufficient activity to obtain drug infusion. Indeed, no differences in the number of inactive hole responses were seen between the different animal groups exposed or not to S1RA.

Chronic pain is often associated to several emotional consequences that impair the quality of life of these patients and difficult the therapeutic approach. Indeed, depressive-like symptoms are often present in patients suffering chronic pain, (Campbell *et al.* 2006), which aggravates the consequences and manifestations of pain (Ong and Keng 2003). Because depression and chronic pain are often associated, an appropriate treatment of both emotional components of the chronic pain and painful symptoms may improve the beneficial effects in these patients (Campbell *et al.* 2006). In a second experiment, we evaluated the consequence of non-contingent S1RA administration on the anhedonic state induced by the sciatic nerve ligation. This emotional feature was assessed by measuring the preference for sucrose solution in the Phecomp food and drink monitoring system that was described in the previous paragraphs. It has been previously described that the consumption and the preference for highly palatable sweet solutions is decreased during in anhedonic state (Papp *et al.* 1991; Strekalova *et al.* 2004; Strekalova and Steinbusch 2010). In accordance, we found that anhedonia induced by the exposure to neuropathic pain diminishes the sucrose intake. In addition, non-contingent administration of the sigma 1 antagonist improved this emotional response. Indeed, animals exposed to partial sciatic nerve ligation receiving S1RA displayed the same preference for sucrose solution as sham-operated mice. These data show that S1RA is able to improve both physical and emotional manifestations of neuropathic pain pointing the high potential interest of this new compound for the treatment this pathology.

Our results revealed the analgesic efficacy of a new σR1 antagonist, S1RA, in the treatment of neuropathic pain. This analgesic effect was observed together with an improvement of the emotional consequences associated to the presence of chronic pain. S1RA administered at the highest dose was devoid of addictive-like effects. The operant responses

evaluated in this animal model have a high predictive value to estimate the clinical benefit/risk ratio of new analgesic compounds to treat chronic pain, such as S1RA.

CONCLUSIONS

Conclusions:

The main conclusions of the work presented in this thesis can be summarized as follows:

1. The cognitive effects of nicotine and physosotigmine were attenuated in the absence of CB_1 cannabinoid receptor activity in the active avoidance paradigm. However, scopolamine cognitive effects are independent from CB_1 receptor activity in this task.

2. The cognitive responses induced by rimonabant were similar in the active avoidance and the two object recognition paradigms and were different to those observed in CB_1 knockout mice.

3. The deleterious effects of the high fat diet on glucose, insulin and cholesterol levels were prevented in CB_1 knockout mice. Interestingly, nicotine reduced body weight and glucose levels, and induced anxiogenic-like effects in wild-type mice, but not in CB_1 knockout animals. The mutant mice also showed lower preference for high palatable drink in the absence of nicotine treatment.

4. The results obtained after nicotine treatment on metabolism and anxiogenic-like responses provide new evidence of the important role played by the endocannabinoid system in the pharmacological responses of nicotine that will be useful to better understand the interactions between nicotine and cannabinoid compounds.

5. $A_{2A}R$ deficient mice showed a significant decrease of the mechanical allodynia and a suppression of thermal hyperalgesia and allodynia induced by a the parcial sciatic nerve ligation. The expression of microglia and astrocytes was enhanced in wild-type mice exposed to sciatic nerve injury and this response was attenuated in knockout animals.

6. The new σR1 antagonist, S1RA, revealed a robust efficacy in the alleviation of neuropathic pain manifestations. The analgesic effects of this compound were observed together with an improvement of the emotional deficits consequences associated to the presence of chronic pain. S1RA administered at the highest dose was devoid of reinforcing effects.

7. We demonstrated the involvement of $A_{2A}Rs$ and σR1 in the control of neuropathic pain and propose these receptors as interesting targets for the development of new drugs for the management of these clinical manifestations of pain.

8. We succeed to validate a new operant animal model with a high predictive value to estimate the clinical benefit/risk ratio of new analgesic compounds to treat chronic pain.

REFERENCES

References

Abel E.L. (1975) Cannabis: effects on hunger and thirst. *Behav.Biol.* **15,** 255-281.

Agudo J., Martin M., Roca C., Molas M., Bura S.A., Zimmer A., Bosch F., & Maldonado R. Deficiency of CB2 cannabinoid receptor in mice improves insulin sensitivity but increases food intake and obesity with age.Diabetologia 2010 Ref Type: *In Press*

Alonso G., Phan V., Guillemain I., Saunier M., Legrand A., Anoal M., & Maurice T. (2000) Immunocytochemical localization of the sigma(1) receptor in the adult rat central nervous system. *Neuroscience* **97,** 155-170.

Amir R., Liu C.N., Kocsis J.D., & Devor M. (2002) Oscillatory mechanism in primary sensory neurones. *Brain* **125,** 421-435.

Andersson K. & Arner P. (2001) Systemic nicotine stimulates human adipose tissue lipolysis through local cholinergic and catecholaminergic receptors. *Int.J.Obes.Relat Metab Disord.* **25,** 1225-1232.

Arendash G.W., Sanberg P.R., & Sengstock G.J. (1995) Nicotine enhances the learning and memory of aged rats. *Pharmacol.Biochem.Behav.* **52,** 517-523.

Ashakumary L. & Vijayammal P.L. (1997) Effect of nicotine on lipoprotein metabolism in rats. *Lipids* **32,** 311-315.

Aso E., Renoir T., Mengod G., Ledent C., Hamon M., Maldonado R., Lanfumey L., & Valverde O. (2009) Lack of CB1 receptor activity impairs serotonergic negative feedback. *J.Neurochem.* **109,** 935-944.

Attaway C.M., Compton D.M., & Turner M.D. (1999) The effects of nicotine on learning and memory: a neuropsychological assessment in young and senescent Fischer 344 rats. *Physiol Behav.* **67,** 421-431.

Balerio G.N., Aso E., Berrendero F., Murtra P., & Maldonado R. (2004) Delta9-tetrahydrocannabinol decreases somatic and motivational manifestations of nicotine withdrawal in mice. *Eur.J.Neurosci.* **20,** 2737-2748.

Balerio G.N., Aso E., & Maldonado R. (2006) Role of the cannabinoid system in the effects induced by nicotine on anxiety-like behaviour in mice. *Psychopharmacology (Berl)* **184,** 504-513.

Ballantyne J.C. & LaForge K.S. (2007) Opioid dependence and addiction during opioid treatment of chronic pain. *Pain* **129,** 235-255.

Baños J.E., Sanchez G., Berrendero F., & Maldonado R. (2003) Neuropathic pain: some clues for future drug treatments. *Mini.Rev.Med.Chem.* **3,** 719-727.

Barbano M.F., Castane A., Martin-Garcia E., & Maldonado R. (2009) Delta-9-tetrahydrocannabinol enhances food reinforcement in a mouse operant conflict test. *Psychopharmacology (Berl)* **205,** 475-487.

Baron R. (2006) Mechanisms of disease: neuropathic pain--a clinical perspective. *Nat.Clin.Pract.Neurol.* **2,** 95-106.

Baron R. (2000) Capsaicin and nociception: from basic mechanisms to novel drugs. *Lancet* **356,** 785-787.

Bastia E., Varani K., Monopoli A., & Bertorelli R. (2002) Effects of A(1) and A(2A) adenosine receptor ligands in mouse acute models of pain. *Neurosci.Lett.* **328,** 241-244.

Becerra L., Breiter H.C., Wise R., Gonzalez R.G., & Borsook D. (2001) Reward circuitry activation by noxious thermal stimuli. *Neuron* **32,** 927-946.

Bellocchio L., Cervino C., Vicennati V., Pasquali R., & Pagotto U. (2008) Cannabinoid type 1 receptor: another arrow in the adipocytes' bow. *J.Neuroendocrinol.* **20 Suppl 1,** 130-138.

Bellocchio L., Lafenetre P., Cannich A., Cota D., Puente N., Grandes P., Chaouloff F., Piazza P.V., & Marsicano G. (2010) Bimodal control of stimulated food intake by the endocannabinoid system. *Nat.Neurosci.* **13,** 281-283.

Bennett G.J. & Xie Y.K. (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* **33,** 87-107.

Benowitz N.L. (1996) Pharmacology of nicotine: addiction and therapeutics. *Annu.Rev.Pharmacol.Toxicol.* **36,** 597-613.

Benyamin R., Trescot A.M., Datta S., Buenaventura R., Adlaka R., Sehgal N., Glaser S.E., & Vallejo R. (2008) Opioid complications and side effects. *Pain Physician* **11,** S105-S120.

Berg D.K., Conroy W.G., Liu Z., & Zago W.M. (2006) Nicotinic signal transduction machinery. *J.Mol.Neurosci.* **30,** 149-152.

