

**First European Workshop on Cannabinoid Research  
Madrid (Spain), April 4-5, 2003**



**organized by the:**

**“Agencia Antidroga de la Comunidad de Madrid”**



**Agencia Antidroga**



**and the**

**“Sociedad Española de Investigación sobre Cannabinoides”**



# Scientific Programme

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**April 4, 2003**

## **9:00 Opening**

- Ilmo. Sr. José Manuel Torrecilla, Gerente de la Agencia Antidroga de la Comunidad de Madrid
- Ilmo. Sr. Angel Nogales, Decano de la Facultad de Medicina de la Universidad Complutense de Madrid
- José Antonio Ramos Atance, Presidente de la Sociedad Española de Investigación sobre Cannabinoides

## **9:30-14:00 1<sup>st</sup> session: Pharmacology and Biochemistry of Cannabinoids** (chairs: Roger G. Pertwee and Julián Romero)

- 9:30 Pharmacology of cannabinoid receptors  
Roger G. Pertwee, UK
- 10:00 The endocannabinoid membrane transporter: An elusive protein  
Vincenzo Di Marzo, Italy
- 10:30 Biochemistry and pharmacology of FAAH, the enzyme responsible for the metabolism of anandamide and related *N*-acyl ethanolamines  
Christopher Fowler, Sweden

## **11:00 Coffee break**

- 11:30 Signaling systems coupled to cannabinoid receptors  
Inés Díaz-Laviada, Spain
- 12:00 Distribution of cannabinoid receptors and ligands in the brain  
Julián Romero, Spain
- 12:30 Cannabinoids modulate neurotransmission  
Bela Szabo, Germany
- 13:00 Novel cannabinoid analogs with therapeutic potential  
M.L. López-Rodríguez, Spain
- 13:30 General Discussion

## **14:00-17:00 Poster presentation (lunch included)**

## **17:00 Coffee break**

## **17:30-20:00 2<sup>nd</sup> Session: Functions of the endocannabinoid signaling system** (chairs: Beat Lutz and Manuel Guzmán)

- 17:30 Role of the endocannabinoid system in nociception and cannabinoid withdrawal  
Jorge Manzanares, Spain
- 18:00 Cannabinoids and memory  
Beat Lutz, Germany

- 18:30 Cannabinoids and motor control  
Javier Fernández-Ruiz, Spain
- 19:00 Cannabinoids and cell death/survival decision  
Manuel Guzmán, Spain
- 19:30 Cannabinoids and neuroendocrine control  
Tibor Wenger, Hungary

## **April 5, 2003**

### **9:00-11:00 2<sup>nd</sup> Session (cont.): Functions of the endocannabinoid signaling system**

- 9:00 Cannabinoids and inflammation  
Carmen Guaza, Spain
- 9:30 The ubiquitous role of endocannabinoids in physiological processes: Examples in neuroprotection, feeding and bone formation  
Raphael Mechoulam, Israel
- 10:00 Cannabinoids and feeding behavior  
Gerard Le Fur, France
- 10:30 General Discussion

### **11:00 Coffee break**

### **11:30-14:00 3<sup>rd</sup> session: Cannabinoids and drugs of abuse (chairs: Daniela Parolaro and Rafael Maldonado)**

- 11:30 Neurobiology of cannabinoid tolerance and dependence  
Daniela Parolaro, Italy
- 12:00 Interactions between cannabinoids and opioids  
Rafael Maldonado, Spain
- 12:30 Endocannabinoids and alcoholism: a substrate for allostasis  
Fernando Rodríguez de Fonseca, Spain
- 13:00 Cannabis and psychiatric pathology  
Luis Núñez Domínguez, Spain
- 13:30 General Discussion

### **14:00 End**

# Lectures

## PHARMACOLOGY OF CANNABINOID RECEPTORS

Roger G. Pertwee

Department of Biomedical Sciences, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, United Kingdom

Mammalian tissues contain at least two types of cannabinoid receptor, CB<sub>1</sub> and CB<sub>2</sub>. These are both coupled through G<sub>i/o</sub> protein, negatively to adenylate cyclase and positively to mitogen-activated protein kinase. The CB<sub>1</sub> receptor is also coupled through G protein to certain types of calcium and potassium channel. CB<sub>1</sub> receptors are found mainly on central and peripheral neurones and one function of these receptors is to inhibit neurotransmitter release. CB<sub>2</sub> receptors are present mainly on immune cells. Their roles are proving more difficult to establish but seem to include the modulation of cytokine release.

Several endogenous ligands for cannabinoid receptors have been identified. These “endocannabinoids” are all eicosanoids, for example arachidonoyl ethanolamide (anandamide), 2-arachidonoyl glycerol (2-AG) and 2-arachidonoyl glyceryl ether (noladin ether). Apart from endocannabinoids, other notable CB<sub>1</sub> and CB<sub>2</sub> receptor agonists include the CB<sub>1</sub>/CB<sub>2</sub> ligands delta-9-tetrahydrocannabinol (THC), HU-210, CP55940 and *R*-(+)-WIN55212, the CB<sub>1</sub>-selective ligands, methanandamide, ACEA, ACPA and O-1812 and the CB<sub>2</sub>-selective ligands, L759633, L759656, JWH-133 and HU-308. Cannabinoid receptor antagonists/inverse agonists have also been developed, the most important of these being SR141716A and LY320135, which are CB<sub>1</sub>-selective, and SR144528 and AM630, which are CB<sub>2</sub>-selective. In contrast to most other cannabinoid receptor agonists, anandamide and methanandamide activate vanilloid (VR1) as well as cannabinoid receptors. Some metabolites of anandamide also have pharmacological activity. Thus, certain *in vivo* effects of anandamide seem to depend on its conversion by fatty acid amide hydrolase to arachidonic acid, and in some tissues, anandamide exhibits much less efficacy at vanilloid receptors than metabolites to which it is converted by lipoxygenases.

Evidence has emerged for a number of novel cannabinoid receptor subtypes. These include:

- SR141716A-sensitive non-CB<sub>1</sub>, non-CB<sub>2</sub>, non-vanilloid-receptors, present in some arteries, that are activated by anandamide and abnormal cannabidiol but not by 2-AG or by established non-eicosanoid CB<sub>1</sub>/CB<sub>2</sub> receptor agonists and that are antagonized by the non-psychotropic plant cannabinoid, cannabidiol;
- SR141716A-sensitive non-CB<sub>1</sub>, non-CB<sub>2</sub>, non-vanilloid-receptors on nerve terminals in the guinea pig ileum that are activated by anandamide;
- SR141716A-sensitive, non-CB<sub>1</sub>, ‘vanilloid-like’ receptors within the brain that are activated by capsaicin, *R*-(+)-WIN55212 and CP55940 and antagonized by capsazepine;
- SR141716A-sensitive, non-CB<sub>1</sub>, G protein-coupled, imidazoline/EDG receptors, at sympathetic nerve terminals and within the brain, that are activated by CP55940, HU-210 and *R*-(+)-WIN55212;
- SR141716A-insensitive non-CB<sub>1</sub>, non-CB<sub>2</sub>, non-vanilloid receptors that are expressed in some brain areas and activated by *R*-(+)-WIN55212 or anandamide but not by THC, HU-210 or CP55940;
- SR141716A-insensitive, SR144528-sensitive, non-vanilloid, ‘CB<sub>2</sub>-like’ peripheral receptors on which palmitoylethanolamide but not anandamide may act to relieve inflammatory pain;
- receptors on nerve terminals in the mouse vas deferens at which AM630 is 20 times more potent as an antagonist of THC than of anandamide;
- SR141716A-insensitive allosteric sites on 5-HT<sub>3</sub> receptors that are more sensitive to THC than to *R*-(+)-WIN55212, anandamide or CP55940 and that when occupied by these cannabinoids, oppose inward neuronal currents triggered by activation of the 5-HT<sub>3</sub> receptor;

- allosteric non-CB<sub>1</sub>, non-CB<sub>2</sub> sites on muscarinic M<sub>1</sub> and M<sub>4</sub> receptors with affinity for anandamide and methanandamide but not for *R*-(+)-WIN55212 or SR141716A;
- SR141716A-insensitive allosteric sites on AMPA GLU<sub>A3</sub> receptors with which anandamide but not *R*-(+)-WIN55212 can interact to inhibit kainate-activated currents.

In addition, results recently obtained in this laboratory from experiments with the mouse isolated vas deferens point to the existence of novel non-CB<sub>1</sub>, non-CB<sub>2</sub> site(s) through which cannabidiol can act to (i) augment electrically-evoked release of contractile transmitters, (ii) oppose the ability of CB<sub>1</sub> receptor agonists and certain non-cannabinoids to inhibit evoked neurotransmitter release and (iii) produce non-competitive antagonism of alpha<sub>1</sub>-adrenoceptor agonists such as phenylephrine.

## THE ENDOCANNABINOID MEMBRANE TRANSPORTER: AN ELUSIVE PROTEIN

Vincenzo Di Marzo

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Five endogenous compounds capable of functionally activating either or both cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors have been identified so far. These are, in chronological order of discovery: i) *N*-arachidonylethanolamine (anandamide, AEA) (Devane et al., *Science*, 1994), and some of its polyunsaturated congeners; ii) 2-arachidonoylglycerol (2-AG) (Mechoulam et al., *Biochem. Pharmacol.*, 1995; Sugiura et al., *BBRC*, 1995); iii) 2-arachidonyl glyceryl ether (noladin) (Hanus et al., *PNAS*, 2001); iv) *O*-arachidonoyl-ethanolamine (virodhamine) (Porter et al., *JPET*, 2002); and v) *N*-arachidonoyl-dopamine (NADA) (Huang et al., *PNAS*, 2002). The metabolism of these five compounds occurs to some extent via different enzymatic reactions, including: i) hydrolysis of the amide or ester bonds (for AEA and 2-AG); ii) direct esterification into membrane (phospho)glycerides (for 2-AG and noladin); iii) oxidation (for AEA and 2-AG); and iv) methylation of the aromatic moiety (for NADA). However, in all cases, endocannabinoid metabolism must be preceded by endocannabinoid cellular uptake, since all the metabolic reactions discovered so far occur in intracellular compartments.

Evidence has accumulated for the existence of a common molecular mechanism facilitating the cellular uptake of all endocannabinoids (Di Marzo et al., *Nature*, 1994; *Biochem. J.*, 1998; Beltramo and Pomelli, *Neuroreport*, 2000; Bisogno et al., *Eur. J. Biochem.* 2001; Fezza et al., *FEBS Letts*, 2001; Porter et al., *JPET*, 2002; Huang et al., *PNAS*, 2002). This mechanism mediates the transport of these compounds in the direction of the gradient of concentrations across the cell membrane, and is: 1) rapid ( $t_{1/2}$ =3-7 min); 2) temperature-dependent; 3) saturable; 4) rather selective for arachidonate-containing endocannabinoids; 5) up-regulated by nitric oxide; and 6) Na<sup>+</sup>- and ATP-independent (Hillard and Jarrahian, *Chem. Phys. Lipids*, 2000; Fowler and Jacobsson, *Prostagl. Leukotr. Ess. Fatty Acids*, 2002). However, to date, no molecular evidence has been reported yet to support the existence of a protein acting as an “endocannabinoid membrane transporter”. Since AEA cellular uptake is dependent on AEA intracellular metabolism, it has been instead suggested (Deutsch et al., *JBC*, 2001) that there is no AEA transporter, and that AEA uptake occurs via passive diffusion through the plasma membrane, a process that can be made rapid by the very efficacious intracellular hydrolysis of this endocannabinoid, which is catalyzed by the fatty acid amide hydrolase (FAAH) (Cravatt et al., *Nature*, 1996). Recent structural data obtained on FAAH crystals, showing that this enzyme is indeed capable of associating with the plasma membrane, seem to support this hypothesis (Bracey et al., *Science*, 2002).

Yet, still very strong, albeit indirect, evidence supports the existence of an endocannabinoid transporter. In particular: 1) numerous synthetic substances have been developed that selectively inhibit anandamide cellular uptake without inhibiting FAAH (De Petrocellis et al., *FEBS Letts*. 2000; Lopez-Rodriguez et al., *J. Med. Chem.*, 2001; Di Marzo et al., *JPET*, 2002; Ortar et al., *Biochem. Pharmacol.* 2003); conversely, several non-FAAH-expressing cells still take up AEA quite rapidly, and some FAAH inhibitors inhibit AEA uptake only at concentrations higher than those required to inhibit AEA hydrolysis; 2) inhibitors of AEA cellular uptake *enhance* those effects of AEA that are mediated by cannabinoid receptors, but *inhibit* the effects of AEA on vanilloid VR1 receptors (due to the fact that the VR1 binding site is on the intracellular side) - if these inhibitors were acting simply by inhibiting FAAH, they should enhance both CB<sub>1</sub>- and VR1-mediated effects of AEA, as demonstrated for some

FAAH inhibitors (De Petrocellis et al., *JBC*, 2001); 3) NADA and noladin are still rapidly taken up by, e.g., RBL-2H3 cells, and yet they are either very stable or refractory to enzymatic hydrolysis, respectively (Fezza et al., *FEBS Letts.*, 2001; Huang et al., *PNAS*, 2002); 4) nitric oxide, peroxynitrite and superoxide anions stimulate AEA cellular re-uptake without affecting FAAH activity (Maccarrone et al., *JBC*, 2000). Furthermore, it must be kept into account that discrepant data are easily obtained in studies on the putative endocannabinoid transporter, since assays of endocannabinoid cellular uptake are extremely sensitive to the experimental procedures used (Bisogno et al., *Eur. J. Biochem.*, 2001).

While waiting for definitive evidence supporting, or ruling out, the existence of the endocannabinoid transporter, selective inhibitors of AEA cellular uptake were shown to be promising therapeutic tools for the alleviation of disorders of both central and peripheral organs. In particular, the compound known as VDM11 has been shown to exert “indirect” endocannabinoid-like effects, but only in those animal models where endocannabinoid signalling was found to be enhanced, possibly to exert a “tonic” beneficial action. Thus, enhanced levels of anti-spastic, anti-diarrhea and anti-tumour endocannabinoids, and/or of cannabinoid receptors, were found in: 1) the spinal cord of mice with chronic relapsing experimental allergic encephalomyelitis (CREAE), during the spastic phase; 2) the small intestine of cholera-toxin treated mice; and 3) malignant thyroid cell-derived carcinomas respectively. Accordingly, VDM11 inhibits: 1) spasticity in mice with CREAE (Baker et al., *FASEB J.*, 2001); 2) cholera toxin-induced intestinal secretion and diarrhea (Izzo et al., *Gastroenterology*, in press); and 3) the growth of malignant thyroid cell-derived carcinoma in athymic mice (M. Bifulco, G. Portella and V. Di Marzo, unpublished results), respectively. By contrast, no enhancement of the levels of neuroprotective endocannabinoids was found in the brain of an animal model of neuronal excitotoxicity, where, in fact, VDM11 induces no neuroprotective action (van der Stelt et al., *J. Neurosci.*, 2001). In an animal model of Huntington’s chorea, where striatal endocannabinoid signalling is impaired, VDM11 has also no beneficial anti-hyperkinetic action (Lastres-Becker et al., *J. Neurochem.*, 2003).

If their pharmacological and therapeutic effects are due to the enhancement of the endogenous levels of endocannabinoids, inhibitors of the endocannabinoid transporter, administered systemically or locally, should increase the tissue and plasma amounts of AEA. Indeed, this was found for AM404 (Beltramo et al., *Science*, 1997; Giuffrida et al., *Eur. J. Pharmacol.*, 2000). However, it is now recognized that this compound activates vanilloid VR1 receptors at doses lower than those necessary to inhibit AEA re-uptake (Zygmunt et al., *Eur. J. Pharmacol.*, 2000; Ross et al., *Br. J. Pharmacol.*, 2001), and that stimulation of these receptors by capsaicin leads to the biosynthesis of AEA (Di Marzo et al., *Eur. J. Pharmacol.*, 2001; Ahluwalia et al., *J. Neurochem.*, 2003). Hence, a direct effect of AM404 on AEA biosynthesis, rather than inactivation, appears more likely. By contrast, VDM11, which is almost inactive at VR1 receptors, enhances AEA levels in cancer cells and tissue and, at the same time, inhibits cancer growth (M. Bifulco, G. Portella and V. Di Marzo, unpublished results). These observations underline the importance of developing ever more selective inhibitors of endocannabinoid cellular uptake in order to understand the true physiopathological relevance of this process, and to ascertain the presence of a transporter.

In conclusion, despite the fact that no molecular evidence has been found yet for the existence of the endocannabinoid membrane transporter, there are today more examples of possible therapeutic uses of inhibitors of this elusive protein than of FAAH inhibitors. An intensive experimental effort should now be made to characterize this molecule, and to develop inhibitors with ever higher potency, selectivity and bio-availability.

## **BIOCHEMISTRY AND PHARMACOLOGY OF FATTY ACID AMIDE HYDROLASE, THE ENZYME RESPONSIBLE FOR THE METABOLISM OF ANANDAMIDE AND RELATED *N*-ACYL ETHANOLAMINES**

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The ability of endocannabinoids to act as signalling molecules requires efficient pathways both for their synthesis and removal. A key enzyme in this respect is fatty acid amide hydrolase (FAAH), which catalyses the metabolism of anandamide to form arachidonic acid. FAAH has a wide substrate-specificity, and can catalyse a variety of biologically active *N*-acyl ethanolamines and *N*-acylamines. In the brain, FAAH has a localisation that is complimentary to that for CB<sub>1</sub> cannabinoid receptors. Within cells, FAAH is located to mitochondrial and microsomal fractions, suggesting that extracellular anandamide must be accumulated within the cell prior to its metabolism.