Bernaards C.M., Twisk J.W., Snel J., van M.W., & Kemper H.C. (2005) In a prospective study in young people, associations between changes in smoking behavior and risk factors for cardiovascular disease were complex. *J.Clin.Epidemiol.* **58,** 1165-1171.

Berrendero F., Kieffer B.L., & Maldonado R. (2002) Attenuation of nicotine-induced antinociception, rewarding effects, and dependence in mu-opioid receptor knock-out mice. *J.Neurosci.* **22,** 10935-10940.

Berrendero F., Mendizabal V., Robledo P., Galeote L., Bilkei-Gorzo A., Zimmer A., & Maldonado R. (2005) Nicotine-induced antinociception, rewarding effects, and physical dependence are decreased in mice lacking the preproenkephalin gene. *J.Neurosci.* **25,** 1103-1112.

Berrendero F., Robledo P., Trigo J.M., Martin-Garcia E., & Maldonado R. (2010) Neurobiological mechanisms involved in nicotine dependence and reward: Participation of the endogenous opioid system. *Neurosci.Biobehav.Rev.*

Berthoud H.R. (2006) Homeostatic and non-homeostatic pathways involved in the control of food intake and energy balance. *Obesity.(Silver.Spring)* **14 Suppl 5,** 197S-200S.

Bicher H.I. & MECHOULAM R. (1968) Pharmacological effects of two active constituents of marihuana. *Arch.Int.Pharmacodyn.Ther.* **172,** 24-31.

Bisogno T., Ligresti A., & Di M., V (2005) The endocannabinoid signalling system: biochemical aspects. *Pharmacol.Biochem.Behav.* **81,** 224-238.

Blondel A., Sanger D.J., & Moser P.C. (2000) Characterisation of the effects of nicotine in the five-choice serial reaction time task in rats: antagonist studies. *Psychopharmacology (Berl).* **149,** 293-305.

Bohme G.A., Laville M., Ledent C., Parmentier M., & Imperato A. (2000) Enhanced long-term potentiation in mice lacking cannabinoid CB1 receptors. *Neuroscience* **95,** 5-7.

Bontempi B., Whelan K.T., Risbrough V.B., Rao T.S., Buccafusco J.J., Lloyd G.K., & Menzaghi F. (2001) SIB-1553A, (+/-)-4-[[2-(1-methyl-2-pyrrolidinyl)ethyl]thio]phenol hydrochloride, a subtype-selective ligand for nicotinic acetylcholine receptors with putative cognitive-enhancing properties: effects on working and reference memory performances in aged rodents and nonhuman primates. *J.Pharmacol.Exp.Ther.* **299,** 297-306.

Braida D. & Sala M. (2000) Cannabinoid-induced working memory impairment is reversed by a second generation cholinesterase inhibitor in rats. *Neuroreport* **11,** 2025-2029.

Brambilla R., Cottini L., Fumagalli M., Ceruti S., & Abbracchio M.P. (2003) Blockade of A2A adenosine receptors prevents basic fibroblast growth factor-induced reactive astrogliosis in rat striatal primary astrocytes. *Glia* **43,** 190-194.

Bray G.A. (2000) Reciprocal relation of food intake and sympathetic activity: experimental observations and clinical implications. *Int.J.Obes.Relat Metab Disord.* **24 Suppl 2,** S8-17.

Bridges D., Thompson S.W., & Rice A.S. (2001) Mechanisms of neuropathic pain. *Br.J.Anaesth.* **87,** 12-26.

Brown A.J. (2007) Novel cannabinoid receptors. *Br.J.Pharmacol.* **152,** 567-575.

Bruijnzeel A.W. & Gold M.S. (2005) The role of corticotropin-releasing factor-like peptides in cannabis, nicotine, and alcohol dependence. *Brain Res.Brain Res.Rev.* **49,** 505-528.

Buccafusco J.J., Jackson W.J., Jonnala R.R., & Terry A.V., Jr. (1999) Differential improvement in memory-related task performance with nicotine by aged male and female rhesus monkeys. *Behav.Pharmacol.* **10,** 681-690.

Bullen C. (2008) Impact of tobacco smoking and smoking cessation on cardiovascular risk and disease. *Expert.Rev.Cardiovasc.Ther.* **6,** 883-895.

Buxbaum D.M. (1972) Analgesic activity of 9 -tetrahydrocannabinol in the rat and mouse. *Psychopharmacologia.* **25,** 275-280.

Byas-Smith M.G., Max M.B., Muir J., & Kingman A. (1995) Transdermal clonidine compared to placebo in painful diabetic neuropathy using a two-stage 'enriched enrollment' design. *Pain* **60,** 267-274.

Calignano A., La R.G., Giuffrida A., & Piomelli D. (1998) Control of pain initiation by endogenous cannabinoids. *Nature* **394,** 277-281.

Calignano A., La R.G., Makriyannis A., Lin S.Y., Beltramo M., & Piomelli D. (1997) Inhibition of intestinal motility by anandamide, an endogenous cannabinoid. *Eur.J.Pharmacol.* **340,** R7-R8.

Campbell J.N., Basbaum A.I., Dray A., Dubner R., Dworkin R.H., & Sang N.C. (2006) Emerging strategies for the treatment of neuropathic pain. IASP Press, Seattle.

Carter M.L. (2004) Spinal cord stimulation in chronic pain: a review of the evidence. *Anaesth.Intensive Care* **32,** 11-21.

Castañé A., Berrendero F., & Maldonado R. (2005) The role of the cannabinoid system in nicotine addiction. *Pharmacol.Biochem.Behav.* **81,** 381-386.

Castañé A., Celerier E., Martin M., Ledent C., Parmentier M., Maldonado R., & Valverde O. (2006) Development and expression of neuropathic pain in CB1 knockout mice. *Neuropharmacology* **50,** 111-122.

Castañé A., Valjent E., Ledent C., Parmentier M., Maldonado R., & Valverde O. (2002) Lack of CB1 cannabinoid receptors modifies nicotine behavioural responses, but not nicotine abstinence. *Neuropharmacology* **43,** 857-867.

Cavuoto P. & Wittert G.A. (2009) The role of the endocannabinoid system in the regulation of energy expenditure. *Best.Pract.Res.Clin.Endocrinol.Metab* **23,** 79-86.

Cendan C.M., Pujalte J.M., Portillo-Salido E., Montoliu L., & Baeyens J.M. (2005) Formalin-induced pain is reduced in sigma(1) receptor knockout mice. *Eur.J.Pharmacol.* **511,** 73-74.

Chait L.D. & Perry J.L. (1992) Factors influencing self-administration of, and subjective response to, placebo marijuana. *Behav.Pharmacol.* **3,** 545-552.

Chaki S., Tanaka M., Muramatsu M., & Otomo S. (1994) NE-100, a novel potent sigma ligand, preferentially binds to sigma 1 binding sites in guinea pig brain. *Eur.J.Pharmacol.* **251,** R1-R2.

Chen H., Hansen M.J., Jones J.E., Vlahos R., Bozinovski S., Anderson G.P., & Morris M.J. (2007) Regulation of hypothalamic NPY by diet and smoking. *Peptides* **28,** 384-389.

Chevaleyre V., Takahashi K.A., & Castillo P.E. (2006) Endocannabinoid-mediated synaptic plasticity in the CNS. *Annu.Rev.Neurosci.* **29,** 37-76.

Choca J.I., Proudfit H.K., & Green R.D. (1987) Identification of A1 and A2 adenosine receptors in the rat spinal cord. *J.Pharmacol.Exp.Ther.* **242,** 905-910.

Ciamei A., Aversano M., Cestari V., & Castellano C. (2001) Effects of MK-801 and nicotine combinations on memory consolidation in CD1 mice. *Psychopharmacology (Berl)* **154,** 126-130.

Cicero T.J., Inciardi J.A., & Munoz A. (2005) Trends in abuse of Oxycontin and other opioid analgesics in the United States: 2002-2004. *J.Pain.* **6,** 662-672.

Clarke J.R., Rossato J.I., Monteiro S., Bevilaqua L.R., Izquierdo I., & Cammarota M. (2008) Posttraining activation of CB1 cannabinoid receptors in the CA1 region of the dorsal hippocampus impairs object recognition long-term memory. *Neurobiol.Learn.Mem.* **90,** 374-381.

Cohen C., Kodas E., & Griebel G. (2005) CB1 receptor antagonists for the treatment of nicotine addiction. *Pharmacol.Biochem.Behav.* **81,** 387-395.

Colburn R.W., Rickman A.J., & DeLeo J.A. (1999a) The effect of site and type of nerve injury on spinal glial activation and neuropathic pain behavior. *Exp.Neurol.* **157,** 289-304.

Collins D.R., Pertwee R.G., & Davies S.N. (1995) Prevention by the cannabinoid antagonist, SR141716A, of cannabinoid-mediated blockade of long-term potentiation in the rat hippocampal slice. *Br.J.Pharmacol.* **115,** 869-870.

Compton D.R., Dewey W.L., & Martin B.R. (1990) Cannabis dependence and tolerance production. *Adv.Alcohol Subst.Abuse* **9,** 129-147.

Cook J.W., Spring B., & McChargue D. (2007) Influence of nicotine on positive affect in anhedonic smokers. *Psychopharmacology (Berl)* **192,** 87-95.

Cook S.A., Lowe J.A., & Martin B.R. (1998) CB1 receptor antagonist precipitates withdrawal in mice exposed to Delta9-tetrahydrocannabinol. *J.Pharmacol.Exp.Ther.* **285,** 1150-1156.

Cota D. (2008) The role of the endocannabinoid system in the regulation of hypothalamic-pituitary-adrenal axis activity. *J.Neuroendocrinol.* **20 Suppl 1,** 35-38.

Coull J.A., Boudreau D., Bachand K., Prescott S.A., Nault F., Sik A., De K.P., & De K.Y. (2003) Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature* **424,** 938-942.

Court J., Martin-Ruiz C., Piggott M., Spurden D., Griffiths M., & Perry E. (2001) Nicotinic receptor abnormalities in Alzheimer's disease. *Biol.Psychiatry* **49,** 175-184.

Court J., Spurden D., Lloyd S., McKeith I., Ballard C., Cairns N., Kerwin R., Perry R., & Perry E. (1999) Neuronal nicotinic receptors in dementia with Lewy bodies and schizophrenia: alpha-bungarotoxin and nicotine binding in the thalamus. *J.Neurochem.* **73,** 1590-1597.

Cravatt B.F., Giang D.K., Mayfield S.P., Boger D.L., Lerner R.A., & Gilula N.B. (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* **384,** 83-87.

Cunha R.A. (2005) Neuroprotection by adenosine in the brain: From A(1) receptor activation to A (2A) receptor blockade. *Purinergic.Signal.* **1,** 111-134.

Damaj M.I., Kao W., & Martin B.R. (2003) Characterization of spontaneous and precipitated nicotine withdrawal in the mouse. *J.Pharmacol.Exp.Ther.* **307,** 526-534.

Dani J.A. & Heinemann S. (1996) Molecular and cellular aspects of nicotine abuse. *Neuron.* **16,** 905-908.

Debonnel G. & de M.C. (1996) Modulation of NMDA and dopaminergic neurotransmissions by sigma ligands: possible implications for the treatment of psychiatric disorders. *Life Sci.* **58,** 721-734.

Decker M.W., Rueter L.E., & Bitner R.S. (2004) Nicotinic acetylcholine receptor agonists: a potential new class of analgesics. *Curr.Top.Med.Chem.* **4,** 369-384.

DeLeo J.A. & Yezierski R.P. (2001) The role of neuroinflammation and neuroimmune activation in persistent pain. *Pain* **90,** 1-6.

Despres J.P., Golay A., & Sjostrom L. (2005) Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. *N.Engl.J.Med.* **353,** 2121-2134.

Di Marzo V, Bifulco M., & De P.L. (2004) The endocannabinoid system and its therapeutic exploitation. *Nat.Rev.Drug Discov.* **3,** 771-784.

Di Marzo V, Fontana A., Cadas H., Schinelli S., Cimino G., Schwartz J.C., & Piomelli D. (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* **372,** 686-691.

Di Marzo V. & Matias I. (2005) Endocannabinoid control of food intake and energy balance. *Nat.Neurosci.* **8,** 585-589.

Di Marzo V (2008) CB(1) receptor antagonism: biological basis for metabolic effects. *Drug Discov.Today* **13,** 1026-1041.

Di Marzo.V, Bifulco M., & De P.L. (2004) The endocannabinoid system and its therapeutic exploitation. *Nat.Rev.Drug Discov.* **3,** 771-784.

Di Marzo V, Fontana A., Cadas H., Schinelli S., Cimino G., Schwartz J.C., & Piomelli D. (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* **372,** 686-691.

Diaz J.L., Zamanillo D., Corbera J., Baeyens J.M., Maldonado R., Pericas M.A., Vela J.M., & Torrens A. (2009) Selective sigma-1 (sigma1) receptor antagonists: emerging target for the treatment of neuropathic pain. *Cent.Nerv.Syst.Agents Med.Chem.* **9,** 172-183.

Dinh T.P., Freund T.F., & Piomelli D. (2002) A role for monoglyceride lipase in 2-arachidonoylglycerol inactivation. *Chem.Phys.Lipids* **121,** 149-158.

Doak G.J. & Sawynok J. (1995) Complex role of peripheral adenosine in the genesis of the response to subcutaneous formalin in the rat. *Eur.J.Pharmacol.* **281,** 311-318.

Dogra S., Beydoun S., Mazzola J., Hopwood M., & Wan Y. (2005) Oxcarbazepine in painful diabetic neuropathy: a randomized, placebo-controlled study. *Eur.J.Pain* **9,** 543-554.

Dong Y. & Benveniste E.N. (2001) Immune function of astrocytes. *Glia* **36,** 180-190.

Dunwiddie T.V. & Masino S.A. (2001) The role and regulation of adenosine in the central nervous system. *Annu.Rev.Neurosci.* **24,** 31-55.

Egerton A., Allison C., Brett R.R., & Pratt J.A. (2006) Cannabinoids and prefrontal cortical function: insights from preclinical studies. *Neurosci.Biobehav.Rev.* **30,** 680-695.

Ennaceur A. & Delacour J. (1988) A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behav.Brain Res.* **31,** 47-59.

Esfandyari T., Camilleri M., Busciglio I., Burton D., Baxter K., & Zinsmeister A.R. (2007) Effects of a cannabinoid receptor agonist on colonic motor and sensory functions in humans: a randomized, placebo-controlled study. *Am.J.Physiol Gastrointest.Liver Physiol* **293,** G137-G145.

Ferre S., Diamond I., Goldberg S.R., Yao L., Hourani S.M., Huang Z.L., Urade Y., & Kitchen I. (2007) Adenosine A2A receptors in ventral striatum, hypothalamus and nociceptive circuitry implications for drug addiction, sleep and pain. *Prog.Neurobiol.* **83,** 332-347.

Fertig J.B. & Allen J.P. (1996) Health behavior correlates of hazardous drinking by Army personnel. *Mil.Med.* **161,** 352-355.

Fibiger H.C., Damsma G., & Day J.C. (1991) Behavioral pharmacology and biochemistry of central cholinergic neurotransmission. *Adv.Exp.Med.Biol.* **295,** 399-414.

Fields H. (2004) State-dependent opioid control of pain. *Nat.Rev.Neurosci.* **5,** 565-575.

Fields H.L., Heinricher M.M., & Mason P. (1991) Neurotransmitters in nociceptive modulatory circuits. *Annu.Rev.Neurosci.* **14,** 219-245.

Fields H.L., Malick A., & Burstein R. (1995) Dorsal horn projection targets of ON and OFF cells in the rostral ventromedial medulla. *J.Neurophysiol.* **74,** 1742-1759.

Finnerup N.B., Otto M., McQuay H.J., Jensen T.S., & Sindrup S.H. (2005) Algorithm for neuropathic pain treatment: an evidence based proposal. *Pain* **118,** 289-305.

Flores C.M. (2000) The promise and pitfalls of a nicotinic cholinergic approach to pain management. *Pain* **88,** 1-6.

Flugsrud-Breckenridge M.R., Gevirtz C., Paul D., & Gould H.J., III (2007) Medications of abuse in pain management. *Curr.Opin.Anaesthesiol.* **20,** 319-324.

Fredholm B.B., IJzerman A.P., Jacobson K.A., Klotz K.N., & Linden J. (2001a) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol.Rev.* **53,** 527-552.

Fredholm B.B., Irenius E., Kull B., & Schulte G. (2001b) Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochem.Pharmacol.* **61,** 443-448.

Fredholm B.B. & Svenningsson P. (2003) Adenosine-dopamine interactions: development of a concept and some comments on therapeutic possibilities. *Neurology* **61,** S5-S9.

Froeliger B., Gilbert D.G., & McClernon F.J. (2009) Effects of nicotine on novelty detection and memory recognition performance: double-blind, placebo-controlled studies of smokers and nonsmokers. *Psychopharmacology (Berl)*.

Fromm G.H., Terrence C.F., & Chattha A.S. (1984) Baclofen in the treatment of trigeminal neuralgia: double-blind study and long-term follow-up. *Ann.Neurol.* **15,** 240-244.

Gallagher M. & Colombo P.J. (1995) Ageing: the cholinergic hypothesis of cognitive decline. *Curr.Opin.Neurobiol.* **5,** 161-168.

Gessa G.L., Mascia M.S., Casu M.A., & Carta G. (1997) Inhibition of hippocampal acetylcholine release by cannabinoids: reversal by SR 141716A. *Eur.J.Pharmacol.* **327,** R1-R2.