A number of different compounds have been found to inhibit the activity of FAAH. Such compounds include non-selective inhibitors like phenylmethylsulfonyl fluoride, substrate analogues like arachidonoyl trifluoromethyl ketone, and, most recently, carbamate analogues such as URB532 (3'-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate). This compound has an ID<sub>50</sub> value of 150 µg/kg following i.p. administration to rats, shows good selectivity for FAAH over CB receptors, and produces antinociceptive and anxiolytic effects that are attenuated by the CB<sub>1</sub> receptor antagonist rimonabant (SR141716A) (Kathuria et al., *Nature Med* 9 [2003] 76-81). This result, together with the findings a) that mice lacking FAAH possess a higher pain threshold than the corresponding wild-type animals (Cravatt et al., *Proc Natl Acad Sci USA* 98 [2001] 9371-6) and b) that the FAAH inhibitor AM374 (palmitoylsulfonyl fluoride) reduces spasticity in a mouse model of multiple sclerosis (Baker et al., *FASEB J* 15 [2001] 300-2) suggest that inhibition of FAAH may be an interesting target for drug development.

Recent data, however, suggest that FAAH inhibitors may already be in clinical use. The non-steroidal anti-inflammatory agents (R)-ibuprofen and (S)-flurbiprofen inhibit the metabolism of FAAH by intact cells in a pH sensitive manner, with IC<sub>50</sub> values of 26 and 14 µM at an extracellular pH value of 6.2 (i.e. at a pH that would be found in inflamed tissue) (Holt and Fowler, in press). Flurbiprofen, when administered intrathecally, reduced inflammatory pain in the formalin test in a manner that is blocked by CB<sub>1</sub> receptor antagonism (Ates et al., *Eur. J. Neurosci.* 17 [2003] 597-604). These authors suggested that this effect was the result of a combination of FAAH inhibition together with a shift in endocannabinoid synthesis secondary to cyclooxygenase inhibition.

## SIGNALLING SYSTEMS COUPLED TO CANNABINOID RECEPTORS

Inés Díaz-Laviada

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Cannabinoids are the constituents of the marijuana plant (*Cannabis sativa*) of which the principal psychoactive ingredient is  $\Delta^9$ -Tetrahydrocannabinol (THC). Cannabinoids exert a wide spectrum of effects in humans through the interaction with specific cannabinoid receptors [reviewed in 1]. Two types of cannabinoid receptors, CB<sub>1</sub> and CB<sub>2</sub>, have been identified and emerging evidences suggest the existence of additional receptors. The CB<sub>1</sub> cannabinoid receptor is highly expressed in central nervous system although there are increasing evidences showing that CB<sub>1</sub> is expressed in peripheral tissues. Tissue distribution of CB<sub>2</sub> is different from that of CB<sub>1</sub> being located mainly in immune tissues and cells. The structure of CB<sub>1</sub> and CB<sub>2</sub> is consistent with heptahelical receptor family also named G protein-coupled receptors (GPCRs), that are single polypeptides with seven  $\alpha$ -helices-domains. Activation of cannabinoid receptors promotes its interaction with G proteins, resulting in guanosine diphosphate/guanosine triphosphate exchange and subsequent dissociation of the  $\alpha$  and  $\beta\gamma$  subunits. These subunits regulate the activity of multiple effector proteins including adenylyl cyclases, ion channels, phosphoinositide 3-kinase, and phospholipases [2].

We have shown that CB<sub>1</sub> cannabinoid receptor is expressed in human prostate gland and prostate tumor cells PC-3 and LNCaP [3]. Moreover, an anandamide specific transporter is also expressed in PC-3 cells. Prostate CB<sub>1</sub> is coupled to different signalling pathways exerting adenylyl cyclase inhibition and extracellular signal-regulated kinase cascade activation. Several mechanisms have been proposed for the coupling of GPCRs receptors to MAP kinase cascade. They include (1) signals initiated by classical G protein effectors, e.g. PKA, PKC, (2) signals triggered by direct interaction between  $\beta$ -arrestins and components of the MAPK cascade, (3) signals initiated by cross-talk between GPCRs and receptor tyrosine kinases (RTKs) by a mechanism named “receptor transactivation”, (4) signals regulated by  $\beta\gamma$  subunits upon dissociation from heterotrimeric G proteins, and (5) signals initiated by cAMP independently of PKA. By using PI3K specific inhibitors, we have seen that activation of ERK by cannabinoids in prostate cells is mediated by a PI3K-dependent mechanism. Cannabinoids also induce a phosphorylation increase in PKB, a protein kinase activated by PI3K.

Other transduction signals activated by cannabinoids in prostate cells include an increase in intracellular calcium concentration.

One of the most interesting research areas in cannabinoid research is the regulation of cellular growth by cannabinoids. The endogenous cannabinoid anandamide and other cannabinoid agonists regulate cellular proliferation in many cell types but the underlying biochemical mechanisms remain unclear. R-(+)-methanandamide (MET) and  $\Delta^9$ -tetrahydrocannabinol (THC) at nanomolar concentration, stimulate epithelial prostate cell growth through a receptor-dependent mechanism. However, high doses of cannabinoids induce cellular apoptosis by a CB<sub>1</sub>- and CB<sub>2</sub>-independent mechanism. This results indicate new effects of cannabinoids in the regulation of prostate cellular growth.

1. Howlett, A. C.; Barth, F.; Bonner, T. I.; Cabral, G.; Casellas, P.; Devane, W. A.; Felder, C. C.; Herkenham, M.; mackie, K.; Martin, B. R.; Mechoulam, R.; Pertwee, R. G. *Pharmacol. Rev.* 2002, 54, 161.
2. McAllister, S. D.; Glass, M. *Prostaglandins Leukot.Essent. Fatty Acids*, 2002, 66, 161.
3. Ruiz-Llorente, L.; Sánchez, M. G.; Carmena, M. J.; Prieto, J. C.; Sánchez-Chapado, M.; Izquierdo, A.; Díaz-Laviada, I. *Prostate* 2003, 54, 95.

*Acknowledgements.* Work in the author's laboratory is supported by grants from Spanish Ministerio de Ciencia y tecnología (Saf 2002/01572) and Agencia Antidroga de la Comunidad de Madrid.

## DISTRIBUTION OF CANNABINOID RECEPTORS AND LIGANDS IN THE BRAIN

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Our knowledge on the distribution of the different constituents of the Endocannabinoid System (ECS) has experienced a great impulse in the few past years. These data provide us with the neuroanatomical basis necessary to explain many of the effects of both natural and synthetic cannabinoids. In parallel with improvements in the methodological approaches used, molecular aspects of this system have been unveiled, so attention has been focused on cannabinoid CB<sub>1</sub> receptor subtype, its endogenous ligands, and the uptake carrier and the degrading enzyme (fatty acid amide hydrolase, FAAH) for the so called “endocannabinoids”. These elements of the ECS are differentially distributed in the brain, with particular aspects regarding their localization and density. Thus, CB<sub>1</sub> receptors are known to be present in many brain regions, with highest densities in motor-related structures, such as cortex, basal ganglia, and cerebellum. Also limbic areas contain high levels of these receptors, which has been thought to be the neuroanatomical substrate for many of the classical effects attributed to cannabinoids. Among concrete cell’s populations, CB<sub>1</sub> receptors are expressed in cortical pyramidal neurons, cerebellar Purkinje neurons, medium-sized striatal neurons, and others.

The search for the endogenous ligands for these receptors was culminated with the discovery of a family of structurally-related compounds, currently referred to as “endocannabinoids”. The quantification of these compounds has revealed as extremely difficult, mainly due to the intrinsic unstability of their chemical structures. In addition, a postmortem massive generation of these compounds has been described. As a consequence, the precise distribution of these compounds has been hard to determine, although some data have been recently obtained. Thus, structures that exhibit highest amounts of anandamide and 2-arachidonoylglycerol include striatum, hippocampus, limbic structures, brain stem and spinal cord. The precise cells that produce these ligands are still unknown, as experimental tools for this kind of determination are still lacking. In this sense, it would be extremely useful to fully characterize the uptake carrier for endocannabinoids, as the study of its distribution would help to reveal which cells produce and release these compounds.

FAAH distribution has been also widely studied. While it seems to be restricted to large cells in the rat CNS (pyramidal cortical and hippocampal neurons, Purkinje neurons, etc), its presence has also been described in glial cells of the human CNS. The precise meaning of this discrepancy is still unknown.

Finally, it is important to notice that the knowledge of the distribution of the components of the ECS is also a crucial tool in understanding the possible roles that this system may play in several pathophysiological processes. Thus, recent data obtained in our laboratory points to a possible leading role of this system in the inflammatory processes associated to the deposition of the beta-amyloid peptide in Alzheimer’s disease. This is based on the selective overexpression of FAAH and CB<sub>2</sub> receptors in the neuritic plaques-associated glia that can be observed in tissue samples from the brains of Alzheimer’s disease patients. The term “selectively” is employed in two senses: first, that while CB<sub>2</sub> receptors and FAAH exhibit up-regulation in glial cells associated with senile plaques, CB<sub>1</sub> receptor density is not modified in the vicinity of these pathological structures. Secondly, FAAH expression appears to be restricted to reactive astrocytes, and CB<sub>2</sub> receptors are only expressed in activated microglial

cells. Whether this up-regulation is specific of AD or is common to other pathologies that exhibit reactive gliosis is being currently investigated in our laboratory.

# CANNABINOIDS MODULATE NEUROTRANSMISSION

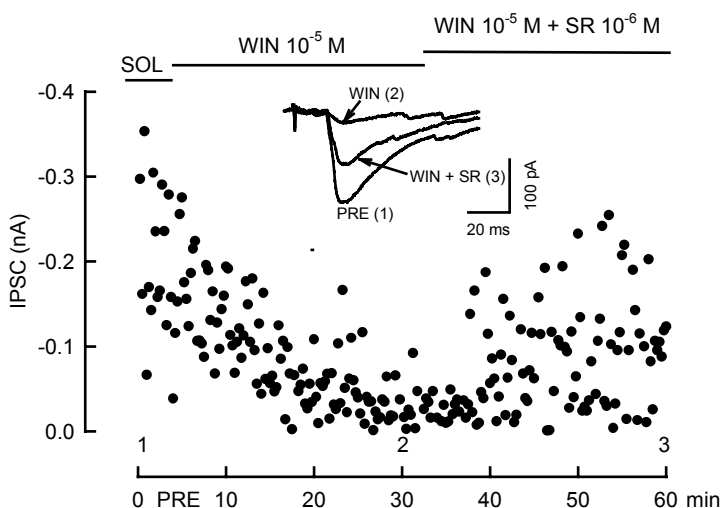
Bela Szabo and Ilka Wallmichrath

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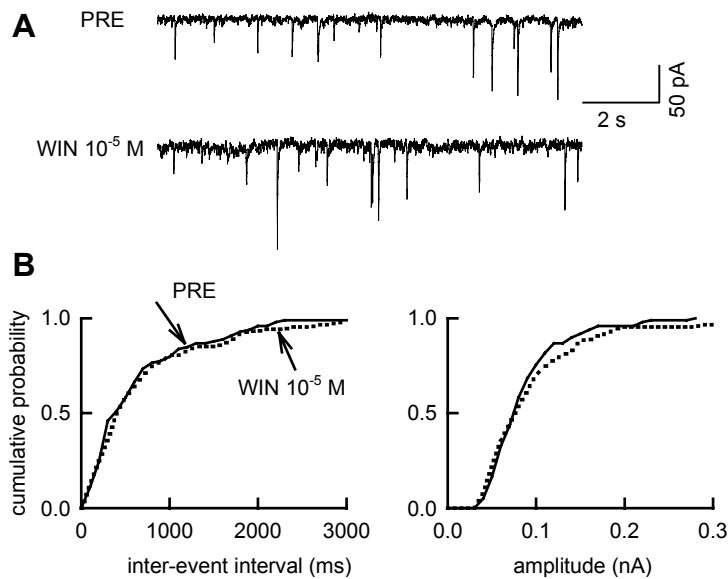
**Introduction.** The neuronal cannabinoid receptor – CB<sub>1</sub>-receptor – is widely distributed in the central and the peripheral nervous system. Anatomical studies suggest that in many regions the CB<sub>1</sub>-receptor is localised in presynaptic axon terminals. Receptors localised in axon terminals are ideally suited to modulate neurotransmission. We have studied the effect of cannabinoids on neurotransmission in several regions of the central and the peripheral nervous system; the underlying hypothesis was that activation of presynaptically localised CB<sub>1</sub>-receptors inhibits neurotransmission.

**Central nervous system.** Brain slices obtained from young rats or mice were superfused. Ion currents in neurons were recorded with the patch clamp technique. Neurotransmission was activated by electrical stimulation. The synthetic mixed CB<sub>1</sub>/CB<sub>2</sub> cannabinoid receptor agonists WIN55212-2 and CP55940 were used as agonists. To verify involvement of CB<sub>1</sub>-receptors, the antagonist SR141716A was employed.

*GABAergic neurotransmission between striatonigral axons and substantia nigra pars reticulata (SNR) neurons.* The highest density of CB<sub>1</sub>-receptors in the brain is observed in the SNR, and the receptors are localised in terminals of striatonigral axons. We studied the effect of cannabinoids on neurotransmission between striatonigral axons and SNR neurons. Striatonigral axons were activated by electrical stimulation in the striatum. WIN55212-2 and CP55940 inhibited inhibitory postsynaptic currents (IPSCs) recorded in SNR neurons (Fig. 1). The results of three kinds of experiments (A-C) suggest that the inhibition of neurotransmission was due to inhibition of GABA release from presynaptic axon terminals. A) WIN55212-2 did not change currents evoked by activation of postsynaptic GABA<sub>A</sub>-receptors in SNR neurons by muscimol. B) WIN55212-2 enhanced the amplitude ratio of IPSCs evoked by paired electrical pulses with an interpulse interval of 100 ms. C) WIN55212-2 did not change the amplitude of miniature IPSCs (mIPSCs) recorded in SNR neurons in the presence of tetrodotoxin (Fig. 2). WIN55212-2 also did not change the frequency of mIPSCs. This latter observation indicates that the cannabinoid agonist did not directly interfere with the vesicular release machinery. Striatonigral neurotransmission was inhibited by depolarisation of SNR neurons from –60 mV to +30 mV for 2-5 s. This phenomenon, also observed at other GABAergic synapses, is termed as depolarisation-induced suppression of inhibition (DSI). DSI at the striatonigral synapse was prevented by the CB<sub>1</sub> antagonist SR141716A; this observation is compatible with the assumption that DSI is mediated by endocannabinoids released from depolarised postsynaptic SNR neurons.



**Fig. 1. Activation of CB<sub>1</sub>-receptors inhibits striatonigral neurotransmission.** Striatonigral neurotransmission was activated by electrical stimulation in the striatum. Resulting inhibitory postsynaptic currents (IPSCs) were recorded in the substantia nigra pars reticulata (SNR). The filled circles represent amplitudes of individual IPSCs. The inset shows IPSCs recorded at time points 1-3. Superfusion of the agonist WIN55212-2 (WIN) after solvent (SOL) decreased the amplitude of IPSCs. The antagonist SR141716A (SR) counteracted the inhibitory effect of WIN.



**Fig. 2. WIN55212-2 (WIN) does not change the sensitivity of postsynaptic GABA<sub>A</sub>-receptors of SNR neurons for endogenous GABA.** Miniature IPSCs (mIPSCs) were recorded in SNR neurons in the presence of tetrodotoxin. **(A)** mIPSCs appeared to be similar before (PRE) and during superfusion of WIN. **(B)** Statistical evaluation verifies that WIN did not change the frequency (inter-event interval) and the amplitude of mIPSCs.

*Glutamatergic neurotransmission in the SNR.* Activation of CB<sub>1</sub>-receptors also inhibited glutamatergic neurotransmission between afferent axons arriving from the Nucleus subthalamicus and SNR neurons. Again, a presynaptic mechanism was identified.

*GABAergic neurotransmission in the corpus striatum.* GABAergic axons in the corpus striatum were electrically stimulated and the resulting IPSCs were recorded in medium spiny neurons (the IPSCs are probably due to release of GABA from axon terminals of fast-spiking parvalbumin-synthesizing interneurons). These IPSCs were presynaptically inhibited by activation of CB<sub>1</sub>-receptors.

*GABAergic neurotransmission in the cerebellar cortex.* GABAergic neurotransmission between basket and Purkinje cells was studied by simultaneously recording action potentials in presynaptic basket cells and IPSCs in synaptically coupled postsynaptic Purkinje cells. Activation of CB<sub>1</sub>-receptors led to a decrease in the amplitude of IPSCs coupled to presynaptic action potentials. The cause of the inhibition was again a decrease in the release of GABA from axon terminals. Interestingly, cannabinoids directly inhibited the vesicular release machinery in the axon terminals of basket cells. These experiments demonstrated presynaptic inhibition by cannabinoids under relatively physiological conditions: the presynaptic neuron was intact and transmitter release was elicited by spontaneously generated action potentials.

**Peripheral nervous system.** Release-inhibiting presynaptic CB<sub>1</sub>-receptors were also identified in neurons belonging to the sympathetic and parasympathetic nervous system.

*Sympathetic nervous system.* In pithed rabbits an artificial sympathetic tone was created by electrically stimulating preganglionic sympathetic neurons with an electrode positioned in the spinal canal. WIN55212-2 and CP55940 dramatically decreased noradrenaline release from sympathetic neurons. Consequently, blood pressure decreased markedly. A decrease in the plasma adrenaline concentration suggested that adrenaline release from the adrenal medulla was decreased as well. This was verified in experiments carried out on adrenal slices: WIN55212-2 inhibited electrically-evoked adrenaline release also in this preparation. In another series of experiments, the cardioaccelerator sympathetic nerves were electrically stimulated. WIN55212-2 and CP55940 inhibited the electrically-evoked increase in heart rate.

*Parasympathetic nervous system.* The Nervus vagus was electrically stimulated in rabbits and the resulting bradycardia was recorded. Activation of CB<sub>1</sub>-receptors markedly inhibited the vagally-evoked bradycardia.

**Summary.** Our experiments show that activation of CB<sub>1</sub> cannabinoid receptors leads to presynaptic inhibition of neurotransmission in the central and the peripheral nervous system. GABAergic, glutamatergic, noradrenergic and cholinergic neurotransmission are all inhibited.

## NOVEL CANNABINOID ANALOGS WITH THERAPEUTIC POTENTIAL

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Cannabinoids are substances widely spread over a variety of organisms. Both plants and animals biosynthesise ligands able to bind cannabinoid receptors.  $\Delta^9$ -THC, the most representative example of cannabinoids present in plants which is the active principle of *C. sativa*, was described in the middle sixties for the first time. But it has been only during this decade when it has been established the presence of endogenous ligands for cannabinoid receptors in many animals, from the simplest leeches to the most evolutioned mammals as humans.