Gifford A.N. & Ashby C.R., Jr. (1996) Electrically evoked acetylcholine release from hippocampal slices is inhibited by the cannabinoid receptor agonist, WIN 55212-2, and is potentiated by the cannabinoid antagonist, SR 141716A. *J.Pharmacol.Exp.Ther.* **277,** 1431-1436.

Gilbert P.E. (1981) A comparison of THC, nantradol, nabilone, and morphine in the chronic spinal dog. *J.Clin.Pharmacol.* **21,** 311S-319S.

Gilron I. (2007) Gabapentin and pregabalin for chronic neuropathic and early postsurgical pain: current evidence and future directions. *Curr.Opin.Anaesthesiol.* **20,** 456-472.

Girdler S.S., Maixner W., Naftel H.A., Stewart P.W., Moretz R.L., & Light K.C. (2005) Cigarette smoking, stress-induced analgesia and pain perception in men and women. *Pain* **114,** 372-385.

Godfrey L., Yan L., Clarke G.D., Ledent C., Kitchen I., & Hourani S.M. (2006) Modulation of paracetamol antinociception by caffeine and by selective adenosine A2 receptor antagonists in mice. *Eur.J.Pharmacol.* **531,** 80-86.

Gold M.S. (2000) Spinal nerve ligation: what to blame for the pain and why. *Pain* **84,** 117-120.

Goldberg M.S., Scott S.C., & Mayo N.E. (2000) A review of the association between cigarette smoking and the development of nonspecific back pain and related outcomes. *Spine* **25,** 995-1014.

Goldstein D.J., Lu Y., Detke M.J., Lee T.C., & Iyengar S. (2005) Duloxetine vs. placebo in patients with painful diabetic neuropathy. *Pain* **116,** 109-118.

Gomez R., Navarro M., Ferrer B., Trigo J.M., Bilbao A., Del A., I, Cippitelli A., Nava F., Piomelli D., & Rodriguez de F.F. (2002) A peripheral mechanism for CB1 cannabinoid receptor-dependent modulation of feeding. *J.Neurosci.* **22,** 9612-9617.

Gotti C. & Clementi F. (2004) Neuronal nicotinic receptors: from structure to pathology. *Prog.Neurobiol.* **74,** 363-396.

Gotti C., Zoli M., & Clementi F. (2006) Brain nicotinic acetylcholine receptors: native subtypes and their relevance. *Trends Pharmacol.Sci.* **27,** 482-491.

Goya P., Jagerovic N., Hernandez-Folgado L., & Martin M.I. (2003) Cannabinoids and neuropathic pain. *Mini.Rev.Med.Chem.* **3,** 765-772.

Greenbaum L., Rigbi A., Teltsh O., & Lerer B. (2009) Role of genetic variants in the CHRNA5-CHRNA3-CHRNB4 cluster in nicotine dependence risk: importance of gene-environment interplay. *Mol.Psychiatry* **14,** 828-830.

Grotenhermen F. (2004) Pharmacology of cannabinoids. *Neuro.Endocrinol.Lett.* **25,** 14-23.

Grotenhermen F. (2003) Pharmacokinetics and pharmacodynamics of cannabinoids. *Clin.Pharmacokinet.* **42,** 327-360.

Guan Z.Z., Zhang X., Blennow K., & Nordberg A. (1999) Decreased protein level of nicotinic receptor alpha7 subunit in the frontal cortex from schizophrenic brain. *Neuroreport.* **10,** 1779-1782.

Guitart X., Codony X., & Monroy X. (2004) Sigma receptors: biology and therapeutic potential. *Psychopharmacology (Berl)* **174,** 301-319.

Guitart X. & Farre A.J. (1998) The effect of E-5842, a sigma receptor ligand and potential atypical antipsychotic, on Fos expression in rat forebrain. *Eur.J.Pharmacol.* **363,** 127-130.

Hampson R.E. & Deadwyler S.A. (1998) Role of cannabinoid receptors in memory storage. *Neurobiol.Dis.* **5,** 474-482.

Hanus L., bu-Lafi S., Fride E., Breuer A., Vogel Z., Shalev D.E., Kustanovich I., & MECHOULAM R. (2001) 2-arachidonyl glyceryl ether, an endogenous agonist of the cannabinoid CB1 receptor. *Proc.Natl.Acad.Sci.U.S.A* **98,** 3662-3665.

Hasko G. & Cronstein B.N. (2004) Adenosine: an endogenous regulator of innate immunity. *Trends Immunol.* **25,** 33-39.

Hasko G. & Pacher P. (2008) A2A receptors in inflammation and injury: lessons learned from transgenic animals. *J.Leukoc.Biol.* **83,** 447-455.

Hasko G., Pacher P., Vizi E.S., & Illes P. (2005) Adenosine receptor signaling in the brain immune system. *Trends Pharmacol.Sci.* **26,** 511-516.

Hatsukami D.K., Stead L.F., & Gupta P.C. (2008) Tobacco addiction. *Lancet* **371,** 2027-2038.

Heese K., Fiebich B.L., Bauer J., & Otten U. (1997) Nerve growth factor (NGF) expression in rat microglia is induced by adenosine A2a-receptors. *Neurosci.Lett.* **231,** 83-86.

Hellewell S.B., Bruce A., Feinstein G., Orringer J., Williams W., & Bowen W.D. (1994) Rat liver and kidney contain high densities of sigma

1 and sigma 2 receptors: characterization by ligand binding and photoaffinity labeling. *Eur.J.Pharmacol.* **268,** 9-18.

Herkenham M., Lynn A.B., Johnson M.R., Melvin L.S., de Costa B.R., & Rice K.C. (1991) Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J.Neurosci.* **11,** 563-583.

Herman R.M., D'Luzansky S.C., & Ippolito R. (1992) Intrathecal baclofen suppresses central pain in patients with spinal lesions. A pilot study. *Clin.J.Pain* **8,** 338-345.

Hildebrand B.E., Panagis G., Svensson T.H., & Nomikos G.G. (1999) Behavioral and biochemical manifestations of mecamylamine-precipitated nicotine withdrawal in the rat: role of nicotinic receptors in the ventral tegmental area. *Neuropsychopharmacology* **21,** 560-574.

Hindley S., Herman M.A., & Rathbone M.P. (1994) Stimulation of reactive astrogliosis in vivo by extracellular adenosine diphosphate or an adenosine A2 receptor agonist. *J.Neurosci.Res.* **38,** 399-406.

Hocking G. & Cousins M.J. (2003) Ketamine in chronic pain management: an evidence-based review. *Anesth.Analg.* **97,** 1730-1739.

Hoffman A.F., Oz M., Yang R., Lichtman A.H., & Lupica C.R. (2007) Opposing actions of chronic Delta9-tetrahydrocannabinol and cannabinoid antagonists on hippocampal long-term potentiation. *Learn.Mem.* **14,** 63-74.

Hogg R.C., Raggenbass M., & Bertrand D. (2003) Nicotinic acetylcholine receptors: from structure to brain function. *Rev.Physiol Biochem.Pharmacol.* **147,** 1-46.

Hohmann A.G. (2002) Spinal and peripheral mechanisms of cannabinoid antinociception: behavioral, neurophysiological and neuroanatomical perspectives. *Chem.Phys.Lipids* **121,** 173-190.

Hohmann A.G. & Herkenham M. (1998) Regulation of cannabinoid and mu opioid receptors in rat lumbar spinal cord following neonatal capsaicin treatment. *Neurosci.Lett.* **252,** 13-16.

Holland P.C. & Bouton M.E. (1999) Hippocampus and context in classical conditioning. *Curr.Opin.Neurobiol.* **9,** 195-202.

Howlett A.C. (2002) The cannabinoid receptors. *Prostaglandins Other Lipid Mediat.* **68-69,** 619-631.

Hukkanen J., Jacob P., III, & Benowitz N.L. (2005) Metabolism and disposition kinetics of nicotine. *Pharmacol.Rev.* **57,** 79-115.

Hussey M.J., Clarke G.D., Ledent C., Hourani S.M., & Kitchen I. (2007) Reduced response to the formalin test and lowered spinal NMDA glutamate receptor binding in adenosine A2A receptor knockout mice. *Pain* **129,** 287-294.

Hutcheson D.M., Tzavara E.T., Smadja C., Valjent E., Roques B.P., Hanoune J., & Maldonado R. (1998) Behavioural and biochemical evidence for signs of abstinence in mice chronically treated with delta-9-tetrahydrocannabinol. *Br.J.Pharmacol.* **125,** 1567-1577.

Ibrahim M.M., Deng H., Zvonok A., Cockayne D.A., Kwan J., Mata H.P., Vanderah T.W., Lai J., Porreca F., Makriyannis A., & Malan T.P., Jr. (2003) Activation of CB2 cannabinoid receptors by AM1241 inhibits experimental neuropathic pain: pain inhibition by receptors not present in the CNS. *Proc.Natl.Acad.Sci.U.S.A* **100,** 10529-10533.

Inui A. (1999) Feeding and body-weight regulation by hypothalamic neuropeptides--mediation of the actions of leptin. *Trends Neurosci.* **22,** 62-67.

Irwin M.S., Gilbert S.E., Terenghi G., Smith R.W., & Green C.J. (1997) Cold intolerance following peripheral nerve injury. Natural history and factors predicting severity of symptoms. *J.Hand Surg.Br.* **22,** 308-316.

Jansen K.L., Elliot M., & Leslie R.A. (1992) sigma receptors in rat brain and testes show similar reductions in response to chronic haloperidol. *Eur.J.Pharmacol.* **214,** 281-283.

Ji R.R. & Woolf C.J. (2001) Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain. *Neurobiol.Dis.* **8,** 1-10.

Jo Y.H., Talmage D.A., & Role L.W. (2002) Nicotinic receptor-mediated effects on appetite and food intake. *J.Neurobiol.* **53,** 618-632.

Josiah D.T. & Vincler M.A. (2006) Impact of chronic nicotine on the development and maintenance of neuropathic hypersensitivity in the rat. *Psychopharmacology (Berl).* **188,** 152-161.

Kaelin-Lang A., Lauterburg T., & Burgunder J.M. (1998) Expression of adenosine A2a receptor gene in rat dorsal root and autonomic ganglia. *Neurosci.Lett.* **246,** 21-24.

Kano M., Ohno-Shosaku T., Hashimotodani Y., Uchigashima M., & Watanabe M. (2009) Endocannabinoid-mediated control of synaptic transmission. *Physiol Rev.* **89,** 309-380.

Kathmann M., Weber B., Zimmer A., & Schlicker E. (2001) Enhanced acetylcholine release in the hippocampus of cannabinoid CB(1) receptor-deficient mice. *Br.J.Pharmacol.* **132,** 1169-1173.

Kaymakcalan S., Turker R.K., & Turker M.N. (1974) Analgesic effect of delta 9-tetrahydrocannabinol in the dog. *Psychopharmacologia.* **35,** 123-128.

Kekuda R., Prasad P.D., Fei Y.J., Leibach F.H., & Ganapathy V. (1996) Cloning and functional expression of the human type 1 sigma receptor (hSigmaR1). *Biochem.Biophys.Res.Commun.* **229,** 553-558.

Kelemen W.L. & Fulton E.K. (2008) Cigarette abstinence impairs memory and metacognition despite administration of 2 mg nicotine gum. *Exp.Clin.Psychopharmacol.* **16,** 521-531.

Kim S.H. & Chung J.M. (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* **50,** 355-363.

Kirkham T.C., Williams C.M., Fezza F., & Di M., V (2002) Endocannabinoid levels in rat limbic forebrain and hypothalamus in relation to fasting, feeding and satiation: stimulation of eating by 2-arachidonoyl glycerol. *Br.J.Pharmacol.* **136,** 550-557.

Kumari V., Gray J.A., ffytche D.H., Mitterschiffthaler M.T., Das M., Zachariah E., Vythelingum G.N., Williams S.C., Simmons A., & Sharma T. (2003) Cognitive effects of nicotine in humans: an fMRI study. *Neuroimage.* **19,** 1002-1013.

Lai J., Hunter J.C., & Porreca F. (2003) The role of voltage-gated sodium channels in neuropathic pain. *Curr.Opin.Neurobiol.* **13,** 291-297.

Lamota L., Bermudez-Silva F.J., Marco E.M., Llorente R., Gallego A., Rodriguez de F.F., & Viveros M.P. (2008) Effects of adolescent nicotine and SR 147778 (Surinabant) administration on food intake, somatic

growth and metabolic parameters in rats. *Neuropharmacology* **54,** 194-205.

Le N.N. & Changeux J.P. (1999) The Ligand Gated Ion Channel Database. *Nucleic Acids Res.* **27,** 340-342.

Ledent C., Valverde O., Cossu G., Petitet F., Aubert J.F., Beslot F., Bohme G.A., Imperato A., Pedrazzini T., Roques B.P., Vassart G., Fratta W., & Parmentier M. (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science* **283,** 401-404.

LeDoux J.E. (2000) Emotion circuits in the brain. *Annu.Rev.Neurosci.* **23,** 155-184.

Lee Y.W. & Yaksh T.L. (1996) Pharmacology of the spinal adenosine receptor which mediates the antiallodynic action of intrathecal adenosine agonists. *J.Pharmacol.Exp.Ther.* **277,** 1642-1648.

Lemmonds C.A., Williams D.K., & Wenger G.R. (2002) Effect of pentobarbital, d-amphetamine, and nicotine on two models of sustained attention in pigeons. *Psychopharmacology (Berl).* **163,** 391-398.

Levin E.D. & Chen E. (2004) Nicotinic involvement in memory function in zebrafish. *Neurotoxicol.Teratol.* **26,** 731-735.

Levin E.D., Christopher N.C., Briggs S.J., & Rose J.E. (1993) Chronic nicotine reverses working memory deficits caused by lesions of the fimbria or medial basalocortical projection. *Brain Res.Cogn Brain Res.* **1,** 137-143.

Levin E.D., Kaplan S., & Boardman A. (1997) Acute nicotine interactions with nicotinic and muscarinic antagonists: working and reference memory effects in the 16-arm radial maze. *Behav.Pharmacol.* **8,** 236-242.

Levin E.D., McClernon F.J., & Rezvani A.H. (2006) Nicotinic effects on cognitive function: behavioral characterization, pharmacological specification, and anatomic localization. *Psychopharmacology (Berl)* **184,** 523-539.

Levin E.D. & Rezvani A.H. (2000) Development of nicotinic drug therapy for cognitive disorders. *Eur.J.Pharmacol.* **393,** 141-146.

Li J., Daughters R.S., Bullis C., Bengiamin R., Stucky M.W., Brennan J., & Simone D.A. (1999) The cannabinoid receptor agonist WIN 55,212-2

mesylate blocks the development of hyperalgesia produced by capsaicin in rats. *Pain* **81,** 25-33.

Li Y., Dorsi M.J., Meyer R.A., & Belzberg A.J. (2000) Mechanical hyperalgesia after an L5 spinal nerve lesion in the rat is not dependent on input from injured nerve fibers. *Pain* **85,** 493-502.

Li Y., Oskouian R.J., Day Y.J., Rieger J.M., Liu L., Kern J.A., & Linden J. (2006) Mouse spinal cord compression injury is reduced by either activation of the adenosine A2A receptor on bone marrow-derived cells or deletion of the A2A receptor on non-bone marrow-derived cells. *Neuroscience* **141,** 2029-2039.

Liberto C.M., Albrecht P.J., Herx L.M., Yong V.W., & Levison S.W. (2004) Pro-regenerative properties of cytokine-activated astrocytes. *J.Neurochem.* **89,** 1092-1100.

Lichtman A.H. & Martin B.R. (1991) Cannabinoid-induced antinociception is mediated by a spinal alpha 2-noradrenergic mechanism. *Brain Res.* **559,** 309-314.

Lim G., Sung B., Ji R.R., & Mao J. (2003) Upregulation of spinal cannabinoid-1-receptors following nerve injury enhances the effects of Win 55,212-2 on neuropathic pain behaviors in rats. *Pain* **105,** 275-283.

Liu J., Gao B., Mirshahi F., Sanyal A.J., Khanolkar A.D., Makriyannis A., & Kunos G. (2000) Functional CB1 cannabinoid receptors in human vascular endothelial cells. *Biochem.J.* **346 Pt 3,** 835-840.

Liu R.H., Mizuta M., & Matsukura S. (2003) Long-term oral nicotine administration reduces insulin resistance in obese rats. *Eur.J.Pharmacol.* **458,** 227-234.

Liu Y.L., Connoley I.P., Wilson C.A., & Stock M.J. (2005) Effects of the cannabinoid CB1 receptor antagonist SR141716 on oxygen consumption and soleus muscle glucose uptake in Lep(ob)/Lep(ob) mice. *Int.J.Obes.(Lond)* **29,** 183-187.

Loram L.C., Harrison J.A., Sloane E.M., Hutchinson M.R., Sholar P., Taylor F.R., Berkelhammer D., Coats B.D., Poole S., Milligan E.D., Maier S.F., Rieger J., & Watkins L.R. (2009) Enduring reversal of neuropathic pain by a single intrathecal injection of adenosine 2A receptor agonists: a novel therapy for neuropathic pain. *J.Neurosci.* **29,** 14015-14025.

Lundqvist T. (2005) Cognitive consequences of cannabis use: comparison with abuse of stimulants and heroin with regard to attention, memory and executive functions. *Pharmacol.Biochem.Behav.* **81,** 319-330.

Mabley J.G., Pacher P., Southan G.J., Salzman A.L., & Szabo C. (2002) Nicotine reduces the incidence of type I diabetes in mice. *J.Pharmacol.Exp.Ther.* **300,** 876-881.