These findings provided the adequate framework for the development of a growing number of studies aimed at elucidating the chemistry and the molecular bases of these compounds in order to rationalize the knowledge about their implication in a wide range of physiological and medical effects, something well known since ancient times, but only from an empirical point of view. This objective made necessary the development of synthetic agents able to bind and activate cannabinoid receptors in a more potent and selective way, compounds which very soon became a tool of primary importance to allow the characterization of the endogenous cannabinoid system (ECS).

The ECS<sup>1</sup> is the primary responsible for all the effects mediated by cannabinoids and it is constituted by two G protein coupled receptors, named CB<sub>1</sub> and CB<sub>2</sub>, its endogenous ligands such as anandamide (AEA), 2-arachidonoylglycerol, 2-arachidonyl glyceryl ether and the recently reported virodhamine, and an inactivation system which degrades these endocannabinoids. This termination system consists of the uptake of anandamide followed by its intracellular metabolism by fatty acid amidohydrolase (FAAH). The ECS is involved in the regulation of a wide variety of physiological functions<sup>2</sup> such as antinociception, brain development, memory, retrograde neuronal communication, control of movement, cardiovascular and immune regulation and cellular proliferation. In consequence, the compounds affecting ECS function are potential therapeutic agents<sup>3</sup> for the treatment of diverse pathologies including neurodegenerative disorders, nociceptive alterations and malignant tumors.

With this objective, a variety of synthetic ligands targeting the four different proteins that constitute the ECS have been developed to date. The main structural features<sup>4</sup> of these compounds, including the most representative structure-activity relationship studies, their potential pharmacological applications as well as their more significative drawbacks and advantages will be overviewed.

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## **ROLE OF THE ENDOGENOUS CANNABINOID SYSTEM IN NOCICEPTION AND CANNABINOID WITHDRAWAL**

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In recent years, an endogenous cannabinoid signalling system has been described to play a regulatory role within the brain and the periphery. This regulatory system is composed of two specific cannabinoid receptors (CB<sub>1</sub> –present in the CNS, peripheral neurons and in certain non-neural tissues, and CB<sub>2</sub> –present preferentially in the immune system), a variety of endogenous cannabinoid ligands (anandamide, 2-arachidonoylglycerol), derivatives of arachidonic acid, and a process of termination of the biological action of endocannabinoids that involves a carrier-mediated uptake system and a degradative enzyme, called fatty acid amidohydrolase (Pertwee, 2001, *Prog Neurobiol* 63:569-611). Within the brain, this endocannabinoid system seems to play a modulatory role in several processes such as pain, control of movement, emesis, appetite, learning and memory, spasticity, tumor growth, stroke, etc...

Cannabinoid antinociceptive activity is produced at central and peripheral levels (Fox et al., 2001, *Pain* 92:91-100). Central antinociceptive action occurs at both supraspinal and spinal levels (Iversen and Chapman, 2002, *Curr. Opin Pharmacol* 2:50–55). Both actions are blocked by CB<sub>1</sub> receptor antagonist (SR-141716A) and  $\mu$  (naltrexone,  $\beta$ -funaltrexamine) and  $\kappa$ -opiate antagonists (nor-binaltorphimine). It is interesting to note that acute administration of cannabinoid agonists results in an increase in the release of endogenous opioid ligands such as enkephalins and dynorphins, a fact that has been confirmed by the increase in opioid gene expression in the brain and spinal cord after subchronic (5 days) treatment with cannabinoid agonists (Manzanares et al., 1999, *TiPS* 20:287–294). This action may support, at least in part, the synergistic antinociceptive activity induced by subeffective doses of opiate and cannabinoid agonists and may allow the reduction of dosage of morphine in the treatment of chronic pain, therefore decreasing the adverse effects of repeated opiate treatment (respiratory depression, inhibition of intestinal transit, etc).

On the other hand, the prolonged exposure to cannabis together with intermittent periods of cessation of drug consumption may induce pronounced changes in the expression of key genes. These neuroplastic adaptations may increase the vulnerability to drug dependence and/or the development of mood disorders. However, little is known of the neurochemical mechanisms involved in cannabinoid tolerance, dependence and withdrawal. Recently, our group has examined the behavioural signs that occur during tolerance development to cannabinoid treatment and hormonal and gene expression alterations induced by spontaneous cannabinoid withdrawal in mice (Oliva et al., *J. Neurochemistry*, (2003, in press).

Cessation of the cannabinoid receptor agonist CP-55,940 results in an increase in motor activity that lasted 72 hours without significant alterations in behavioural signs of abstinence. Corticosterone plasma concentrations dramatically increased 24 and 72 hours. Similarly, an increase (40%) in cannabinoid [<sup>35</sup>S]GTP $\gamma$ S binding autoradiography was detected on days 1 and 3 of abstinence. Spontaneous cannabinoid withdrawal produced time-related significant alterations in gene transcription :1) decreased (20%) tyrosine hydroxylase (TH) mRNA levels in the ventral tegmental area and increased (50%) in substantia nigra, 2) increased

proenkephalin (PENK) gene expression more than 100% in caudate-putamen, nucleus accumbens, olfactory tubercle and piriform cortex, and 3) increased (20-40%) proopiomelanocortin (POMC) gene expression in the arcuate nucleus of the hypothalamus.

In summary, these results indicate that spontaneous cannabinoid withdrawal produced an integrated (behaviour, endocrine and gene transcription) response. This cannabinoid withdrawal syndrome include mild alterations in ambulatory and exploratory activity, transient and long lasting pronounced molecular changes in the functional activity of the HPA axis, cannabinoid receptors and key genes (TH, PENK and POMC) involved in the control of motivation, reward and stress. This behavioural plasticity induced by cannabinoid withdrawal in mice led us to speculate that in other animal species these and/or other behavioral and neurochemical alterations may possibly occur. Although this remains to be determined, neurochemical changes in gene expressions, similar to those found in this study, may potentially alter vulnerability to other drugs of abuse or to schizoaffective disorders occurring in cannabis abusers.

Supported by FISS (01/1438) to J. Manzanares.

## CANNABINOIDS AND MEMORY

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To investigate the role of the endocannabinoid system in amygdala-dependent learning, CB1-deficient mice were studied in an auditory fear-conditioning tests, which is believed to be an amygdala-dependent learning. Fear conditioning is a behavioral test, which allows the dissection of distinct phases of memory processing, such as acquisition, consolidation and recall/extinction. Animals are trained to associate a previously neutral stimulus (in this case a loud tone; conditioned stimulus, CS) with an aversive stimulus (a mild electric foot shock; unconditioned stimulus, US). At different time points after training (conditioning), animals are exposed to the tone (which has now the attribute of a negative experience). A natural reaction of fear is a typical behavior called freezing, described as the lack of any movement except for respiration. Freezing behavior can be observed and quantified, and the percentage of time showing freezing during the tone presentation gives a value of the tone-shock association which the animal formed at moment of conditioning (acquisition and consolidation of aversive memory). However, when the CS (tone) is repeatedly presented without US (shock), the fear reaction (freezing) gradually decreases. This reaction is called extinction. This behavior is believed to reflect a new form of learning rather than a simple “erasure” of the original fear memory.

In this test, at the first exposition to the tone after conditioning (24 hours), CB1<sup>-/-</sup> mice showed the same levels of freezing behavior as compared to CB1<sup>+/+</sup> control littermates, indicating that CB1 is not required for proper acquisition or consolidation of fear memories. However, whereas CB1<sup>+/+</sup> normally decreased freezing to the tone during subsequent tone exposures, this behavior was completely impaired in CB1<sup>-/-</sup> mice, indicating a crucial role of CB1 in extinction of aversive memories. Given the lack of CB1 during the entire life in CB1<sup>-/-</sup> mice, which might lead to potential developmental defects, and which in turn could underlie the impaired extinction observed, a pharmacological approach was undertaken to temporally dissect the activation of CB1 during fear memory processing. Treatment of wild-type mice with the CB1-specific antagonist SR141716A at moment of conditioning had no effect on memory acquisition, but at moment of extinction during the tone presentation, the drug mimicked the phenotype of CB1<sup>-/-</sup> mice, revealing an acute physiological activation of CB1 during 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, but not in prefrontal cortex, another region known to modulate extinction. Moreover, electrophysiological studies revealed that endocannabinoids and CB1 were crucially involved in long-term depression of GABAergic inhibitory currents in the basolateral amygdala.

Although the universality of these results for aversive situations other than fear conditioning has still be shown, and although it remains to be investigated whether or not it is possible to enhance extinction by pharmacological stimulation of the endogenous cannabinoid system, this newly described biological function of endogenous cannabinoids and CB1 might lead to the development of novel therapeutic strategies for the treatment of patients with inadequate reaction to potentially dangerous situations (for example phobics and patients with posttraumatic stress disorders and certain forms of chronic pain).

At this point, it is interesting to note that the pharmacological treatments of animals with CB1 agonists were not able to give insights into the mechanisms on how the endogenous cannabinoid system is involved in learning and memory. All these investigations pointed to a role of the endogenous cannabinoid system in short-term memory. Subsequent experiments with CB1 antagonists came to the conclusion that lack of cannabinoid signaling leads to an enhanced memory performance, i.e. not acquisition but rather consolidation is increased. It was discussed that the endogenous cannabinoid system is important for the process of “forgetting”. Using CB1-deficient mice as a model system, an involvement of the endogenous cannabinoid system in extinction was revealed.

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## CANNABINOIDS AND MOTOR CONTROL

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One of the most characteristic and therapeutically-promising functions of the endocannabinoid signaling system is the control of movement. It is well-documented that the administration of plant-derived, synthetic or endogenous cannabinoids affected movement usually producing motor inhibition (Consroe, *Neurobiol. Dis.* 5, 534-551, 1998; Romero et al., *Pharmacol. Ther.* 95, 137-152, 2002). The magnitude of this inhibition seems to depend on the type of cannabinoid, dose used and time after administration at which the effects were tested. This cannabinoid-induced motor inhibition was paralleled by changes in the activity of GABA, dopamine and glutamate, all of which are neurotransmitters involved in the control of movement in the basal ganglia (Sañudo-Peña et al., *Life Sci.* 65, 703-713, 1999; Fernández-Ruiz et al., *Prostag. Leukot. Essent. Fatty Acids* 66, 257-267, 2002). The motor effects of cannabinoids are likely to be mediated by the activation of cannabinoid CB<sub>1</sub> receptors, and, in part, by vanilloid VR1 receptors, which are densely distributed in the basal ganglia (Romero et al., *Pharmacol. Ther.* 95, 137-152, 2002; Fernández-Ruiz et al., *Prostag. Leukot. Essent. Fatty Acids* 66, 257-267, 2002). CB<sub>1</sub> receptors are located presynaptically on striatal GABAergic projection neurons that reach the substantia nigra, globus pallidus and entopeduncular nucleus, and also in the neurons of the subthalamonigral glutamatergic pathway (Herkenham et al., *Brain Res.* 547, 267-274, 1991; Tsou et al., *Neuroscience* 83, 393-411, 1998), whereas VR1 receptors are mainly located onto nigrostriatal dopaminergic neurons (Mezey et al., *PNAS* 97, 3655-3660, 2000). The fact that cannabinoids affect movement and the prominent presence of CB<sub>1</sub> and VR1 receptors in the basal ganglia have encouraged the research on the possible therapeutic usefulness of cannabinoids to improve movement in motor degenerative disorders. Two disorders, Parkinson's disease (PD) and Huntington's disease (HD), have attracted most interest. Three aspects have been examined in these disorders: (i) the changes in endocannabinoid signaling system in the basal ganglia, (ii) the ability of cannabinoids and related compounds to alleviate motor symptoms, and (iii) the protective effects of cannabinoids against the degenerative processes that affect the basal ganglia structures.

Despite several studies in rat models of PD that showed a certain efficiency of cannabinoid agonists combined with dopamine-acting drugs in attenuating the hypokinetic signs of these animals (Sañudo-Peña et al., *Life Sci.* 65, 703-713, 1999), experiences with humans and non-human primates have indicated that cannabinoid agonists produce an enhancement rather than an amelioration in the hypokinetic symptoms typical of PD (Consroe, *Neurobiol. Dis.* 5, 534-551, 1998), in concordance with the hypokinetic profile of cannabinoid agonists. Based on this and also on the evidence that the endocannabinoid transmission becomes hyperactive in postmortem basal ganglia from PD patients (Lastres-Becker et al., *Eur. J. Neurosci.* 14, 1827-1832, 2001) or from animal models of this disease (Romero et al., *Life Sci.* 66, 485-494, 2000; Di Marzo et al., *FASEB J.* 14, 1432-1438, 2000), it has been claimed a beneficial effect of CB<sub>1</sub> receptor antagonists rather than agonists in this disease (Brotchie, *Ann. Neurol.* 47, S105-S114, 2000). Unfortunately, the results obtained using SR141716, a selective CB<sub>1</sub> receptor antagonist, have resulted to date controversial (Di Marzo et al., *FASEB J.* 14, 1432-1438, 2000; Meschler et al., *Psychopharmacol.* 156, 79-85, 2001) and further analyses will be necessary. In addition to this potential role of SR141716 in reducing hypokinesia in PD, another studies have recently demonstrated that plant-derived cannabinoid agonists, such as

$\Delta^9$ -tetrahydrocannabinol or cannabidiol, might be neuroprotective in PD since they were able to delay/arrest the degeneration of nigrostriatal dopaminergic neurons, although these effects of cannabinoids are possibly caused by their antioxidant properties rather than by activating CB<sub>1</sub> receptors (Lastres-Becker, Ramos, Mechoulam, Fernández-Ruiz, unpublished results).

In contrast with PD, the studies in HD have demonstrated that the endocannabinoid signaling system becomes hypoactive in the basal ganglia in this hyperkinetic disorder (Fernández-Ruiz et al., *Prostag. Leukot. Essent. Fatty Acids* 66, 257-267, 2002). Thus, decreases in CB<sub>1</sub> receptors have been reported in postmortem basal ganglia: (i) from HD patients (Glass et al., *Neuroscience* 97, 505-519, 2000), (ii) from transgenic mice, overexpressing a mutated form of huntingtin, as in the human pathology (Denovan-Wright and Robertson, *Neuroscience* 98, 705-713, 2000; Lastres-Becker et al., *Brain Res.* 929, 236-242, 2002), and (iii) from rats with striatal atrophy caused by administration of mitochondrial toxins, mainly 3-nitropropionic acid (Page et al., *Exp. Brain Res.* 130, 142-150, 2000; Lastres-Becker et al., *Synapse* 44, 23-35, 2002). HD is characterized by progressive involuntary choreiform movements due to the degeneration of striatal projection GABAergic neurons. As CB<sub>1</sub> receptors are located on these neurons, it was expected that they decrease during the course of striatal injury. However, these losses already appear at very early phases when cell death does not exist or is minimal, which has led to the opinion that they might be involved in the pathogenesis itself. To further explore this hypothesis, we have examined whether CB<sub>1</sub> receptor activation, starting at early phases of 3-nitropropionic acid-induced striatal degeneration, might influence the progress of striatal injury. Our data indicated that  $\Delta^9$ -tetrahydrocannabinol was effective in reducing the magnitude of striatal degeneration caused by the toxin (Lastres-Becker, Fernández-Ruiz, Brouillet, unpublished results). In addition, we have also shown that substances that increase the activity of endocannabinoid transmission, such as the inhibitor of endocannabinoid uptake, AM404, were able to reduce hyperkinesia and recover the neurochemical deficits in a rat model of HD (Lastres-Becker et al., *Synapse* 44, 23-35, 2002), although it appears that the contribution of the activation of VR1 receptors is important in this beneficial effect of AM404 (Lastres-Becker et al., *J. Neurochem.* 84, 1097-1109, 2003).

Therefore, the involvement of the endocannabinoid signaling system in the control of movement appears relevant, not only in physiological conditions but also in the different pathologies affecting the basal ganglia function. Thus, recent evidence prove that the endocannabinoid signaling system is altered in the basal ganglia in PD, HD and possibly also in other motor disorders, which support that compounds that selectively target the different proteins of this system might be of therapeutic value in alleviating motor signs or in delaying/arresting basal ganglia degeneration. This lecture will review all this evidence trying to establish the future lines for research on the therapeutic potential of the endocannabinoid signaling system in motor disorders.

Supported by a grant from CAM-PRI (08.5/0063/2001)

## **CANNABINOIDS AND CELL SURVIVAL/DEATH DECISION**

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Cannabinoids produce a wide spectrum of central and peripheral effects, some of which may have clinical application. Recently these compounds have been shown to control the survival/death decision of a number of cell types. As the most prominent cannabinoid actions occur in brain, the present communication will focus mainly on neural cell fate.

Most of the experimental evidence indicates that cannabinoids may protect neurons from toxic insults such as glutamatergic excitotoxicity, traumatic injury, ischaemia and oxidative damage. Several mechanisms have been suggested to contribute to cannabinoid-induced neuroprotection, including (i) inhibition of glutamatergic neurotransmission, (ii) antioxidant capacity, (iii) modulation of glial cell function (attenuation of cytokine, reactive oxygen species and nitric oxide production; enhancement of nutrient supply to neurons; prevention of astrocyte and oligodendrocyte death; blockade of gap junction communication); and (iv) inhibition of endothelin-1-induced vasoconstriction. Protection of astrocytes and oligodendrocytes by cannabinoids may rely mostly on the activation of the phosphatidylinositol 3-kinase/Akt survival pathway. Cannabinoids have also been reported to inhibit neural progenitor cell differentiation to neuronal phenotype both in culture and in vivo, a process that depends at least partially on the inhibition of the extracellular signal-regulated kinase cascade.

In contrast to their protective action on non-transformed neural cells, cannabinoids induce apoptosis of transformed neural (glioma) cells in culture and regression of malignant gliomas in rats and mice by a mechanism that may involve sustained ceramide accumulation and extracellular signal-regulated kinase activation. Impairment of angiogenesis may contribute as well to the anti-tumour action of cannabinoids. Breast, prostate, lymphoid, thyroid and skin cancer cells are also sensitive to cannabinoid-induced growth inhibition, although the underlying mechanisms are still unknown.

The effects of cannabinoids on the cell survival/death decision are therefore very complex and may depend on experimental factors such as type of cell examined, stage of cell differentiation, drug concentration, and timing of drug delivery. In any event, the neuroprotective effect of cannabinoids might have potential clinical relevance for the treatment of neurodegenerative disorders such as multiple sclerosis, Parkinson disease and Huntington disease, as well as of neurological processes such as close head injury and brain ischaemia/stroke. Their growth-inhibiting action on transformed cells might be useful for the management of malignant tumours (e.g. gliomas). Ongoing investigation is in search for cannabinoid-based therapeutic strategies devoid of non-desired psychotropic effects.

## CANNABINOIDS AND NEUROENDOCRINE CONTROL

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Cannabis as a drug of abuse was used as far back as 1000 years BC. The hemp as a medicine has been used for several centuries, too. It had been a discrepancy concerning the effects of hemp on reproductive and endocrine organs. There was a period when it was considered as a potent sexual stimulator or in contrast it was thought to depress sexual activity.

It is of interest to investigate the effects of cannabinoids on different neuroendocrine functions because of different reasons:

- The marijuana as a medicine is an actual medico-political question.
  - Several hypothalamic regulatory functions follow important changes at puberty when many young people start to use the marijuana as a drug of abuse.
  - The endocannabinoids take part in the regulation of hypothalamo-pituitary functions particularly in the regulation of adenohipophyseal secretory mechanisms.
- CB<sub>1</sub> central cannabinoid receptors (CB<sub>1</sub> receptors) are distributed in the hypothalamus and in the adenohipophysis, too.
  - Endocannabinoids are present in both hypothalamus and adenohipophysis
  - CB<sub>1</sub> receptor mRNA transcripts in adenohipophysis have been demonstrated.
  - The anandamide (AEA) content increases significantly immediately before the onset of puberty in rodents.
  - CB<sub>1</sub> receptor inactivated (K.O.) mice have lower basal level of luteinizing hormone (LH) and testosterone.

The experimental data has shown that both exogenous (THC) and endogenous (AEA) cannabinoids decrease pituitary LH levels. THC administration delays puberty and causes irregular vaginal cycles in rats. Cannabinoids modulate prolactin (PRL) secretion, too. Ovarian steroids in certain steroid-sensitive brain areas, especially in the hypothalamus modify sensitivity to cannabinoid treatment. Cannabinoids increase the activity of the hypothalamo-pituitary-adrenal axis in both wild type and CB<sub>1</sub> receptor K.O. mice. Pituitary adenomas express CB<sub>1</sub> receptors and synthesize AEA.

All these data and experimental observations emphasize the role of the endocannabinoid system in neuroendocrine regulations. Furthermore, both exogenous and endogenous cannabinoids may alter neuroendocrine functions not only by direct action on hypothalamus but on pituitary, too.

## CANNABINOIDS AND INFLAMMATION

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Cannabinoids (CBs) can modulate responses to immune challenge and inflammation in the periphery and the central nervous system. For years, cannabinoids have been considered, both *in vitro* and *in vivo*, as immunosuppressive agents. However, currently this picture is changing toward the concept that CBs exert an immunomodulatory role mainly, via interaction with specific receptors expressed in cells involved in immune/inflammatory reactions. CBs exhibit very complex effects on the immune system and influenced almost every component of immune machinery, but their effects often depend on CB ligand, doses, cell type and local environment. Nevertheless, in general CBs produce a negative action in a variety of parameters of the immune response, particularly in those related to inflammation. The aim of this presentation is to improve understanding of the modulatory effects of CBs on the immune system and specifically in neuroinflammation.

Brain macrophages (microglia), astrocytes and endothelial cells are involved in the intracerebral immune response and exert their activities, in part, by secreting cytokines, neurotropic and neurotoxic factors. CB receptors are expressed in glial cells and endocannabinoids are produced not only by neurons, but also by activated astrocytes and microglia. Exogenous and endogenous CBs interfere with the production of pro and anti-inflammatory cytokines as well as other inflammatory mediators such as nitric oxide. Since inflammatory responses in the brain must be under tight regulation to prevent the accumulation of potentially cytotoxic mediators, the inhibition of inflammatory gene expression in glial cells by CBs may be a critical feature contributing to their neuroprotective properties.

In experimental animal models of peripheral or central chronic inflammation, like rheumatoid arthritis and multiple sclerosis respectively, CBs have been useful as therapeutical agents by attenuating disease symptomatology. Theiler's virus infection of the central nervous system (CNS) induces an immune-mediated demyelinating disease in susceptible mouse strains and serves as a relevant infection model for human multiple sclerosis. In this model, treatment with CBs during established disease, significantly improved in a long-lasting way the neurological deficits. At a histological level, cannabinoids reduced microglial activation, abrogated major histocompatibility complex class II antigen expression, and decreased infiltrating T cells in the spinal cord. Both recovery of motor function and reduction of inflammation paralleled extensive remyelination. Therefore, there is a high potential for further research, in particular, the pharmacological modulation of endocannabinoid system would be a good target for the development of cannabinoid-based therapy in immune/inflammatory disorders.

## **THE UBIQUITOUS ROLE OF ENDOCANNABINOIDS IN PHYSIOLOGICAL PROCESSES: EXAMPLES IN NEUROPROTECTION, FEEDING AND BONE FORMATION.**

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Research over the last decade has led to the surprising finding that the endocannabinoids are involved in biological processes in almost every major mammalian system. In the present lecture we shall discuss some of our results in 3 different areas of physiological importance:

**NEUROPROTECTION:** Brain injury triggers the accumulation of harmful mediators, including reactive oxygen intermediates that lead to secondary damage. Protective mechanisms to attenuate the secondary damage are also set in motion. These include processes in which adenosine, melatonin, female sex hormones and others play a role. Are the cannabinoids to be included in the list?

We have observed that the levels of the endocannabinoid 2-arachidonoyl glycerol (2-AG) sharply increase after closed head injury (CHI). In order to test whether this phenomenon has physiological significance we administered synthetic 2-AG after CHI and found significant reduction of brain edema, better clinical recovery, reduced infarct volume and reduced hippocampal death compared with controls. The neuroprotective effect was attenuated by the CB1 receptor antagonist SR141716 indicating that the mechanism of the process is apparently a cannabinoid one. This assumption is strengthened by the observation that in CB1 knock out mice CHI causes more severe damage than in wild type mice. And in these mice administration of 2-AG did not lead to any improvement.

**FEEDING and SUCKLING:** Several groups, including ours, have reported that endocannabinoids enhance appetite. Indeed short term starvation causes increase in 2-AG levels and enhanced appetite; prolonged starvation (about 10 days) causes lowering of 2-AG levels. This effect may be of ecological significance – animals may survive better if during a period of lack of food their appetite is kept at relatively low levels.

2-AG was found in animal and human milk, which suggested that the endocannabinoid system could be involved in suckling and neonatal development. Indeed when the CB1 antagonist was administered to newly born mouse pups on their first day the animals did not suck and died within a few days due to lack of food. In order to show that the devastating effects on suckling and growth were due to blocking of the cannabinoid system and not to some toxic effect of the antagonist, THC was co-administered. The anti-suckling effect of the antagonist was eliminated, showing that the endocannabinoid system was involved in suckling.

Pups of CB1 knock-out mice did not suck on the first day of life, but they started to suck later and the antagonist had only a partial effect. Our data suggest that CB1 knock-out neonates possess a compensatory mechanism which helps them overcome the lack of CB1 receptors.

**BONE REMODELING.** There are 2 striking features among the multitude of clinical characteristics of osteoporosis: gonadal failure favors bone loss and obesity protects from bone loss. And the peptide leptin is known to negatively regulate both osteoblastic and endocannabinoid activity. The role of endocannabinoids in the control of body weight and

reproduction as well as its relationship to leptin led us to look at the possibility that the endocannabinoid system is involved in bone remodeling. In preliminary observations we have noted that reverse transcription polymerase chain reaction of differentiating osteoblastic precursor cells demonstrates increase in progressive increase in mRNA levels of CB2 but not of CB1. In addition normal mice treated systematically with 2-AG showed a dose dependent increase in trabecular bone formation. On the basis of these preliminary data we assume that endocannabinoids stimulate bone formation.

## CANNABINOIDS AND FEEDING BEHAVIOR

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Rimonabant, a CB<sub>1</sub> antagonist, has shown high selectivity for CB<sub>1</sub> receptors in rat brain compared to CB<sub>2</sub> receptors in rat spleen, and this selectivity has also been seen in CHO-CB<sub>1</sub> compared to CHO-CB<sub>2</sub> human membranes. The compound also selectively antagonises pharmacological responses elicited by the cannabinoid receptor agonists such as WIN 55212-2.

A great deal of interest has been centred on the role of CB<sub>1</sub> receptors and eating behavior as they appear to be largely distributed in brain areas involved in the control of feeding behavior (i.e. lateral hypothalamus, limbic system) and additionally seem to be implicated in food intake control.

Recent results show that endocannabinoids in the hypothalamus may tonically activate CB<sub>1</sub> receptors to maintain food intake, and may increase the incentive value of food. Further evidence shows that the CB<sub>1</sub> receptors may be involved in the motivational aspects of eating by enhancing the satisfaction derived from eating through activation of the meso-limbic dopaminergic system. All this evidence would seem to indicate that specific CB<sub>1</sub> antagonists like rimonabant should have some effect in body weight control. Pharmacological studies have shown that it reduces the consumption of a palatable (sucrose) drinking solution in rats, that it decreases body weight gain in obese compared to lean Zucker rats, and additionally reduces ethanol consumption in C57BL/6 mice and palatable food consumption in marmosets. Thus, in general, rimonabant appears to reduce the appetite or rewarding properties of food and drink in numerous pharmacological models. Recent studies in a diet-induced obesity model in mice, widely used for research on the human obesity syndrome, show that the compound, during a 5-week treatment, induced a transient reduction in food intake with a marked but sustained reduction of body weight. Rimonabant had no effect in CB<sub>1</sub> receptor knockout mice, which confirmed the implication of CB<sub>1</sub> receptors in the activity of the compound.

Clinical studies in progress have shown that it reduces hunger, caloric intake and body weight in obese patients. The results of a Phase IIb study show that the compound at doses of 5, 10, and 20 mg once daily was able to reduce significantly the body weight in obese patients over a period of 16 weeks when compared to placebo, with a very good safety profile. Phase III studies with the compound are in progress. In additional clinical studies, the compound has been evaluated in smoking cessation, and Phase II studies have shown an increased abstinence with the compound with a prevention of the secondary weight gain often seen in this situation.

## NEUROBIOLOGY OF CANNABINOID TOLERANCE AND DEPENDENCE

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Exposure to drugs of abuse can cause experience-dependent changes in their behavioral effects. Different animal models have been used to clarify the consequences of chronic exposure to cannabinoid agonists.

Following the chronic administration of cannabinoids, tolerance develops to most of their pharmacological effects (i.e. antinociception, locomotion, hypothermia, catalepsy etc.) with a rapid development, and a marked decrease of the acute response is already observed after few administrations of cannabinoid agonists. Pharmacodynamic events seem to play a crucial role on cannabinoid tolerance, although pharmacokinetic mechanisms could also occur. Among the molecular mechanisms of cannabinoid tolerance, a significant decrease in the total number of CB1 cannabinoid receptors and its mRNA levels has been reported in several brain structures such as the striatum, limbic system, cortex and cerebellum. Another cellular event underpinning cannabinoid tolerance is alteration in G proteins expression or their functional activity as demonstrated by the reduction of cannabinoid agonist-stimulated [<sup>35</sup>S]-GTPγS binding observed in most brain regions of rats chronically treated with cannabinoids. Compensatory changes in cAMP pathway have been reported although alteration in other intracellular signaling events cannot be ruled out. Recent data on the gene changes associated with chronic exposure to cannabinoids revealed that several genes were permanently affected, including cannabinoid receptor-coupled signaling pathways, synaptic and membrane structure, motility and neuron growth.

Since the advent of the cannabinoid receptor antagonist SR141716A, cannabinoid agonists have also been shown to produce physical dependence that can be precipitated by administration of the antagonist to cannabinoid-tolerant animals. In rodents, a large number of somatic signs (wet dog shakes, head shakes, facial rubbing, body tremor, etc.) and an absence of vegetative manifestations characterize cannabinoid withdrawal. Cannabinoid CB1 receptors mediate somatic manifestations of cannabinoid withdrawal. Thus, SR141716A administration in CB1 receptor knock-out mice receiving chronic THC treatment fails to precipitate any manifestation of cannabinoid abstinence. Analysis of the molecular adaptations induced at the receptor level in cannabinoid abstinent animals revealed that 24 hours after SR141716-precipitated withdrawal the receptor down-regulation was still present but the [<sup>35</sup>S]-GTPγS binding returned to the control level. Moreover cannabinoid withdrawal syndrome is associated with compensatory changes in the cAMP pathway, selectively localized in the cerebellum, as demonstrated by the increased adenylyl cyclase activity and cAMP levels measured in abstinent animals. Finally, a marked inhibition in extracellular levels of corticotropin releasing factor in the mesolimbic system and mesolimbic dopamine activity have been reported during cannabinoid withdrawal.

Last, besides tolerance and physical dependence, repeated exposure to cannabinoids results in a progressive and enduring enhancement in the motor stimulant effects of subsequent drug challenge referred to as behavioral sensitization, suggesting that cannabinoids could trigger neurobiological alterations not dissimilar from those observed with more harmful abused drugs.

## **INTERACTIONS BETWEEN OPIOIDS AND CANNABINOIDS**

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Several studies have shown functional relationships between the endogenous cannabinoid and opioid systems. However, acute  $\Delta^9$ -tetrahydrocannabinol (THC) pharmacological responses and physical dependence were not modified in knock-out mice with single deletion of mu, delta or kappa opioid receptors. To further investigate the neurobiological basis of cannabinoid dependence, we have now evaluated THC responses in double mu and delta opioid receptor knock-out mice. Antinociception and hypolocomotion induced by acute THC administration remain unaffected whereas the acute hypothermic effects were slightly attenuated in these double mutants. During chronic THC treatment, knock-out mice developed slower tolerance to the hypothermic effects but the development of tolerance to antinociceptive and hypolocomotor effects was almost unaffected. The rewarding properties of THC were abolished in knock-out mice. Interestingly, the somatic manifestations of THC withdrawal were also significantly attenuated in mutant mice, suggesting that a cooperative action of mu and delta opioid receptors is required for the entire expression of THC dependence.

## ENDOCANNABINOIDS AND ALCOHOLISM: A SUBSTRATE FOR ALLOSTASIS?

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The elements of the endogenous cannabinoid system (ECS), specially cannabinoid CB1 receptors, are the pharmacological target of natural cannabinoids. However, since the ECS is a key regulatory mechanism of transmitters systems involved in acute reward and dependence, the ECS may be involved in the biological substrates of major addictive processes. The CB1 receptor regulates the synthesis and release of these transmitters, including monoamines, acetylcholine, glutamate and neuropeptides. Thus, the mesolimbic DA system is clearly involved in the rewarding properties of cannabinoids as well as in the motivational consequences of cannabinoid withdrawal. An alteration in mesolimbic CRF function is also related to the dysphoric effects of cannabinoid abstinence. Bidirectional interactions between the endogenous cannabinoid and opioid systems are crucial for cannabinoid motivational properties and the development of cannabinoid tolerance and dependence. Additionally, the endogenous cannabinoid system is associated with the neuroadaptions underlying ethanol dependence and withdrawal. Lastly, relapse processes may be modulated by cannabinoid drugs, confirming the proposed role for the ECS as a major transmitter system involved in addiction.

We have recently explored the evolution of the different elements of the endogenous cannabinoid system (CB1 receptor, Amido Hydrolase (FAAH), Monoacyl glycerol lipase, N-acyltransferase, anandamide transporter and the endocannabinoids anandamide, palmithylethanolamide and oleylethanolamide) along the different stages of the addictive cycle to ethanol (first exposure, induction of dependence, abstinence, extinction and relapse). The studies have been done in rats self-administering ethanol. We will focus on the analysis of the evolution of these molecular targets on the cerebellum striatum hippocampus and extended amygdala /hypothalamic circuitry to evaluate how the endocannabinoid system contributes to the allostatic mechanisms that lead to the addictive phenotype. Acute, chronic and relapse from ethanol exposure modulates the different elements of the ECS in a differential way. While acute exposure to ethanol does not affect FAAH and NAT activities, it changes the ability of cannabinoid receptors to couple to its transduction system. This effect is associated with a decreased release of the endocannabinoid anandamide. Chronic ethanol exposure reversed the effects of ethanol on CB1 receptors, normalizing the effects on anandamide production. Chronic effects of ethanol on CB1 receptors changed the sensitivity of ethanol self-administering animals to the inhibitory effects of the cannabinoid antagonist SR141716A. This effect was persistent since SR141716A suppressed cue-induced reinstatement to ethanol self-administration. These data indicates that drugs interacting with CB1 receptors are a relevant target for the treatment of alcoholism.

## **CANNABIS AND PSYCHIATRIC PATHOLOGY**

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Over the last ten years we have witnessed a great step forward in the analysis of the different fields related to cannabis derivatives. However, certain deficit is noticeable in the research done with respect to the psychological effects of those substances in humans.

In the last two or three years a small but significant number of articles have been published, which analyse the effect that prolonged consumption of cannabis derivatives has on humans, precisely the area where a major controversy existed.

Aspects such as the chronic use of cannabis as related to schizophrenia, or cognitive deterioration, or depression and anxiety, or the existence of cannabis dependence, have all been evaluated recently. These studies show a thorough methodology, with large samples and very close follow-ups, all of them being features deeply criticized in the past.

The results are fairly clear and show that the prolonged consumption of cannabis derivatives significantly increases the risk of developing any of the above mentioned disorders. Nonetheless, these conclusions require further analysis in order to confirm or reject them. Moreover, several studies have been carried out concerning cannabis psychosis -another unsolved controversy- the results of which would support the existence of such disorder, independently from schizophrenia.