Maldonado R. & Berrendero F. (2010) Endogenous cannabinoid and opioid systems and their role in nicotine addiction. *Curr.Drug Targets.* **11,** 440-449.

Mallet P.E. & Beninger R.J. (1998) The cannabinoid CB1 receptor antagonist SR141716A attenuates the memory impairment produced by delta9-tetrahydrocannabinol or anandamide. *Psychopharmacology (Berl)* **140,** 11-19.

Malmberg A.B. & Basbaum A.I. (1998) Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. *Pain* **76,** 215-222.

Manchikanti L. (2007) National drug control policy and prescription drug abuse: facts and fallacies. *Pain Physician.* **10,** 399-424.

Mancuso G., Warburton D.M., Melen M., Sherwood N., & Tirelli E. (1999) Selective effects of nicotine on attentional processes. *Psychopharmacology (Berl)* **146,** 199-204.

Mano-Otagiri A., Iwasaki-Sekino A., Ohata H., Arai K., & Shibasaki T. (2009) Nicotine suppresses energy storage through activation of sympathetic outflow to brown adipose tissue via corticotropin-releasing factor type 1 receptor. *Neurosci.Lett.* **455,** 26-29.

Martin B.R. & Lichtman A.H. (1998) Cannabinoid transmission and pain perception. *Neurobiol.Dis.* **5,** 447-461.

Martin M., Ledent C., Parmentier M., Maldonado R., & Valverde O. (2002) Involvement of CB1 cannabinoid receptors in emotional behaviour. *Psychopharmacology (Berl)* **159,** 379-387.

Martin T.J. & Ewan E. (2008) Chronic pain alters drug self-administration: implications for addiction and pain mechanisms. *Exp.Clin.Psychopharmacol.* **16,** 357-366.

Martin W.J., Loo C.M., & Basbaum A.I. (1999) Spinal cannabinoids are anti-allodynic in rats with persistent inflammation. *Pain* **82,** 199-205.

Matias I., Cristino L., & Di M., V (2008) Endocannabinoids: some like it fat (and sweet too). *J.Neuroendocrinol.* **20 Suppl 1,** 100-109.

Matsuda L.A., Lolait S.J., Brownstein M.J., Young A.C., & Bonner T.I. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* **346,** 561-564.

Mazzari S., Canella R., Petrelli L., Marcolongo G., & Leon A. (1996) N-(2-hydroxyethyl)hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cell activation. *Eur.J.Pharmacol.* **300,** 227-236.

McCleane G. & Smith H.S. (2007) Opioids for persistent noncancer pain. *Anesthesiol.Clin.* **25,** 787-7ii.

McClernon F.J., Gilbert D.G., & Radtke R. (2003) Effects of transdermal nicotine on lateralized identification and memory interference. *Hum.Psychopharmacol.* **18,** 339-343.

MECHOULAM R. & Hanus L. (2000) A historical overview of chemical research on cannabinoids. *Chem.Phys.Lipids* **108,** 1-13.

Mei J. & Pasternak G.W. (2001) Molecular cloning and pharmacological characterization of the rat sigma1 receptor. *Biochem.Pharmacol.* **62,** 349-355.

Mei J. & Pasternak G.W. (2002) Sigma1 receptor modulation of opioid analgesia in the mouse. *J.Pharmacol.Exp.Ther.* **300,** 1070-1074.

Millan M.J. (1999) The induction of pain: an integrative review. *Prog.Neurobiol.* **57,** 1-164.

Millns P.J., Chapman V., & Kendall D.A. (2001) Cannabinoid inhibition of the capsaicin-induced calcium response in rat dorsal root ganglion neurones. *Br.J.Pharmacol.* **132,** 969-971.

Molina-Holgado F., Gonzalez M.I., & Leret M.L. (1995) Effect of delta 9-tetrahydrocannabinol on short-term memory in the rat. *Physiol Behav.* **57,** 177-179.

Moragrega I., Carrasco M.C., Vicens P., & Redolat R. (2003) Spatial learning in male mice with different levels of aggressiveness: effects of

housing conditions and nicotine administration. *Behav.Brain Res.* **147,** 1-8.

Moreau J.L. & Huber G. (1999) Central adenosine A(2A) receptors: an overview. *Brain Res.Brain Res.Rev.* **31,** 65-82.

Moss D.E. & Johnson R.L. (1980) Tonic analgesic effects of delta 9-tetrahydrocannabinol as measured with the formalin test. *Eur.J.Pharmacol.* **61,** 313-315.

Munro S., Thomas K.L., & bu-Shaar M. (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature* **365,** 61-65.

Nishizaki T., Nagai K., Nomura T., Tada H., Kanno T., Tozaki H., Li X.X., Kondoh T., Kodama N., Takahashi E., Sakai N., Tanaka K., & Saito N. (2002) A new neuromodulatory pathway with a glial contribution mediated via A(2a) adenosine receptors. *Glia* **39,** 133-147.

Ong K.S. & Keng S.B. (2003) The biological, social, and psychological relationship between depression and chronic pain. *Cranio.* **21,** 286-294.

Osei-Hyiaman D., DePetrillo M., Pacher P., Liu J., Radaeva S., Batkai S., Harvey-White J., Mackie K., Offertaler L., Wang L., & Kunos G. (2005) Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *J.Clin.Invest* **115,** 1298-1305.

Ossipov M.H., Lai J., Malan T.P., Jr., & Porreca F. (2000) Spinal and supraspinal mechanisms of neuropathic pain. *Ann.N.Y.Acad.Sci.* **909,** 12-24.

Ozaki S., Narita M., Narita M., Iino M., Miyoshi K., & Suzuki T. (2003) 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.* **116,** 89-97.

Ozaki S., Narita M., Narita M., Iino M., Sugita J., Matsumura Y., & Suzuki T. (2002) 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. *J.Neurochem.* **82,** 1192-1198.

Ozaki S., Narita M., Narita M., Ozaki M., Khotib J., & Suzuki T. (2004) Role of extracellular signal-regulated kinase in the ventral tegmental area in the suppression of the morphine-induced rewarding effect in mice with sciatic nerve ligation. *J.Neurochem.* **88,** 1389-1397.

Pagotto U., Marsicano G., Cota D., Lutz B., & Pasquali R. (2006) The emerging role of the endocannabinoid system in endocrine regulation and energy balance. *Endocr.Rev.* **27,** 73-100.

Papp M., Willner P., & Muscat R. (1991) An animal model of anhedonia: attenuation of sucrose consumption and place preference conditioning by chronic unpredictable mild stress. *Psychopharmacology (Berl).* **104,** 255-259.

Patapoutian A., Peier A.M., Story G.M., & Viswanath V. (2003) ThermoTRP channels and beyond: mechanisms of temperature sensation. *Nat.Rev.Neurosci.* **4,** 529-539.

Pechlivanova D.M. & Georgiev V.P. (2002) Interaction of angiotensin II and adenosine A1 and A2A receptor ligands on the writhing test in mice. *Pharmacol.Biochem.Behav.* **72,** 23-28.

Pertwee R.G. (2009) Emerging strategies for exploiting cannabinoid receptor agonists as medicines. *Br.J.Pharmacol.* **156,** 397-411.

Pertwee R.G. (2008) Ligands that target cannabinoid receptors in the brain: from THC to anandamide and beyond. *Addict.Biol.* **13,** 147-159.

Picciotto M.R. & Zoli M. (2002) Nicotinic receptors in aging and dementia. *J.Neurobiol.* **53,** 641-655.

Piomelli D. (2003) The molecular logic of endocannabinoid signalling. *Nat.Rev.Neurosci.* **4,** 873-884.

Poirier B., Bidouard J.P., Cadrouvele C., Marniquet X., Staels B., O'Connor S.E., Janiak P., & Herbert J.M. (2005) The anti-obesity effect of rimonabant is associated with an improved serum lipid profile. *Diabetes Obes.Metab* **7,** 65-72.

Pomerleau O.F., Turk D.C., & Fertig J.B. (1984) The effects of cigarette smoking on pain and anxiety. *Addict.Behav.* **9,** 265-271.

Puente B.D., Nadal X., Portillo-Salido E., Sanchez-Arroyos R., Ovalle S., Palacios G., Muro A., Romero L., Entrena J.M., Baeyens J.M., Lopez-Garcia J.A., Maldonado R., Zamanillo D., & Vela J.M. (2009) Sigma-1

receptors regulate activity-induced spinal sensitization and neuropathic pain after peripheral nerve injury. *Pain*.

Puighermanal E., Marsicano G., Busquets-Garcia A., Lutz B., Maldonado R., & Ozaita A. (2009) Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. *Nat.Neurosci.* **12,** 1152-1158.

Puma C., Deschaux O., Molimard R., & Bizot J.C. (1999) Nicotine improves memory in an object recognition task in rats. *Eur.Neuropsychopharmacol.* **9,** 323-327.

Racz I., Nadal X., Alferink J., Banos J.E., Rehnelt J., Martin M., Pintado B., Gutierrez-Adan A., Sanguino E., Bellora N., Manzanares J., Zimmer A., & Maldonado R. (2008a) Interferon-gamma is a critical modulator of CB(2) cannabinoid receptor signaling during neuropathic pain. *J.Neurosci.* **28,** 12136-12145.

Racz I., Nadal X., Alferink J., Banos J.E., Rehnelt J., Martin M., Pintado B., Gutierrez-Adan A., Sanguino E., Manzanares J., Zimmer A., & Maldonado R. (2008b) Crucial role of CB(2) cannabinoid receptor in the regulation of central immune responses during neuropathic pain. *J.Neurosci.* **28,** 12125-12135.

Rashid M.H. & Ueda H. (2002) Neuropathy-specific analgesic action of intrathecal nicotinic agonists and its spinal GABA-mediated mechanism. *Brain Res.* **953,** 53-62.

Ravinet T.C., Arnone M., Delgorge C., Gonalons N., Keane P., Maffrand J.P., & Soubrie P. (2003) Anti-obesity effect of SR141716, a CB1 receptor antagonist, in diet-induced obese mice. *Am.J.Physiol Regul.Integr.Comp Physiol* **284,** R345-R353.

Ravinet T.C., Delgorge C., Menet C., Arnone M., & Soubrie P. (2004) CB1 cannabinoid receptor knockout in mice leads to leanness, resistance to diet-induced obesity and enhanced leptin sensitivity. *Int.J.Obes.Relat Metab Disord.* **28,** 640-648.

Rezvani A.H., Bushnell P.J., & Levin E.D. (2002) Effects of nicotine and mecamylamine on choice accuracy in an operant visual signal detection task in female rats. *Psychopharmacology (Berl)* **164,** 369-375.

Rezvani A.H. & Levin E.D. (2001) Cognitive effects of nicotine. *Biol.Psychiatry* **49,** 258-267.

Rigbi A., Yakir A., Sarner-Kanyas K., Pollak Y., & Lerer B. (2010) Why do young women smoke? VI. A controlled study of nicotine effects on attention: pharmacogenetic interactions. *Pharmacogenomics.J.*

Rinaldi-Carmona M., Barth F., Heaulme M., Shire D., Calandra B., Congy C., Martinez S., Maruani J., Neliat G., Caput D., & . (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett.* **350,** 240-244.

Rodriguez de Fonseca F., Fernandez-Ruiz J.J., Murphy L., Eldridge J.C., Steger R.W., & Bartke A. (1991) Effects of delta-9-tetrahydrocannabinol exposure on adrenal medullary function: evidence of an acute effect and development of tolerance in chronic treatments. *Pharmacol.Biochem.Behav.* **40,** 593-598.

Roh D.H., Kim H.W., Yoon S.Y., Seo H.S., Kwon Y.B., Kim K.W., Han H.J., Beitz A.J., Na H.S., & Lee J.H. (2008) 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* **109,** 879-889.

Samovilova N.N. & Vinogradov V.A. (1992) Subcellular distribution of (+)-[3H]SKF 10,047 binding sites in rat liver. *Eur.J.Pharmacol.* **225,** 69-74.

Sang C.N. (2000) NMDA-receptor antagonists in neuropathic pain: experimental methods to clinical trials. *J.Pain Symptom.Manage.* **19,** S21-S25.

Sansone M., Castellano C., Palazzesi S., Battaglia M., & mmassari-Teule M. (1993) Effects of oxiracetam, physostigmine, and their combination on active and passive avoidance learning in mice. *Pharmacol.Biochem.Behav.* **44,** 451-455.

Sawynok J. (1998) Adenosine receptor activation and nociception. *Eur.J.Pharmacol.* **347,** 1-11.

Sawynok J. & Liu X.J. (2003) Adenosine in the spinal cord and periphery: release and regulation of pain. *Prog.Neurobiol.* **69,** 313-340.

Sawzdargo M., Nguyen T., Lee D.K., Lynch K.R., Cheng R., Heng H.H., George S.R., & O'Dowd B.F. (1999) Identification and cloning of three novel human G protein-coupled receptor genes GPR52, PsiGPR53 and GPR55: GPR55 is extensively expressed in human brain. *Brain Res.Mol.Brain Res.* **64,** 193-198.

Schmidt A., Lebel L., Koe B.K., Seeger T., & Heym J. (1989) Sertraline potently displaces (+)-[3H]3-PPP binding to sigma sites in rat brain. *Eur.J.Pharmacol.* **165,** 335-336.

Scholz J. & Woolf C.J. (2002) Can we conquer pain? *Nat.Neurosci.* **5 Suppl,** 1062-1067.

Schreiber S., Backer M.M., & Pick C.G. (1999) The antinociceptive effect of venlafaxine in mice is mediated through opioid and adrenergic mechanisms. *Neurosci.Lett.* **273,** 85-88.

Sebastiao A.M. & Ribeiro J.A. (1996) Adenosine A2 receptor-mediated excitatory actions on the nervous system. *Prog.Neurobiol.* **48,** 167-189.

Sebert M.E. & Shooter E.M. (1993) Expression of mRNA for neurotrophic factors and their receptors in the rat dorsal root ganglion and sciatic nerve following nerve injury. *J.Neurosci.Res.* **36,** 357-367.

Sindrup S.H. & Jensen T.S. (1999) Efficacy of pharmacological treatments of neuropathic pain: an update and effect related to mechanism of drug action. *Pain* **83,** 389-400.

Skuza G. & Wedzony K. (2004) Behavioral pharmacology of sigma-ligands. *Pharmacopsychiatry* **37 Suppl 3,** S183-S188.

Smith H.S. (2008) Opioid-related issues "popping" up again. *Pain Physician* **11,** S1-S4.

Smith P.B., Welch S.P., & Martin B.R. (1994) Interactions between delta 9-tetrahydrocannabinol and kappa opioids in mice. *J.Pharmacol.Exp.Ther.* **268,** 1381-1387.

Solowij N., Stephens R.S., Roffman R.A., Babor T., Kadden R., Miller M., Christiansen K., McRee B., & Vendetti J. (2002) Cognitive functioning of long-term heavy cannabis users seeking treatment. *JAMA* **287,** 1123-1131.

Spring B., Cook J.W., Appelhans B., Maloney A., Richmond M., Vaughn J., Vanderveen J., & Hedeker D. (2008) Nicotine effects on affective response in depression-prone smokers. *Psychopharmacology (Berl)* **196,** 461-471.

Stahl S.M. (1998) Basic psychopharmacology of antidepressants, part 1: Antidepressants have seven distinct mechanisms of action. *J.Clin.Psychiatry* **59 Suppl 4,** 5-14.

Stella N., Schweitzer P., & Piomelli D. (1997) A second endogenous cannabinoid that modulates long-term potentiation. *Nature* **388,** 773-778.

Strekalova T., Spanagel R., Bartsch D., Henn F.A., & Gass P. (2004) Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration. *Neuropsychopharmacology.* **29,** 2007-2017.

Strekalova T. & Steinbusch H.W. (2010) Measuring behavior in mice with chronic stress depression paradigm. *Prog.Neuropsychopharmacol.Biol.Psychiatry.* **34,** 348-361.

Suzuki T., Kishimoto Y., Ozaki S., & Narita M. (2001) Mechanism of opioid dependence and interaction between opioid receptors. *Eur.J.Pain.* **5 Suppl A:63-5.,** 63-65.

Svendsen K.B., Jensen T.S., & Bach F.W. (2004) Does the cannabinoid dronabinol reduce central pain in multiple sclerosis? Randomised double blind placebo controlled crossover trial. *BMJ* **329,** 253.

Svenningsson P., Le M.C., Fisone G., & Fredholm B.B. (1999) Distribution, biochemistry and function of striatal adenosine A2A receptors. *Prog.Neurobiol.* **59,** 355-396.

Taiwo Y.O. & Levine J.D. (1990) Direct cutaneous hyperalgesia induced by adenosine. *Neuroscience* **38,** 757-762.

Takahashi K.A. & Castillo P.E. (2006) The CB1 cannabinoid receptor mediates glutamatergic synaptic suppression in the hippocampus. *Neuroscience* **139,** 795-802.

Tal M. & Bennett G.J. (1994) Extra-territorial pain in rats with a peripheral mononeuropathy: mechano-hyperalgesia and mechano-allodynia in the territory of an uninjured nerve. *Pain* **57,** 375-382.

Tandrup T., Woolf C.J., & Coggeshall R.E. (2000) Delayed loss of small dorsal root ganglion cells after transection of the rat sciatic nerve. *J.Comp Neurol.* **422,** 172-180.

Tanga F.Y., Raghavendra V., & DeLeo J.A. (2004) Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. *Neurochem.Int.* **45,** 397-407.