New details are coming to be known, which might influence the effects that the consumption of cannabis derivatives has on the mind (personality features, genetic aspects, etc.), although these are areas which also require further research.

All this is happening at a moment in which there is an increasing number of consumers as well as a higher percentage of THC in each intake. This situation involves a higher risk of developing undesirable psychological effects, precisely when studies on humans are more and more intensive in the search of safety parameters, necessary to establish the possibilities of cannabis derived substances as therapeutic agents.

Under no circumstances should we forget such undesirable effects when we take up a position in the debate on legalization of cannabis.

## **POSTER PRESENTATIONS**

## P-1

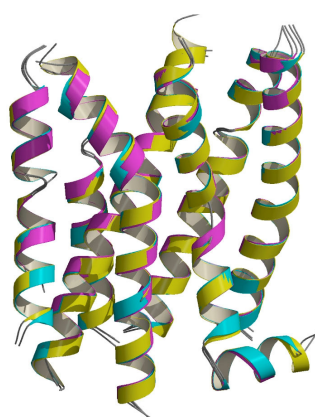
### CANNABINOID RECEPTORS: MOLECULAR MODELLING OF THE PROTEIN LIGAND COMPLEX.

Cristina Montero, Nuria Campillo, Pilar Goya, Juan Antonio Páez

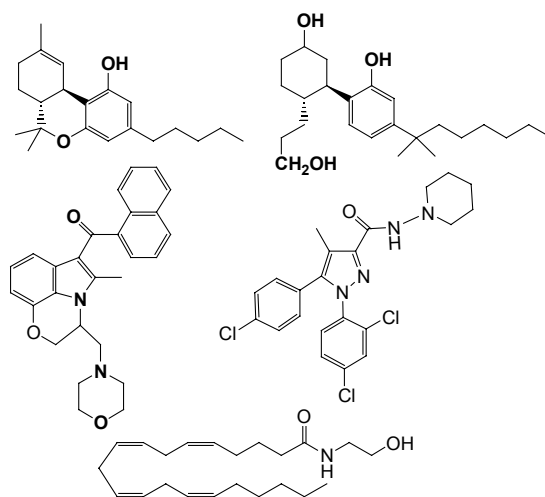
Instituto de Química Médica (CSIC). Juan de la Cierva 3, 28006-Madrid

The cannabinoid receptors are involved in a vast variety of biological processes and thus the potential therapeutic applications claimed for cannabinoids include treatment of nausea in cancer chemotherapy, anorexia of AIDS, neurological disorders and spinal injury symptoms, glaucoma, malignant gliomas, vascular effects and analgesia. These effects are mediated by two types of cannabinoid receptors that have been identified, CB1 cloned in 1990, and CB2 cloned in 1993 [1-3].

The 3D models of both CB1 and CB2 receptors belonging to the superfamily of GPCRs have been built by homology modeling[4] as template using the X-ray structure of Bovine Rhodopsin (1f88) determined by Miyano et al [5] (figure 1).



**Figure 1.** Structural superposition of CB1, CB2 models and 1f88.



**Figure 2.** Cannabinoid ligands.

The properties of the cannabinoid system has been studied by means of docking techniques, using the 3D models of both cannabinoid CB1 and CB2 receptors built by homology modeling and well known reference agonist and antagonist compounds.

An approach based on the flexibility of the structures have been used to the model of the receptor-ligand complexes, taking into account the five structural groups of cannabinoid ligands, as classical, non classical, endocannabinoids, aminoalkylindoles and diarylpyrazoles (figure 2).

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## P-2

### TRIFLUOROMETHYLKETONE DERIVATIVES AS FATTY ACID AMIDE HYDROLASES INHIBITORS: SYNTHESIS AND PHARMACOLOGICAL EVALUATION

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**Introduction-** Fatty acid amides, including endocannabinoids, are important lipid transmitters implicated in a wide diversity of physiological effects. For instance, anandamide, one of the major endocannabinoids, acts via cannabinoid and vanilloid receptors to produce analgesia and stimulation of appetite. Oleamide is a sleeping inducer and palmitoylethanolamide exhibits anti-inflammatory and analgesic properties without directly interacting with cannabinoid receptors.

All of these lipids are cleaved by amidases. Two amidases are now assumed to hydrolyze the fatty acid amides. Fatty acid amide hydrolase (FAAH), also named anandamide hydrolase, is the most characterized one<sup>1</sup>. It has been cloned from several species, FAAH<sup>-/-</sup> knockout animals were obtained<sup>2</sup> and very recently a 2.8 Angström X-Ray crystal structure<sup>3</sup> has been reported. The second one proposed as acid amide hydrolase (AAH) has been partially characterized<sup>4</sup>. It can be distinguished from the FAAH by its pH optimum, sensitivity to pharmacological agents, as well as substrate specificity.

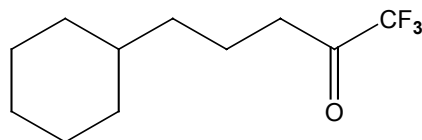
**Aim** – The aim of our study was to synthesize trifluoromethylketone inhibitors derived from phenylpropionic acid and to evaluate the inhibition potential towards FAAH and AAH. Finally, their affinity for the cannabinoid receptors has been determined.

#### Results

**Chemistry:** The trifluoromethylketones were synthesized from the corresponding acid chloride by reaction with trifluoroacetic anhydride and pyridine at  $-60^{\circ}\text{C}$ , under  $\text{N}_2$ <sup>5</sup>.

**Pharmacology:** The inhibition of FAAH was determined by monitoring the hydrolysis of [<sup>3</sup>H]-anandamide on rat brain homogenates whilst inhibition of AAH, we evaluated the degradation of [<sup>14</sup>C]-palmitoylethanolamine on solubilized proteins from the 12000 x g pellet of rat lung. The affinity for the cannabinoid receptors was determined by the specific binding displacement of [<sup>3</sup>H]-CP 55-940 on homogenates from CHO cells expressing either CB<sub>1</sub> or CB<sub>2</sub> receptors.

Among the 13 compounds synthesized in our laboratory, the 1,1,1 trifluoromethyl-5-cyclohexylpentan-2-one (CL9) was the most potent inhibitor of FAAH and moreover it was not inhibitor of AAH. In fact, at 100  $\mu\text{M}$ , the percentages of inhibition were of 99,7% and 31,6% respectively to FAAH and AAH. All the compounds did not bind to cannabinoid receptors.



CL9

In conclusion, the synthesized trifluoromethylketone compounds seem to be selective inhibitors of the FAAH.

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### P-3

#### THE PRECLINICAL PROFILE OF SLV319, A NEW SELECTIVE CANNABINOID CB<sub>1</sub> RECEPTOR ANTAGONIST.

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The newly synthesized CB compound SLV319 selectively binds to the hCB<sub>1</sub> receptor *in vitro* (pK<sub>i</sub>=8.0; hCB<sub>2</sub> pK<sub>i</sub> <5.0), the nearest affinity was on the Histamine<sub>2</sub> receptor with a pK<sub>i</sub> of 5.5. In a functional assay SLV319 antagonizes the CB<sub>1</sub> agonist, WIN-55212-2, induced cAMP accumulation in human CHO cells (pA<sub>2</sub>=8.5). SLV319 also antagonizes WIN-55212-2 induced inhibition of K<sup>+</sup>-induced ACh release from hippocampal slices (pA<sub>2</sub>=8.1) and 4-AP-induced glutamate release from synaptosomes (pA<sub>2</sub>=8.0). These results indicate that SLV319 is a selective CB<sub>1</sub> receptor antagonist *in vitro*. *In vivo*, SLV319 antagonized the WIN-55212-2 induced hypotensive (ED<sub>50</sub>= 2.4 mg/kg po) and hypothermic (ED<sub>50</sub>= 0.5 mg/kg po) responses. After systemic administration SLV319, increased extracellular ACh levels in hippocampus using microdialysis (LED=4.6 mg/kg po). In rats trained to discriminate WIN-55212-2 from vehicle, SLV319 fully blocked the WIN-55212-2 induced discriminative stimulus (ED<sub>50</sub>=8.3 mg/kg po). These data show that also *in vivo* SLV319 behaves as a CB<sub>1</sub> receptor antagonist.

Daily treatment with SLV319 dose-dependently reduced weight gain in both Wistar and Zucker rats (LED = 3 mg/kg po). In the social recognition task, treatment of adult rats with SLV319 (1, 3, 10 mg/kg, po) following the first exposure to a juvenile, reduced the time spent exploring the juvenile rat upon the second encounter (LED = 10 mg/kg po), suggesting that SLV319 had enhanced the consolidation of the information gained at the first exposure. These results indicate that SLV319 may have therapeutic potential in reducing weight gain and may improve memory consolidation.

Together these results indicate that SLV319 acts as a CB<sub>1</sub> selective antagonist with good oral availability and may have potential as a therapy for obesity and for the restoration of cognitive deficits related to consolidation processes.

Supported by Solvay Pharmaceuticals BV.

#### P-4

### QUANTIFICATION OF PALMITOYLETHANOLAMIDE : A COMPARISON BETWEEN HPLC WITH FLUOROMETRIC DETECTION AFTER DERIVATIZATION AND HPLC-MS

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✦ *Both authors contributed equally to this study.*

Since the discovery of anandamide as the natural mediator of cannabinoid receptors in 1992, fatty acid amides received a renewed interest, whether they are or not endocannabinoids. Among them, palmitoylethanolamide or *N*-palmitoylethanolamine (PEA) is an intriguing lipid derivative, unable to recognize directly the cannabinoid receptors. However, PEA is able to potentiate anandamide responses on both cannabinoid and vanilloid receptors. On cannabinoid receptors, the proposed mechanism effect is the so called “entourage effect” meaning interfering with the inactivation pathways of anandamide without affecting the receptors. On vanilloid receptors, if PEA has a modest effect on its own, it potentiates the anandamide-induced calcium entry, via an unknown mechanism.

As PEA concentrations increase in several patho-physiological situations, there is a need to achieve a quantification of this derivative. The aim of this study was to compare two analytical procedures : HPLC-MS and HPLC with fluorometric detection after derivatization.

As PEA's structure does not exhibit any chromophore or fluorophore, a derivatization has been made. Dansyl chloride in excess has been added to PEA in presence of dimethylaminopyridine to achieve in high yield the dansyl ester of PEA (**1**). After a preparative TLC, the dansyl ester of PEA has been analysed through an HPLC system consisting of a mixture of acetonitrile:water 98:2, Ro-SilC18 column and a flow of 1ml/min. Under these conditions, the retention peak was 12.67 min. The fluorometric detection was performed with an excitation wavelength and an emission wavelength of 350 and 500 nm respectively. With this procedure, the limit of detection of the dansyl ester of PEA is  $10^{-7}$  M, which corresponds to  $2.10^{-11}$  to  $4.10^{-8}$  moles of PEA, a value in accordance to the physiological levels. The synthesized internal standard was the dansyl ester of octanol.

The HPLC-MS allows a direct detection of PEA. A gradient HPLC was performed starting with a mixture 30 % of an aqueous phase (A) containing 1 % acetonitrile and 1 % acetic acid and 70 % acetonitrile, followed by a constant gradient from 30 to 2 % of A and 70 to 98 % acetonitrile in a period of 30 min. Using a C-18 column, the retention peak of PEA was 17.5 min. A deuterated internal standard has been used. The limit of detection is inferior to 0.1 pmol. One of the advantages of this method is to study in a single run several endocannabinoids such as anandamide, 2-arachidonoylglycerol, oleoylethanolamine and PEA. In conclusion, the HPLC-MS method seems to be attractive to study the variations of PEA concentrations in several patho-physiological processes, together or not with other endocannabinoids.

**P-5**

**ANANDAMIDE EFFECTS ON MOTOR BEHAVIOR AND NIGROSTRIATAL DOPAMINERGIC ACTIVITY ARE MEDIATED BY THE ACTIVATION OF VANILLOID VR1 RECEPTORS**

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The administration of anandamide produced hypokinesia in rats in parallel to a decrease in dopamine (DA) synthesis in nigrostriatal dopaminergic neurons (Romero et al., Brain Res. 694, 223-232 (1995)). It was hypothesized that this effect was mediated through the activation of CB<sub>1</sub> receptors, but not in a direct way because these receptors are not located onto dopaminergic neurons, but onto striatal projection neurons connected with them. However, two recent discoveries: (i) that anandamide is also able to activate vanilloid VR1 receptors (Zygmunt et al., Nature 400, 452-457 (1999)), and (ii) that VR1 receptors are located onto nigrostriatal dopaminergic neurons (Mezey et al., PNAS 97, 3655-3660 (2000)), have allowed to re-evaluate these observations and suspect that the activation of VR1 rather than CB<sub>1</sub> receptors might be involved in anandamide-induced hypokinesia and decreased DA activity in nigrostriatal neurons. To validate this hypothesis, we carried out two different experiments. First, we explored whether the effects of anandamide by reducing motor activity and by decreasing DA transmission were reversed by capsazepine, a selective antagonist of VR1 receptors. The data demonstrated that anandamide reduced ambulation, stereotypies and exploration, measured in the open-field test, whereas it increased the time spent in inactivity. All these effects were completely reversed by capsazepine, which had no effect by itself. In the same way, anandamide caused a significant decrease in nigrostriatal DA activity, reflected by a reduction in DOPAC contents in the caudate-putamen, which was also reversed by capsazepine. As a second objective, we explored whether anandamide is able to directly influence dopaminergic function by examining *in vitro* DA release using perfused striatal fragments. Our data confirmed that anandamide decreased K<sup>+</sup>-stimulated DA release from nigrostriatal terminals, and that effects were VR1 receptor-mediated. In summary, anandamide behaves as a hypokinetic substance, thus producing motor depression in the open-field test, presumably related to a decrease in nigrostriatal DA activity. However, contrarily to that expected, these effects were completely reversed by capsazepine, a selective VR1 receptor antagonist, thus indicating a predominant role of these receptors, which are located onto dopaminergic neurons, in mediating hypokinetic effects of anandamide. *In vitro* studies support this finding.

Supported by a grant from CAM-PRI (08.5/0063/2001)

**P-6**

**CHRONIC CANNABINOID ADMINISTRATION AFFECTS SEROTONIN TRANSMISSION IN THE CEREBRAL CORTEX BUT NOT IN OTHER BRAIN REGIONS.**

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Recent evidence has suggested that endocannabinoids, in addition to their classic actions on dopamine, GABA and glutamate neurotransmission, might also affect serotonin (5HT) transmission. To further explore this question, we have analyzed the contents of 5HT and of its intraneuronal metabolite, 5-hydroxyindolacetic acid (5HIAA), in the raphe nuclei, the brain region where cell bodies of central serotonergic neurons are located, and in various brain areas receiving serotonergic afferences from the raphe nuclei, of adult rats that were subjected to a chronic treatment with the cannabinoid agonist,  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC), or with vehicle. Results indicated that 5HT and 5HIAA contents were not affected by chronic cannabinoid administration in most of the brain regions analyzed, including the raphe nuclei, caudate-putamen, nucleus accumbens, substantia nigra, ventral-tegmental area, globus pallidus, amygdala, hippocampus and hypothalamus. However, there was a marked increase in the frontal cortex, affecting 5HT but not 5HIAA contents, and, then, decreasing 5HIAA/5HT ratio which can be used as an index for the activity of these neurons. As this effect was seen in a region where changes in serotonergic transmission have been implicated in the development of depressant states, our data might indicate that the endocannabinoid system might be a potential target for the treatment of this neuropsychiatric disease.

Supported by CAM-PRI (08.5/0079/2000)

**P-7**

**EFFECTS OF RM-BETA-CD ON SUBLINGUAL ABSORPTION OF THC**

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Over the years delta-9-tetrahydrocannabinol (THC) has been reported to counter a broad range of symptoms, including AIDS-associated wasting, anorexia, multiple sclerosis, pain and movement disorders. The therapeutic use of THC is limited, however, due to its poor aqueous solubility/dissolution characteristics and significant first-pass metabolism.

In the present study, a sublingual formulation of THC was developed by forming an inclusion complex with randomly methylated beta-cyclodextrin (RM-beta-CD) in order to improve the bioavailability of THC.

Phase solubility studies demonstrated that RM-beta-CD forms an inclusion complex with THC. The solid THC/RM-beta-CD inclusion complex was prepared by freeze-drying method. Dissolution studies demonstrated that RM-beta-CD significantly increases the dissolution rate of THC, thus making the development of a sublingual THC formulation possible.

The bioavailability of THC after sublingual administration of the THC/RM-beta-CD formulation was studied in rabbits, and blood samples were analysed for THC by GC-MS method.

## P-8

### **PRELIMINARY DATA ABOUT THE INFLUENCE OF WEANING ON THE ANTINOCICEPTIVE AND BEHAVIOURAL EFFECTS OF WIN 55,212-2 IN RATS.**

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There is evidence indicating that the endocannabinoid system may play different functional roles at different developmental stages in rodents. The atypical location of cannabinoid CB<sub>1</sub> receptors during the perinatal period seems to be related to a specific involvement of the endocannabinoid system in brain development (Fernández Ruiz et al. 2000, TINS 23: 14-20). In addition, the endocannabinoid system appears to play a role in milk suckling, and hence in neonatal development (Fride et al. 2003, Eur J Pharmacol 461: 27-34). It has been shown that the weaning process in rats is critical for the development of the  $\delta$ -opioid receptor (Goody & Kitchen 2001, J Pharmacol Exp Ther 296: 744-748) and there are well known functional interactions between the cannabinoid and the opioid systems (Manzanas et al. 1999, TIPS 20: 287-294). On the basis of all these evidences, we expected that weaning rat pups would exert an influence on functional responses mediated by CB<sub>1</sub> receptors. The aim of this study was to investigate the effects of the weaning process on the antinociceptive (tail immersion, water at 50°C) and behavioural (open field activity) responses to the cannabinoid receptor agonist WIN 55,212-2 (WIN) in Wistar rats of both genders. For this purpose, we used preweaning rats (20 days of age), 25-day old weaned rats (weaning at day 22) and 25-day old non-weaned rats. In order to select the adequate dose and pre-test time, we performed a first experiment using two doses of WIN (3 and 10 mg/kg) and two pre-test times, 30 and 60 min, in 25-day old weaned rats. From the data obtained in this experiment we chose the dose of 3 mg/kg for the cannabinoid agonist and 60 min as a suitable pre-test time, for the rest of the study. The results indicate that the level of analgesia was slight (around 12-13 % maximum possible effect, MPE), although significant, at day 25 (weaned and non-weaned rats), and significantly higher at this age than at day 20 (0-3.5 % MPE). In the open field, WIN significantly and markedly decreased rearing behaviour (vertical activity) in all the experimental groups ( $P_s < 0.05$ ). The cannabinoid agonist also decreased ambulation (horizontal activity) in pre-weanling and in non-weaned 25-day old rats of both sexes, as well as in 25-day-old weaned males ( $P_s < 0.05$ ). However, the drug did not affect the horizontal activity of 25-day old weaned females. The results demonstrate that the weaning process induces a transient reduction in the sensitivity of female rats to the effects of WIN on horizontal activity. The data also suggest that, during the peri-weanling period, there is a differential maturation of the CB<sub>1</sub> receptors mediating motor and analgesic responses to WIN, and that rearing behaviour (vertical activity) is particularly sensitive to the effects of cannabinoids from early postnatal ages. Similarly to the influence of maternal milk on functional activation of  $\delta$ -opioid receptors in postnatal rats (Goody & Kitchen 2001), there may be a relationship between the presence of 2-arachidonoyl glycerol in maternal milk (Fride et al. 2003) and the differential effect of an exogenous cannabinoid agonist (WIN) on motor activity of weaned and nonweaned rats of the same age. The sexual dimorphism observed in this latter effect might be related to the previously reported sex differences in the developmental pattern of striatal CB<sub>1</sub> receptors (Rodríguez de Fonseca et al. 1993, NeuroReport 4: 135-138). We are currently performing additional experiments on WIN-stimulated [<sup>35</sup>S] GTP  $\gamma$  S binding for a direct assessment of the effect of weaning on the functional activity of brain CB<sub>1</sub> receptors.

This study was supported by the Ministerio de Ciencia y Tecnología (BFI2000-0611).

## P-9

### THE ROLE OF CB<sub>2</sub> RECEPTORS IN PERIPHERAL SOMATOSENSORY PROCESSING

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CB<sub>2</sub> receptors are located outside of the central nervous system (CNS) and have been shown to inhibit acute nociception and inflammatory hyperalgesia (Malan *et al.*, Chem. Phys. Lipids 121, 191-200, 2002). CB<sub>2</sub> cannabinoid receptors are predominantly expressed in immune tissues, however CB<sub>2</sub>-like receptors may be present on peripheral nerves (Griffin *et al.*, Eur. J. Pharmacol. 339, 53-61, 1997). This study determined effects of peripheral injection of the CB<sub>2</sub> selective agonist JWH-133 and antagonist SR144528 on nociceptive transmission in non-inflamed rats and rats with carrageenan-induced hindpaw inflammation. Extracellular recordings of convergent dorsal horn neurones were made in anaesthetised (1% halothane in 66% N<sub>2</sub>O / 33% O<sub>2</sub>) male Sprague Dawley rats (230-300g) (Chapman *et al.*, Anaesthesiology 81, 1429-1435, 1994). Spinal neuronal responses to mechanical stimulation (6, 8, 12, 21, 45 and 80g von Frey monofilaments) of the peripheral receptive field were quantified (firing frequency Hz) during a 10sec stimulus duration. Effects of peripheral injection of JWH-133 (15µg in 50µl), SR144528 (10µg in 50µl) and SR144528+JWH-133 on mechanically-evoked neuronal responses were measured in non-inflamed and rats with established carrageenan-induced hindpaw inflammation (3 hours post intraplantar injection of 100µl of 2% carrageenan). Data are mean maximal effects ±sem; statistical analysis: repeated measures ANOVA, Dunnett's *post hoc* test and Mann-Whitney test.

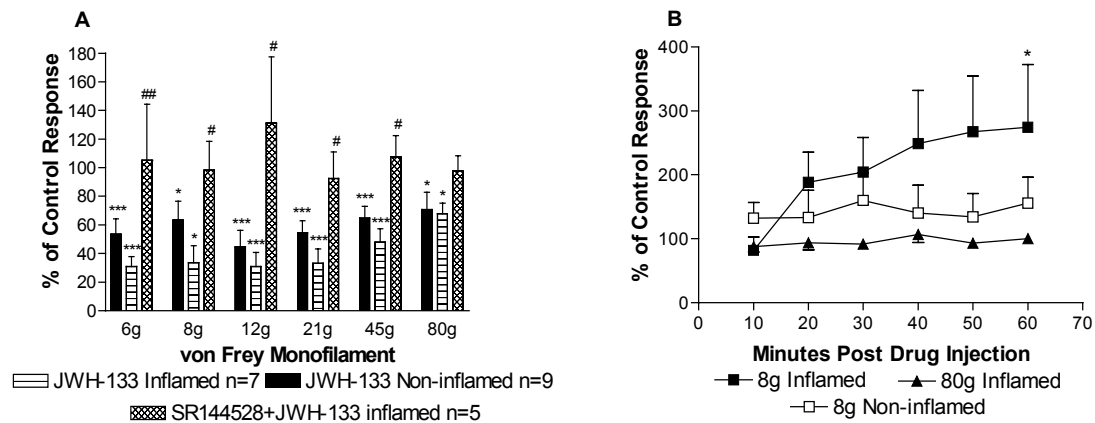


Fig.1. A. Effects of peripheral injection of JWH-133 (15µg in 50µl) on mechanically-evoked responses of spinal neurones at 20minutes post injection in non-inflamed rats and rats with hindpaw inflammation. B. Effects of peripheral injection of SR144528 (10µg in 50µl) on mechanically-evoked responses of spinal neurones in non-inflamed rats and rats with hindpaw inflammation

Peripheral injection of JWH-133 significantly attenuated mechanically-evoked responses of spinal neurones in non-inflamed rats and rats with hindpaw inflammation (Fig. 1A). Inhibitory effects of JWH-133 on mechanically-evoked responses were significantly blocked by SR144528 in rats with hindpaw inflammation (Fig. 1A). Peripheral injection of SR144528 alone significantly facilitated innocuous (8g) but not noxious (80g) mechanically-evoked responses of spinal neurones in rats with hindpaw inflammation (Fig. 1B). SR144528 had no effect on mechanically-evoked responses of spinal neurones in non-inflamed rats (Fig. 1B). Peripheral JWH-133 significantly attenuated both innocuous and noxious mechanically-evoked responses of spinal neurones in non-inflamed rats. This effect was blocked by SR144528, indicating the contribution of the peripheral CB<sub>2</sub> receptors. JWH-133 produced a greater inhibition of mechanically-evoked responses in rats with hindpaw inflammation. The basis for this finding may be an up-regulation of CB<sub>2</sub> receptors on inflammatory cells under these conditions. Activation of CB<sub>2</sub> receptors on mast, or other immune, cells by JWH-133 may decrease the release of pro-inflammatory / pro-nociceptive molecules that sensitise the peripheral afferent endings. Alternatively, direct activation of CB<sub>2</sub> receptors on primary afferent fibres may account for JWH-133-mediated antinociception. SR144528-mediated facilitation of innocuous-evoked responses of spinal neurones in rats with hindpaw inflammation may indicate a CB<sub>2</sub> mediated endocannabinoid tone under these conditions. These data demonstrate that CB<sub>2</sub> receptor agonists have anti-nociceptive effects in animal models of acute and inflammatory pain.

This study was supported jointly by the University of Nottingham and GlaxoSmithKline

## **P-10**

### **CANNABINOID CB1 RECEPTOR IMMUNOREACTIVITY IN ILEAL MYENTERIC NEURONS FROM AGED GUINEA-PIGS.**

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**Introduction.** In the last few years, the presence of CB1 receptors has been demonstrated in myenteric neurons from several gastrointestinal regions of different animal species by immunohistochemical methods. CB1 receptors are expressed early during the development of the gastrointestinal tract, suggestive of an important role of the endogenous cannabinoid system in ontogeny. It has been shown that the enteric nervous system is subjected to some degree of age-related neurodegeneration, although not much is known about the selectivity of this process for the different neuronal populations.

**Objective.** To determine, in guinea-pig ileum, whether the myenteric neuronal population immunoreactive for CB1 receptor is selectively affected by age-related neurodegeneration.

**Methods.** Ileal tissues were obtained from female guinea-pigs of three different ages: 2-3 months (young), 9-10 months (adult), 24-26 months (old). Longitudinal muscle-myenteric plexus preparations (LM-MP) were carefully isolated and processed for double immunohistochemical labelling. Primary antibodies were: biotinilated anti-HuC/D (1:500), to label the whole myenteric neuronal population; rabbit anti-CB1 (1:100, kindly donated by Dr Mackie, Washington University), to label neurons expressing CB1 receptor. Secondary antibodies were: streptavidin-conjugated Alexa Fluor 488 (1:1000) and CY5-donkey anti-rabbit (1:100). Preparations were observed under fluorescence and confocal microscopy. Immunoreactive neurons for each marker were counted and neuronal density both inside and outside the ganglia was statistically compared by the t-student test.

**Results.** A large proportion of neurons showed CB1 immunoreactivity both inside and outside the ganglia in all three ages of animals studied. Inside the ganglia neuronal density of myenteric neurones with CB1 receptor immunoreactivity decreased in an age-related manner, in parallel to the total neuronal population. Outside the ganglia, both total and CB1-positive myenteric neurons were more numerous in the adult group. When compared to the total population, the proportion of CB1-positive myenteric neurons in LM-MP preparations from both adult and old animals showed a slight but statistically significant increase.

**Conclusions.** In agreement with studies from other laboratories, our results support that myenteric neurones are subjected to neurodegenerative processes leading to a decrease in the neuronal counting. CB1-positive neurons seem to follow a similar degree of neurodegeneration. CB1 receptor is confirmed to be widely expressed in the myenteric plexus, and the slight increase in the proportion of this neuronal population suggests that CB1 receptors could be important for myenteric control of gastrointestinal motility still in old ages.

**Acknowledgements.** Supported by Universidad Rey Juan Carlos (PGRAL-2001/11 and PGRAL-2001/3).

## P-11

### THE EFFECTS OF RUTHENIUM RED ON THE INHIBITORY ACTIONS OF NOLADIN ETHER, THC & HU210 ON SENSORY NEUROTRANSMISSION IN THE RAT ISOLATED MESENTERIC ARTERIAL BED.

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In the rat mesenteric arterial bed calcitonin gene-related peptide (CGRP) is released upon activation of sensory nerves producing vasodilatation (Kawasaki *et al.*, 1988). We have previously reported that the endocannabinoid noladin ether,  $\Delta^9$ -tetrahydrocannabinol (THC) and HU210 (Ralevic & Kendall, 2001; Duncan *et al.*, 2002a,b) inhibit sensory neurotransmission via a non-CB<sub>1</sub>/CB<sub>2</sub> mechanism in this preparation. Zygmunt *et al.* (2002) recently reported that THC released CGRP from sensory nerves in rat mesenteric arterial segments and this release was sensitive to the vanilloid receptor channel inhibitor ruthenium red. In this study we investigated whether ruthenium red could reverse the inhibitory actions of these cannabinoid receptor agonists on sensory neurotransmission in the rat isolated mesenteric arterial bed.

Male Wistar rats (250-300g) were killed by exposure to CO<sub>2</sub> and decapitation. Mesenteric beds were isolated and perfused with oxygenated Krebs' solution containing guanethidine (5 $\mu$ M) to block sympathetic neurotransmission (Ralevic & Kendall, 2001). After 30 min equilibration, preparations were precontracted with methoxamine (10-100 $\mu$ M) and three consecutive frequency-response curves to electrical field stimulation (EFS, 1-12Hz, 60V, 0.1ms, 30s) (EFS control, EFSI and EFSII) were constructed in each preparation. The agonist or vehicle (0.01% ethanol) was added after EFS control, 15 min before EFSI. Ruthenium red (1 $\mu$ M) was added at the start of the equilibration period. Data are expressed as mean $\pm$ s.e.m. and analysed by ANOVA with Tukey's post-hoc test or by Student's unpaired t test.

In the presence of 1 $\mu$ M noladin ether, the sensory neurogenic relaxation response, at a submaximal frequency 8Hz was reduced from 57.33 $\pm$ 6.83%, EFS control, to 23.3 $\pm$ 3.78%, EFSII (n=4, P<0.05). In the presence 1 $\mu$ M ruthenium red, the response at 8Hz was reduced from 46.82 $\pm$ 6.44%, EFS control, to 17.96 $\pm$ 2.47%, EFSII (n=6, P<0.01). THC (1 $\mu$ M) inhibited the response at 8Hz from 53.08 $\pm$ 4.98%, EFS control, to 11.14 $\pm$ 1.58%, EFSII (n=4, P<0.001); ruthenium red had no effect on this (8Hz EFS control 54.51 $\pm$ 5.78 to EFSII 25.26 $\pm$ 4.86%, n=12, P<0.01). HU210 (1 $\mu$ M) also inhibited the response at 8Hz; it was reduced from 57.97 $\pm$ 5.37%, EFS control, to 18.37 $\pm$ 4.08%, EFSII (n=7, P<0.001). In the presence of ruthenium red, the inhibitory effect of HU210 at 8Hz was abolished; 49.15 $\pm$ 6.96%, EFS control, to 40.63 $\pm$ 3.65%, EFSII (n=4, P>0.05).

These data show that the inhibitory actions of noladin ether and THC are not reversed by ruthenium red. The inhibition of sensory neurotransmission by HU210, however, is ruthenium red sensitive. The site of action for noladin ether and THC is different from that of HU210 and does not appear to require the presence of functional vanilloid receptors.

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## P-12

### INFLUENCE OF ENDOTHELIUM IN THE VASORELAXATION PRODUCED BY CANNABINOIDS IN RAT AORTA

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**INTRODUCTION.** The vascular effects of cannabinoids in the rat isolated vasculature are very complex. Cannabinoids elicited vasodilatation of isolated small arteries. The possible mechanism in the vasculature has not been established. The effect may be produced by interaction with the CB1 cannabinoid receptor, VR1 vanilloid receptor, or mediated by endothelium-derived factors (NO, PGs, EDHF).

**Objective.** In the present study, the vasodilator actions of cannabimimetic compounds in the rat isolated aorta were investigated, to establish differences in responsiveness between natural and synthetic cannabinoids derivatives and the endothelium-dependent vasodilation.

**METHODS.** The descending aorta of Wistar rats was used. The isolated aortic rings were introduced in an organ baths (under 2g of tension) with Krebs solution (37° C), and bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> gas mixture. A submaximal contraction was induced with one dose of phenylephrine (Phe) 10<sup>-6</sup> M. Cumulative concentration-response curves of all the compounds studied were constructed (10<sup>-9</sup> M -10<sup>-4</sup> M). Vasodilatation produced by carbachol (10<sup>-5</sup> M) was tested after each experiment, considering intact arteries (+E) when relaxation was upper 50% and denuded arteries (-E) when relaxation was lower than 10%. Isometric tension was recorded in a polygraph (Grass Mod. 7D) through force-displacement transducers Grass FT07. Results are expressed in % of relaxation of precontracted-Phe tone (Mean ± s.e.m.).

Drugs: Anandamide, Carbachol, Methanandamide, Phenylephrine and WIN 55,212-2.

#### RESULTS:

% Relaxation (+E)	10 <sup>-9</sup> M	10 <sup>-8</sup> M	10 <sup>-7</sup> M	10 <sup>-6</sup> M	10 <sup>-5</sup> M	10 <sup>-4</sup> M
Methanandamide	2.6±1.5*	10.6±1.5*	20.1±1.1*	28.8±3.2*	45.2±4.9*	71.0±4.8*
Win 55,212-2	1.8±1.3	4.9±1.4	6.2±2.1	11.8±2.7	26.3±4.4	41.7±6.0
Anandamide	2.2±2.2*	6.2±3.8*	15.6±6.5*	27.6±8.2*	41.3±9.2*	56.6±9.7*

% Relaxation (-E)	10 <sup>-9</sup> M	10 <sup>-8</sup> M	10 <sup>-7</sup> M	10 <sup>-6</sup> M	10 <sup>-5</sup> M	10 <sup>-4</sup> M
Methanandamide	0.0±0.0	1.8±1.3	3.5±1.2	3.8±1.2	8.6±1.7	10.9±1.8
Win 55,212-2	0.0±0.0	0.9±1.0	2.1±1.5	5.9±0.9	8.8±1.4	17.6±1.8
Anandamide	2.2±0.7	3.0±0.7	6.5±1.6	11.7±1.9	15.0±2.2	20.4±3.1

\* P < 0.05 (+E vs -E)

**CONCLUSIONS :** The data shows that the presence of functional endothelium is crucial for the vasorelaxation produced by cannabinoid compounds (natural and synthetic). The natural compounds are more potent than synthetic ones as vasodilators in rat aorta.

**ACKNOWLEDGMENT:** This work was supported by CAM (Ref.08.9/0007.1/99)

### **P-13**

#### **EVIDENCE FOR CANNABINOID-INDUCED CONTRACTION IN THE RAT ISOLATED AORTA**

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Preliminary experiments showed that the vasorelaxant effects of two endocannabinoids, anandamide and N-arachidonoyl-dopamine (NADA), were potentiated by the CB<sub>1</sub> antagonist SR141716 (1 μM) in the rat aorta. This could indicate the existence of a SR141716-sensitive cannabinoid-mediated vasoconstrictor response in this tissue. To test this hypothesis, the contractile responses to a number of cannabinoids were tested in the rat aorta.

Male Wistar rats (250-350g) were killed by cervical dislocation and exsanguination. The thoracic aorta was dissected and all connective and adipose tissue were stripped from the vessel. The aorta was mounted in oxygenated Krebs-Henseleit solution at 37°C and connected, using hooks and thread to a force transducer. Vessels were set at a baseline tension of 1.0 g and allowed to equilibrate for an hour. The contractile integrity of each vessels was tested by its ability to contract in the presence of 60 mM KCl. Following equilibration, cannabinoids were added to the bath to obtain the appropriate concentrations from initial stock solutions dissolved in ethanol.