Taylor B.K. (2009) Spinal inhibitory neurotransmission in neuropathic pain. *Curr.Pain Headache Rep.* **13,** 208-214.

Terranova J.P., Michaud J.C., Le F.G., & Soubrie P. (1995) Inhibition of long-term potentiation in rat hippocampal slices by anandamide and WIN55212-2: reversal by SR141716 A, a selective antagonist of CB1 cannabinoid receptors. *Naunyn Schmiedebergs Arch.Pharmacol.* **352,** 576-579.

Terranova J.P., Storme J.J., Lafon N., Perio A., Rinaldi-Carmona M., Le F.G., & Soubrie P. (1996) Improvement of memory in rodents by the selective CB1 cannabinoid receptor antagonist, SR 141716. *Psychopharmacology (Berl)* **126,** 165-172.

Thakur G.A., Tichkule R., Bajaj S., & Makriyannis A. (2009) Latest advances in cannabinoid receptor agonists. *Expert.Opin.Ther.Pat* **19,** 1647-1673.

Tham S.M., Angus J.A., Tudor E.M., & Wright C.E. (2005) Synergistic and additive interactions of the cannabinoid agonist CP55,940 with mu opioid receptor and alpha2-adrenoceptor agonists in acute pain models in mice. *Br.J.Pharmacol.* **144,** 875-884.

Thornton-Jones Z.D., Vickers S.P., & Clifton P.G. (2005) The cannabinoid CB1 receptor antagonist SR141716A reduces appetitive and consummatory responses for food. *Psychopharmacology (Berl)* **179,** 452-460.

Tizabi Y., Overstreet D.H., Rezvani A.H., Louis V.A., Clark E Jr, Janowsky D.S., & Kling M.A. (1999) Antidepressant effects of nicotine in an animal model of depression. *Psychopharmacology (Berl)* **142,** 193-199.

Tsou K., Brown S., Sanudo-Pena M.C., Mackie K., & Walker J.M. (1998) Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience* **83,** 393-411.

Tucci S.A., Rogers E.K., Korbonits M., & Kirkham T.C. (2004) The cannabinoid CB1 receptor antagonist SR141716 blocks the orexigenic effects of intrahypothalamic ghrelin. *Br.J.Pharmacol.* **143,** 520-523.

Ulugol A., Ozyigit F., Yesilyurt O., & Dogrul A. (2006) The additive antinociceptive interaction between WIN 55,212-2, a cannabinoid agonist, and ketorolac. *Anesth.Analg.* **102,** 443-447.

Vadalouca A., Siafaka I., Argyra E., Vrachnou E., & Moka E. (2006) Therapeutic management of chronic neuropathic pain: an examination of pharmacologic treatment. *Ann.N.Y.Acad.Sci.* **1088,** 164-186.

Valjent E. & Maldonado R. (2000) A behavioural model to reveal place preference to delta 9-tetrahydrocannabinol in mice. *Psychopharmacology (Berl)* **147,** 436-438.

Valjent E., Mitchell J.M., Besson M.J., Caboche J., & Maldonado R. (2002) Behavioural and biochemical evidence for interactions between Delta 9-tetrahydrocannabinol and nicotine. *Br.J.Pharmacol.* **135,** 564-578.

Vanegas H. & Schaible H.G. (2004) Descending control of persistent pain: inhibitory or facilitatory? *Brain Res.Brain Res.Rev.* **46,** 295-309.

Vicens P., Carrasco M.C., & Redolat R. (2003) Effects of early training and nicotine treatment on the performance of male NMRI mice in the water maze. *Neural Plast.* **10,** 303-317.

Viveros M.P., Marco E.M., & File S.E. (2006) Nicotine and cannabinoids: parallels, contrasts and interactions. *Neurosci.Biobehav.Rev.* **30,** 1161-1181.

Wagner R., Janjigian M., & Myers R.R. (1998) Anti-inflammatory interleukin-10 therapy in CCI neuropathy decreases thermal hyperalgesia, macrophage recruitment, and endoneurial TNF-alpha expression. *Pain* **74,** 35-42.

Wall T.L., Schoedel K., Ring H.Z., Luczak S.E., Katsuyoshi D.M., & Tyndale R.F. (2007) Differences in pharmacogenetics of nicotine and alcohol metabolism: review and recommendations for future research. *Nicotine.Tob.Res.* **9 Suppl 3,** S459-S474.

Ward S.J. & Dykstra L.A. (2005) The role of CB1 receptors in sweet versus fat reinforcement: effect of CB1 receptor deletion, CB1 receptor antagonism (SR141716A) and CB1 receptor agonism (CP-55940). *Behav.Pharmacol.* **16,** 381-388.

Wasner G., Schattschneider J., Binder A., & Baron R. (2004) Topical menthol--a human model for cold pain by activation and sensitization of C nociceptors. *Brain* **127,** 1159-1171.

Watkins L.R., Milligan E.D., & Maier S.F. (2001) Glial activation: a driving force for pathological pain. *Trends Neurosci.* **24,** 450-455.

Weidenfeld J., Bodoff M., Saphier D., & Brenner T. (1989) Further studies on the stimulatory action of nicotine on adrenocortical function in the rat. *Neuroendocrinology* **50,** 132-138.

Weissman A., Milne G.M., & Melvin L.S., Jr. (1982) Cannabimimetic activity from CP-47,497, a derivative of 3-phenylcyclohexanol. *J.Pharmacol.Exp.Ther.* **223,** 516-523.

Welch S.P., Dunlow L.D., Patrick G.S., & Razdan R.K. (1995) Characterization of anandamide- and fluoroanandamide-induced antinociception and cross-tolerance to delta 9-THC after intrathecal administration to mice: blockade of delta 9-THC-induced antinociception. *J.Pharmacol.Exp.Ther.* **273,** 1235-1244.

Wieseler-Frank J., Maier S.F., & Watkins L.R. (2005) Central proinflammatory cytokines and pain enhancement. *Neurosignals.* **14,** 166-174.

Wilens T.E. & Decker M.W. (2007) Neuronal nicotinic receptor agonists for the treatment of attention-deficit/hyperactivity disorder: focus on cognition. *Biochem.Pharmacol.* **74,** 1212-1223.

Wilkie G.I., Hutson P.H., Stephens M.W., Whiting P., & Wonnacott S. (1993) Hippocampal nicotinic autoreceptors modulate acetylcholine release. *Biochem.Soc.Trans.* **21,** 429-431.

Wilson R.I. & Nicoll R.A. (2002) Endocannabinoid signaling in the brain. *Science* **296,** 678-682.

Wolff M., Heugel P., Hempelmann G., Scholz A., Muhling J., & Olschewski A. (2007) Clonidine reduces the excitability of spinal dorsal horn neurones. *Br.J.Anaesth.* **98,** 353-361.

Wood J.N., Boorman J.P., Okuse K., & Baker M.D. (2004) Voltage-gated sodium channels and pain pathways. *J.Neurobiol.* **61,** 55-71.

Woodruff-Pak D.S. (2003) Mecamylamine reversal by nicotine and by a partial alpha7 nicotinic acetylcholine receptor agonist (GTS-21) in rabbits tested with delay eyeblink classical conditioning. *Behav.Brain Res.* **143,** 159-167.

Yamamoto H., Miura R., Yamamoto T., Shinohara K., Watanabe M., Okuyama S., Nakazato A., & Nukada T. (1999) Amino acid residues in the transmembrane domain of the type 1 sigma receptor critical for ligand binding. *FEBS Lett.* **445,** 19-22.

Yang X., Criswell H.E., & Breese G.R. (1996) Nicotine-induced inhibition in medial septum involves activation of presynaptic nicotinic

cholinergic receptors on gamma-aminobutyric acid-containing neurons. *J.Pharmacol.Exp.Ther.* **276,** 482-489.

Zarrindast M.R., Bakhsha A., Rostami P., & Shafaghi B. (2002) Effects of intrahippocampal injection of GABAergic drugs on memory retention of passive avoidance learning in rats. *J.Psychopharmacol.* **16,** 313-319.

Zimmermann H. (2000) Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch.Pharmacol.* **362,** 299-309.

APPENDIX

Galeote L, Berrendero F, Bura SA, Zimmer A, Maldonado R. Prodynorphin gene disruption increases the sensitivity to nicotine self-administration in mice. Int J Neuropsychopharmacol. 2009; 12(5): 615-25.

Parlato R, Cruz H, Otto C, Murtra P, Parkitna JR, Martin M, et al. Effects of the cell type-specific ablation of the cAMP-responsive transcription factor in noradrenergic neurons on locus coeruleus firing an withdrawal behavior after chronic exposure to morphine. J Neurochem. 2010; 115(3): 563-73.

Agudo J, Martin M, Roca C, Molas M, Bura AS Zimmer A, et al. Deficiency of CB2 cannabinoid receptor in mice improves insulin sensitivity but increases food intake and obesity with age. Diabetologia. 2010; 53(12): 2629-40.