Δ<sup>9</sup>-Tetrahydrocannabinol (THC) caused contraction in the aorta (pEC<sub>50</sub> 4.42 ± 0.42, R<sub>max</sub> 0.40 ± 0.22 g tension), while neither anandamide nor NADA caused an increase in tension. To further characterise the effects of THC, aortae from animals pre-treated with pertussis toxin (PTX, 15 μg/kg i.p. 3 days before death) or WIN55,212-2 (WIN, 4 mg/kg/day i.p. for 14 days) were assessed. Both treatments abolished the THC contractile response (R<sub>max</sub> PTX 0.01 ± 0.03 g, *P*<0.01; WIN 0.03 ± 0.05 g, *P*<0.01). Pre-incubation of aortic rings with SR141716 at 1 μM caused a reduction of the contractile response to THC (R<sub>max</sub> 0.06 ± 0.04 g, *P*<0.05). In the presence of indomethacin, aortae did not contract to THC (R<sub>max</sub> -0.02 ± 0.05 g, *P*<0.01).

These results have demonstrated, for the first time, THC-mediated constriction of the rat aorta. The mechanisms underlying this response appear to involve a Gi/o-protein coupled receptor, possibly the CB<sub>1</sub> or a similar receptor sensitive to chronic adaptation to WIN55,212-2 exposure. On the evidence of the blockade due to indomethacin, THC may cause contraction in the aorta through production of a prostanoid vasoconstrictor.

#### P-14

### INFLUENCE OF DIFFERENT VEHICLES USUALLY USED AS CANNABINOID SOLVENTS IN ENDOTHELIAL CELL LINING.

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**Introduction:** In a wide variety of cannabinoid research protocols either polysorbates or ethanol have been used as adequate vehicles. Ideally, these vehicles should have no pharmacological effects, but it has been reported that agents as Tween 20, Tween 80 or Cremophor EL impaired the vasorelaxation mediated by endothelium. Besides, ethanol might also influence vascular function at both the level of smooth muscle and the endothelium. (*Experientia* 1995; 51:1055-59. Since endogenous cannabinoid system has been implicated in the control of vascular tone, and it has been reported that anandamide caused vasorelaxation mediated by an unidentified endothelial mechanism (*Pharm Ther* 2002; 95:191-202), correct selection of the vehicle for the vascular research protocols, seems to be a crucial issue.

**Aim:** To determine the influence of three different vehicles usually used for cannabinoid solubilization on the endothelium-dependent vasorelaxant responses in rat aorta.

**Material and Methods:** Aorta rings (3-4 mm long) from male Wistar rats (250-300g) were fixed vertically between two stainless steel hooks in a tissue bath containing Krebs-Henseleit buffer at 37°C and bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The upper wire was connected to an isometric force-displacement transducer for tension measurements. 1) After an equilibration period of 90 min. (resting tension of 2 g) and once tested for the presence of functional endothelium, the arteries were precontracted with phenylephrine (Phe, 10<sup>-6</sup> M) and concentration-response curves were carried out by cumulative addition of the different solvents: Tween 80 (1.3 ml/l, 0.65 ml/l), 1:1:18 (Tween 80:ethanol:saline) and DMSO 0.5%. Control rings were similarly treated with Phe but no further additions were made. 2) At the end of each vehicle concentration-response curve, 10 μM of carbachol was added to verify the existence of functional endothelium. 3) The rings were reequilibrated for 30-40 min. and a third 10 μM-carbachol relaxation was generated to determinate if the vehicle produced an inhibition or a destruction of the endothelial cell lining. Results are expressed as % of relaxation of Phe-induced tone. Data are given as the mean ± s.e.m (8-12 rings). A two-way ANOVA (Bonferroni/Dunn *post-hoc* test) was used ( $P \leq 0.05$  = statistically significant).

**Results:** Among all the solvents tested, the vehicles 1:1:18 and Tween 80 (1.3 ml/l) produced a significant reduction (E<sub>max</sub>: 36.41%±3.42 and 29.63%±4.93 respectively,  $P < 0.05$ ) in the Phe-precontracted aorta respect to the control arteries (E<sub>max</sub>: 12.85%±2.94). After the concentration-response curve of each vehicle, only DMSO 0.5% was able to maintain a 10μM-carbachol-relaxation similar to the control arteries (E<sub>max</sub>: 91.70%±6.71 vs 88.11%±5.96, n.s.). After the reequilibration period, all the solvents tested caused a endothelium-dependent relaxation similar to the controls, suggesting that these vehicles provoked an inhibition of endothelium-dependent relaxation but no destruction of this cell lining occurs.

**Conclusion:** DMSO 0.5% seems the most adequate vehicle for cardiovascular research protocols in cannabinoid investigations because of the lack of modification in endothelium-dependent relaxation.

This project is funded by Universidad Rey Juan Carlos (PIGE-02-16)

## P-15

### EFFECT OF A CANNABINOID AGONIST (WIN 55,212-2) IN ILEUM FROM AGED GUINEA-PIGS

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**Introduction.** Cannabinoid agonists acting upon CB1 receptors have been shown to inhibit both the contractile activity of longitudinal muscle-myenteric plexus (LM-MP) preparations in response to electrical stimulation, and the peristaltic response to either electrical or mechanical stimuli, thus meaning that the endogenous cannabinoid system could be involved in the modulation of gastrointestinal motility. Many authors have described age-induced alterations of enteric motility, but data are still controversial.

**Objective.** To determine the effect of aging on the cannabinoid-mediated inhibition of the electrically stimulated contractile activity of guinea-pig ileum LM-MP preparations.

**Methods.** Ileal LM-MP were isolated from female guinea-pigs of four different ages: 21 days (just weaned), 2-3 months (young), 9-10 months (adult), 24 months (old). Preparations were placed in an organ bath and a resting tension of 1 g was applied. Contractile activity of the preparations was recorded by means of an isometric transducer connected to a personal computer. Electrical stimulation consisted of square pulses of 2 ms, elicited at 0.3 Hz. Preparations in which electrical stimulation did not induce twitches of 0.5 g or more, were discarded. The threshold (minimum voltage that induced a stable response) of the electrically-induced contractions was valued for each age. The cannabinoid agonist WIN 55,212-2 was added to the bath at increasing cumulative concentrations ( $10^{-8}$  to  $2.4 \times 10^{-6}$  M). The effect of the cannabinoid was measured as the % of inhibition of contraction three minutes after each administration. Age-related changes were analysed statistically by ANOVA test.

**Results.** Voltage threshold to get stable contractions of LM-MP preparations was similar for the four groups of age. The cannabinoid agonist WIN 55,212-2 inhibited the electrically-induced contractions in a dose-dependent manner in all ages considered. This inhibitory effect was significantly increased in the group of tissues obtained from old animals ( $p < 0.001$ ).

**Conclusions.** From our results it seems that the basal function of the LM-MP preparations does not change significantly with age, meanwhile the response of the preparations to cannabinoid agonists seems to be increased in aged animals. This effect could be due to changes in the expression of the cannabinoid receptor and could participate in some of the alterations reported in functionality of gastrointestinal motility of aged animals. Whether this sensibility of ileum from aged animals is selective of the cannabinoid receptors or is extensible to other inhibitory or/and excitatory systems remains to be elucidated.

**Acknowledgements.** Supported by PGRAL-2001/11.

## **P-16**

### **CANNABIDIOL IS AN ORAL EFFECTIVE THERAPEUTIC AGENT BOTH IN ACUTE INFLAMMATION AND IN CHRONIC FCA-INDUCED ARTHRITIS**

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Cannabidiol is the major non-psychoactive component of marijuana and does not bind to either cannabinoid receptor subtypes. Recent evidences showed that cannabidiol blocked the progression of arthritis induced in mice by collagen, a murine model for rheumatoid arthritis, when administered after onset of clinical symptoms (Malfait et al., Proc. Natl. Acad. Sci. USA 97, 9561-9566, 2000). Furthermore we have recently reported its efficacy in a rat model of acute inflammation induced by i.pl. carrageenan; particularly, cannabidiol inhibited paw edema and thermal hyperalgesia in a dose- and time-dependent fashion when orally given after the induction of inflammation once a day for three days at different doses (5-40 mg/kg) (Costa et al., Proceedings of 12<sup>th</sup> Annual Symposium on the Cannabinoids, 44, 2002).

In the present work we report that the anti-hyperalgesic effect of cannabidiol is fully reversed by the simultaneous i.p. administration of the selective VR1 receptor antagonist capsazepine (10 mg/kg) and only partially anagonized by p.o. administration of the specific CB2 antagonist SR144528 (3 mg/kg, one hour before cannabidiol); on the contrary the simultaneous i.p. treatment with the specific CB1 antagonist SR141716A (0.5 mg/kg) does not reverse the anti-hyperalgesic action of cannabidiol. The anti-inflammatory efficacy of cannabidiol seems unrelated to inhibition of cyclooxygenase (COX) activity, the typical target of non steroidal anti-inflammatory drugs, but related to inhibition of endothelial isoform of nitric oxide synthase (eNOS); in fact cannabidiol, when assayed in vitro, inhibits the eNOS activity in a concentration-related fashion (0.01-100  $\mu$ M) whereas it does not affect either the activity of COX1 or that of COX2, in a concentration range 0.01-1  $\mu$ M, but at higher concentrations (10-100  $\mu$ M) it increases these activities.

We have assayed the therapeutic efficacy of cannabidiol in a rat model of unilateral arthritis induced by an i.pl. injection of Freund's complete adjuvant (FCA) which is characterized by development of hindlimb edema and thermal and mechanical hyperalgesia. Cannabidiol was orally administered after onset of inflammatory symptoms (7 days) at 10, 20 and 40 mg/kg per day for 7 days. Cannabidiol elicits the disappearance both of thermal and mechanical hyperalgesia from the lowest dose (10 mg/kg) and reduces the edema volume in a dose-related manner with a maximal effect elicited by 40 mg/kg (45%). The role of nitric oxide, prostaglandins and free radicals in the development and maintenance of arthritis is well known, so we studied the ability of cannabidiol to affect these inflammatory mediators. At the end of treatment (14 days after the adjuvant injection, time peak of arthritis clinical symptoms) plasmatic PGE2 level and production of nitric oxide and lipid peroxides in paw tissues have been evaluated. The cannabis compound seems to decrease the PGE2 content and inhibits the increase in nitric oxide and lipid peroxide level so that even the lowest dose brings these mediators down to that of non arthritic.

The present study shows that cannabidiol could have a beneficial therapeutic action on unilateral arthritic disease. Future studies will be addressed to clarify the mechanism underlying this anti-arthritic effect.

## P-17

### EFFECTS OF SYSTEMICALLY ADMINISTERED CANNABIDIOL AND $\Delta^9$ -THC ON FORMALIN-EVOKED NOCICEPTIVE BEHAVIOUR IN RATS

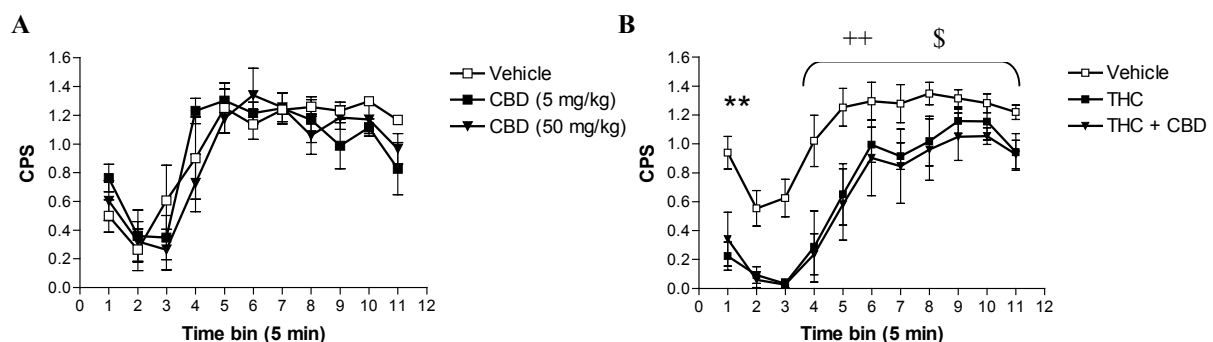
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Cannabinoids demonstrate clear potential as analgesic agents (for review see Pertwee 2001 *Prog. Neurobiol.* **63**, 569-611; Iversen and Chapman 2002 *Curr. Opin. Pharmacol.* **2**, 50-55). There is a considerable body of evidence demonstrating the effectiveness of  $\Delta^9$ -THC in animal models of nociception, and recently there has been increased interest in the other naturally occurring constituents of cannabis. The present study investigated the effects of cannabidiol and  $\Delta^9$ -THC, administered systemically alone, or in combination, on formalin-evoked nociceptive behaviour in rats.

In experiment 1, male Lister-hooded rats (250-300g) received CBD (5 or 50 mg/kg) or vehicle (Ethanol: Cremophor: Saline; ratio=1:1:18, 1 ml/kg). In experiment 2, rats received  $\Delta^9$ -THC (1 mg/kg),  $\Delta^9$ -THC (1 mg/kg) + CBD (5 mg/kg), or vehicle. Rats were injected i.p. 30 min prior to intra-plantar formalin injection (50 $\mu$ l, 2.5%) into the right hind paw. Nociceptive behaviour was tracked and scored for 60 min post-formalin using Ethovision software with the composite pain score technique (CPS-WST<sub>0,1,2</sub>; Watson *et al.* 1997 *Pain* **70**, 53-58). Grooming, rearing, defecation and paw diameter were also measured. In a separate group of rats, effects of  $\Delta^9$ -THC or CBD on locomotor activity 30 min post-injection were assessed over 60 min using activity monitor chambers (Medical Physics, Nottingham University).

In both experiments, peripheral injection of formalin evoked robust licking, flinching, shaking and elevation of the injected paw. Early (3-8 min) and late (18-60 min) phases of nociceptive behaviour were observed in vehicle-treated rats (Figure 1A & 1B). CBD (5 or 50 mg/kg) had no significant effect on the CPS over the 60 min trial (Figure 1A). CBD significantly ( $P < 0.01$ ) reduced defecation in a dose-related manner. Systemic injection of both  $\Delta^9$ -THC and  $\Delta^9$ -THC + CBD significantly reduced the CPS during the first phase (3-8 min) and second phase (18-60 min) compared with vehicle-treated rats (Figure 1B). There was no significant difference between the effects of  $\Delta^9$ -THC alone and  $\Delta^9$ -THC + CBD. Administration of CBD,  $\Delta^9$ -THC or  $\Delta^9$ -THC + CBD had no significant effect on paw oedema, rearing or grooming. Neither CBD nor  $\Delta^9$ -THC significantly affected locomotor activity.



**Figure 1.** Effect of **A:** CBD (5 or 50 mg/kg, i.p.) or **B:** THC (1 mg/kg, i.p) and THC (1 mg/kg)+CBD (5 mg/kg) on formalin-evoked nociceptive behaviour in rats. Data are means  $\pm$  s.e.m. (n = 5-11). Two-way ANOVA results, **A:** ( $F_{2, 20} = 0.76$ ;  $P = 0.47$ ); **B:** ( $F_{2, 20} = 42.8$ ;  $P = 0.0001$ ). \*\* $P < 0.01$  comparing THC or THC+CBD with vehicle-treated rats at T<sub>1</sub>; + $P < 0.01$  comparing THC+CBD with vehicle-treated rats from T<sub>4</sub>-T<sub>11</sub>; \$ $P < 0.05$  comparing THC with vehicle-treated controls from T<sub>4</sub>-T<sub>11</sub>.

In conclusion,  $\Delta^9$ -THC, but not CBD, displays antinociceptive activity in the rat formalin test. Combined administration of CBD does not alter the antinociceptive effect of  $\Delta^9$ -THC.

**P-18**

**THE VR1/CB1 AGONIST ARVANIL INDUCES APOPTOSIS THROUGH A FADD/CASPASE-8 DEPENDENT PATHWAY**

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N-Acyl vanillyl amides (N-AVAMs) represent a new class of hybrid molecules that target both the Vanilloid Receptor type I (VR-1) and the cannabinoid CB<sub>1</sub> receptor. Arvanil is one of the most representative N-AVAMs described so far and it has been shown to inhibit proliferation in human breast cancer cells and NF-κB activation in lymphoid cells. Because some endocannabinoids may elicit immunomodulatory and proapoptotic effects we were interested to investigate the effects of Arvanil in the apoptotic pathway in lymphoid cells. This study shows that Arvanil induce apoptosis (DNA fragmentation measured by the TUNEL method) in Jurkat cells (IC<sub>50</sub>=10 μM) but not in primary peripheral blood T lymphocytes. This proapoptotic effect was greatly enhanced in culture conditions where Arvanil was not quenched by albumin, which is present in the serum. Moreover, by using a cytofluorimetric approach and double staining experiments we detected that Arvanil-induced apoptosis was initiated at the S-phase of the cell cycle and inhibited by competitive peptides for caspase-8 and -3 cleavages.

Kinetic experiments with western blots and fluorimetry show that Arvanil first activates caspase -8, and then caspase-7 and -3 suggesting the triggering of an apoptotic pathway typical of type I cells. This was confirmed by measuring the role of the mitochondria in Arvanil-treated cells, and we observed that i) cyclosporine A did not protect cells from Arvanil-induced apoptosis, and ii) the transmembrane potential of the mitochondria was not collapsed in response to this compound. In addition, Arvanil failed to induce apoptosis in a Jurkat cell line stably transfected with a negative dominant form of the adapter molecule FADD, which, for this reason, is unable to form the death-inducing signalling complex (DISC).

Since Jurkat cells do not express either the VR1 or the CB<sub>1</sub> receptors, and because Arvanil is not a CB<sub>2</sub> receptor agonist, we suggest that Arvanil can induce apoptosis by a novel pathway and could serve as lead compound for the development of novel, potent anti-inflammatory and antitumoral drugs.

## P-19

### ENDOCANNABINOID CONTROL OF COLORECTAL CANCER GROWTH

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The endocannabinoids, anandamide and 2-arachidonoylglycerol (2-AG), or their metabolically stable analogues, inhibit cancer cell proliferation by acting at cannabinoid receptors (CBRs) (De Petrocellis et al., *Proc. Natl. Acad. Sci. USA*, 1998; Melck et al., *Endocrinology*, 2000; Bifulco et al., *FASEB J*, 2001; Bifulco and Di Marzo; *Nat. Med.*, 2002 for review). We studied their role in human colorectal carcinoma (CRC) by determining: 1) the levels of endocannabinoids, cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors, and fatty acid amide hydrolase (FAAH, which catalyses endocannabinoid hydrolysis), in colorectal cancers, adenomatous polyps, and neighbouring healthy mucosa; 2) the effects of endocannabinoids, and of substances inhibiting their inactivation, on the proliferation of human CRC cells.

Tissues were obtained from 21 patients by biopsy during colonoscopy. Endocannabinoids were measured by LC-MS. CB<sub>1</sub>, CB<sub>2</sub> and FAAH expression was analysed by RT-PCR and Western immunoblotting. CRC cell lines (CaCo-2 and DLD-1) were used to test the anti-proliferative effects of synthetic and endogenous cannabinoids, as well as of selective inhibitors of FAAH and of the anandamide membrane transporter (AMT).

Healthy mucosa, adenomas, CRC tissue and cell lines contain anandamide and 2-AG, and express CBRs and FAAH. The levels of the endocannabinoids were 3- and 2-fold higher in adenomas and CRCs than normal mucosa. Instead, consistent differences in CB<sub>1</sub>, CB<sub>2</sub> and FAAH expression were observed.

Anandamide, 2-AG and the CBR agonist HU-210 potently inhibited CaCo-2 cell proliferation. This effect was blocked by the CB<sub>1</sub> antagonist SR141716A, but not by the CB<sub>2</sub> antagonist SR144528, and was mimicked by the selective CB<sub>1</sub> agonists ACEA and NADA, but not by the CB<sub>2</sub>-selective BML-190. In DLD-1 cells, both CB<sub>1</sub> and CB<sub>2</sub> receptors mediated inhibition of proliferation. Selective inhibitors of endocannabinoid hydrolysis by FAAH (arachidonoylserotonin), or of endocannabinoid cellular uptake via the AMT (VDM11 and VDM13), enhanced CaCo-2 cell endocannabinoid levels and blocked cell proliferation, this latter effect being antagonized by SR141716A. CaCo-2 cell differentiation into non-invasive cells resulted in increased FAAH expression and in no responsiveness to (endo)cannabinoids.

Endocannabinoid levels are enhanced in transformed colon mucosa cells possibly to counteract cancer cell proliferation via CBRs. Inhibitors of endocannabinoid inactivation may prove useful, non-psychotropic therapeutic agents against the growth of colorectal tumour as well as other types of cancers. In support of this possibility, we have found in a separate study (V. Di Marzo, G. Portella and M. Bifulco, manuscript in preparation) that the growth of transformed thyroid cells in athymic mice (Bifulco et al., *FASEB J*, 2001) is inhibited efficaciously by the AMT inhibitor VDM11 via enhancement of tumor endocannabinoid levels.

## P-20

### INHIBITION OF HUMAN GLIOMA CELL GROWTH BY THE NON PSYCHOACTIVE CANNABIDIOL

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Recently, there has been renewed interest in cannabinoids as anticancer agents. A number of reports have appeared indicating an increased rate of apoptosis of C6 glioma cells exposed to THC and inhibition of human glioma cells by a selective CB2 cannabinoid compound (Sánchez et al., FEBS letter 6-10, 436 1998; Sánchez et al., Cancer research 5784-5789, 61 2001). Very intriguing is the demonstration of in vivo efficacy of THC and similar agonists when administered intratumorally to rats harboring intracranial C6 gliomas. ( Galve-Roperh et al, Nature Medicine 313-319, 3 2000).

Because the psychoactivity of cannabinoid compounds limit their medicinal usage, great effort has to be directed toward finding an agent that separates these activities. One such strategy is based on the possibility to use cannabinoid compounds which show little or no psychotropic effects. For these reasons cannabidiol (CBD) appears to be, among the bioactive constituents of *Cannabis sativa*, one of those with the highest potential for therapeutic use.

Therefore we undertook the present study to evaluate the in vitro antiproliferative effects of CBD on human glioma cell lines.

CBD was tested for its ability to alter the baseline proliferation rate of human U373 and U87 astrogloma cell lines. The addition of CBD to the culture medium led to a dramatic drop of mitochondrial oxidative metabolism in glioma cells (MTT test), in a concentration-dependent manner (4-30  $\mu$ M), already evident 24 h after CBD exposure with an  $IC_{50}$  of 25 $\mu$ M.

We next performed experiments aimed to verify the cannabinoid or vanilloid receptors involvement on the antiproliferative effect of CBD. Incubation of glioma cell line with SR141716 (CB1 receptor antagonist), SR144528 (CB2 receptor antagonist) or capsazepine (vanilloid receptor antagonist) did not antagonized, neither alone nor in combination, the growth inhibition induced by CBD.

To test whether the CBD-induced reduction in viability resulted from induction of apoptosis, we determined the level of apoptotic cells in control and drug-treated cells. Flow-cytometric analysis revealed an appearance of apoptosis after propidium iodide-staining on about 40% of the total cell population using the  $IC_{50}$  concentration.

Concluding, in our conditions, the non-psychoactive CBD acts to produce a significant antitumor activity inducing apoptosis on human glioma cells and its effects result independent from cannabinoid/vanilloid receptors activation. The present results further confirm the possible application of cannabinoid compounds as antineoplastic agents.

CBD was supplied by GW Pharmaceuticals (UK)

## P-21

### A METABOLICALLY STABLE ANANDAMIDE ANALOGUE INHIBITS ONGOING TUMOUR GROWTH AND TUMOUR METASTASIS

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Stimulation of cannabinoid CB<sub>1</sub> receptors by the endocannabinoid analogue, Met-Fluoro-Anandamide (Met-F-AEA), inhibits the growth of a rat thyroid cancer cell-derived tumor in athymic mice by inhibiting the activity of the oncogene product p21<sup>ras</sup> (Bifulco et al., *FASEB J.*, 2001). Here, by using this and another in vivo animal model, we investigated whether CB<sub>1</sub> stimulation can also inhibit the progress of ongoing tumors, and interfere with the processes of angiogenesis and metastasis, which are also partly controlled by p21<sup>ras</sup>.

Met-F-AEA blocked the growth of tumors previously induced in nude mice by the subcutaneous injection of rat thyroid cells transformed in vitro by a v-K-*ras* containing retrovirus (Ki-MSV). The effect of Met-F-AEA was counteracted by the selective CB<sub>1</sub> receptor antagonist SR141716A. Since CB<sub>1</sub> stimulation in v-K-*ras* transformed cells does not lead to apoptosis, we investigated the hypothesis that the in vivo anti-cancer effect was due, in part, to inhibition of angiogenesis. Indeed, Met-F-AEA significantly inhibited, in tumors as well as transformed cells, the expression of both the vascular endothelial growth factor (VEGF), an angiogenetic factor known to be up-regulated by p21<sup>ras</sup>, and of one of its receptors, the flt-1/VEGFR-1. These effects were also antagonized by SR141716A. Conversely, the levels of the cyclin-dependent kinase inhibitor p27(kip1), an endogenous suppressor of cancer growth, angiogenesis and metastasis, which is down-regulated by p21<sup>ras</sup>, were increased by Met-F-AEA in a way sensitive to SR141716A.

We found that Met-F-AEA inhibits *in vitro* the growth of a metastasis-derived thyroid cancer cell line more potently than a primary rat thyroid cancer cell line. We suggest that this effect is possibly due to the stronger up-regulation of CB<sub>1</sub> receptor expression induced by Met-F-AEA in the metastasis-derived cell line. These latter observations indicate that CB<sub>1</sub> receptor stimulation interferes with metastatic processes, a hypothesis that we tested in a widely used model of metastatic infiltration in vivo, the Lewis lung (3LL) carcinoma in C57Bl/6 mice. After 21 days from the injection of 3LL cells into the left paw of mice, Met-F-AEA reduced significantly the number of metastatic nodes, and this effect was again attenuated by SR141716A.

Our findings indicate that drugs that activate the cannabinoid CB<sub>1</sub> receptor might be used therapeutically to retard cancer growth in vivo not only by suppressing VEGF signalling and angiogenesis, as recently shown concurrently and independently from our group also for skin tumors (Casanova et al., *J. Clin. Invest.*, 2003) and gliomas (Blazquez et al., *FASEB J.*, 2003), but also by inhibiting tumour metastatic spreading.

Supported by a grant from the Associazione Italiana per la Ricerca sul cancro

## P-22

### NEUROPROTECTIVE EFFECT OF WIN-55212 IN A *IN VIVO* MODEL OF HYPOXIC-ISCHEMIC ENCEPHALOPATHY IN NEWBORN RATS IS BOTH CB<sub>1</sub> -DEPENDENT AND –INDEPENDENT.

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**Background:** cannabinoid agonists are neuroprotective in *in vivo* models of acute brain ischemia in adult rats. This effect was mediated by CB<sub>1</sub> receptors. In newborn rats, *in vivo* studies have been limited to excitotoxic insults. Nevertheless, excitotoxicity is only one of the factors involved in hypoxic-ischemic brain damage in newborns.

**Aim:** to investigate the neuroprotective effect of WIN-55212 in an *in vivo* model of hypoxic-ischemic insult in newborn rats, and to determine the role of CB<sub>1</sub> receptors in such effect.

**Methods:** left common carotid artery was ligated in anaesthetised 7-day-old Wistar rats (P7), which were then asphyxiated by inhaling 100% nitrogen during 10 min. Pups recovered from asphyxia received s.c. vehicle (n=23), the cannabinoid agonist R(+)-WIN-55212-2, 0.1 mcg/kg (n=18), or WIN plus the CB<sub>1</sub> antagonist SR-141716, 3 mg/kg (n=10). Pups undergoing sham operation remained as controls (n=12). Pups brain coronal sections were obtained at 14<sup>th</sup> day (P14) and observed under optical microscopy after Nissl or Fluoro-Jade B staining, to quantify in CA1 area of hippocampus and parietal cortex the amount of surviving or degenerating neurones, respectively.

**Results:** Acute asphyxia led to early neuronal loss, accounting for 19% in hippocampus and 29% in cortex (both ANOVA p<0.05 vs. control), as well as to delayed neuronal loss (proportion of degenerating neurones in vehicle group: 13% in hippocampus and 20% in cortex; in control: 4.8% and 3.1%, respectively, ANOVA p<0.05). WIN-55212 administration prevented neuronal loss (neuronal count similar to that of control) as well as neuronal degeneration (proportion of degenerating neurones: 4.3% in hippocampus and 4.1% in cortex, ANOVA p<0.05 vs. vehicle). Co-administration of SR141716 did not modify the protective effect of WIN-55212 on early neuronal death (neuronal count similar to control and WIN-55212 groups), but abolished the WIN-55212-induced prevention of delayed neuronal death (proportion of degenerating neurones after SR141716 co-administration: 16.1% in hippocampus and 16.7% in cortex, both ANOVA p<0.05 vs. control or WIN-55212, p>0.05 vs. vehicle).

**Conclusions:** administration of WIN-55212 after an acute severe asphyxia in newborn rats is neuroprotective, reducing both early and delayed neuronal loss. Neuroprotective effect is due to two parallel mechanisms, either CB<sub>1</sub>-dependent or –independent.

(Supported in part by a grant of the Sociedad Española de Neonatología; SR-141716 kindly supplied by Sanofi, Inc.).

**P-23**

**CHANGES IN THE EXPRESSION PATTERN OF CANNABINOID CB<sub>1</sub> AND CB<sub>2</sub> RECEPTORS AND FATTY ACID AMIDE HYDROLASE IN SELECTED AREAS OF THE BRAINS OF ALZHEIMER'S DISEASE PATIENTS**

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The endocannabinoid system is still poorly understood. Recently, the basic elements that constitute it, i.e. membrane receptors, endogenous ligands and mechanisms for termination of the signaling process, have been partially characterized. However, there is a considerable lack of information concerning the distribution, concentration and function of those components in the human body particularly during pathological events. We have studied the status of some of the components of the endocannabinoid system, fatty acid amide hydrolase and cannabinoid CB<sub>1</sub> and CB<sub>2</sub> receptors, in post mortem brains from patients with Alzheimer's disease. Using specific polyclonal antibodies, we have performed immunohistochemical analysis in hippocampus and entorhinal cortex sections from brains of Alzheimer's disease patients. Our results show that both fatty acid amide hydrolase and cannabinoid CB<sub>2</sub> receptors are abundantly and selectively expressed in neuritic plaque-associated astrocytes and microglia, respectively, while the expression of CB<sub>1</sub> receptors remains unchanged. In addition, the hydrolase activity seems to be elevated in the plaques and surrounding areas. Thus, some elements of the endocannabinoid system may be postulated as possible modulators of the inflammatory response associated to this neurodegenerative process and as possible targets for new therapeutic approaches.

Supported by Comunidad Autónoma de Madrid (08.5/0005.1/2001), to J.R., Fondo de Investigaciones Sanitarias (00/0260 and 00/0251), to J.R. and R.T., and NIDA (DA09155), to C.J.H.

**P-24**

**INVOLVEMENT OF GLIAL ACTIVATION IN THE PROTECTIVE EFFECTS OF HU-210, A NON-SELECTIVE CANNABINOID AGONIST, AGAINST 6-HYDROXYDOPAMINE-INDUCED NEURONAL DEATH**

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Alterations in function of substantia nigra glia in Parkinson's disease (PD) include the release of cytokines such as IL-1 $\beta$  that cause dopaminergic cell death or increase neuronal vulnerability to neurotoxins. Because cannabinoids inhibit IL-1 $\beta$  synthesis and release from glia, and have anti-inflammatory and neuroprotective actions *in vitro* and *in vivo*, we tested the hypotheses that: (i) IL-1 $\beta$  is an essential mediator in the process of neuronal death, and (ii) cannabinoids protect from neuroinflammatory stimuli that lead to dopamine neuron cell loss and contribute to PD pathogenesis. Hypothesis 1: Cell survival was measured after (i) direct exposure of cultured cerebellar granular neurons to 6-hydroxydopamine (6OHDA), or (ii) exposure of these cells to glial conditioned medium obtained from mixed glial cultures treated with 6OHDA. In this last case, mixed glial cultures were obtained from C57BL/6 (wild type, WT) or IL-1 $\beta$  deficient C57BL/6 (IL-1 $\beta$  knock out, KO) neonatal mice. As expected, direct exposure to 6OHDA induced neuronal death. The same effect was observed when neurons were exposed to conditioned media obtained from WT-glial cells exposed to 6OHDA. In contrast, cell survival increased when the neurons were exposed to conditioned media obtained from glial cultures from KO-IL-1 $\beta$  mice. Hypothesis 2: cerebellar granular neurons or mixed glial cultures were pre-treated with the non-selective cannabinoid agonist HU-210 before incubation with 6OHDA, and cell survival was quantitated in all cases. When neurons were treated with 6OHDA and incubated with HU-210, we observed a slight neuroprotective effect, which was not dose-dependent. However, HU-210 had a stronger neuroprotective effect if it was added to cultured glial cells (WT or KO-IL-1 $\beta$ ) and the conditioned media added to cerebellar granular neurons. The effects were dose-dependent, and therefore likely to be cannabinoid-receptor mediated. In conclusion, our data indicate that glia-derived IL-1 $\beta$  plays an important role in toxin-mediated neuronal death and that cannabinoids can confer neuroprotective changes in glia.

**P-25**

**THE CANNABINOID AM404, ANANDAMIDE REUPTAKE BLOCKER, CONTROLS ANTIPARKINSONIAN EFFECTS IN RATS**

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Modulation of the endocannabinoid system has been proposed that might be useful in treating Parkinson's disease. We have observed that, in the unilateral 6-OHDA-lesion model of Parkinson's disease in rats, systemic administration of AM404, anandamide reuptake blocker, exerts antiparkinsonian effects within a wide dose range. Local injections of AM404 into denervated striatum reduced motor deficits in a dose-dependent manner, these effects being mediated by an antagonistic effect upon D2 dopamine receptors together with a positive interaction with 5-HT<sub>1B</sub> serotonin receptors. Motor 5-HT<sub>1B</sub>-mediated effects were blocked by D1 receptor antagonism, pointing to a positive interaction between striatal 5-HT<sub>1B</sub> and D1 receptors after AM404. The findings suggest that systemic administration of AM404: i) exerts antiparkinsonian effects through stimulation of 5-HT<sub>1B</sub> serotonin receptors that positively modulate D1 dopamine receptors, and ii) might enhance anandamide activity in parkinsonian striatum which could have beneficial effects on altered motor function. Hence, anandamide reuptake blocking might be of therapeutic value in the control of symptoms of Parkinson's disease.

*Study supported to EFE by Plan Andaluz de Investigacion (CVI-127), Plan Nacional para las drogas y Laboratorios Dr. Esteve (Barcelona).*

**P-26**

**SENSORIMOTOR GATING IN MICE IS DISRUPTED AFTER AM404, ANANDAMIDE REUPTAKE BLOCKER**

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Prepulse inhibition (PPI) is a phenomenon wherein the startle response is reduced when the startling stimulus is preceded by a low intensity prepulse. It represents a normal sensorimotor gating response that is typically impaired in schizophrenic patients. In rats, PPI is selectively disrupted (decreased PPI) by dopamine agonists such as apomorphine as well as psychotomimetics. Disruption of PPI is attenuated by antipsychotic drugs, hence it is considered as a valid predictor of “psychosis-like” in animal models. It is known that cannabinoid CB1 agonists such as WIN 55,212-2 reduces sensorimotor gating in rats, suggesting that the CB1 receptor and the cannabinoid system are involved in information processing. However, PPI changes in rats are masked by decreased startle amplitude responses. In this study, the effects of AM404 (0, 2.5 and 5 mg/kg IP) on PPI and startle response in Swiss mice have been studied. The PPI protocol was based on standard methodologies following acoustic stimuli (pulse, 120 dB; prepulses of 70 and 80dB). The findings indicated that AM404 disrupted PPI at the highest dose tested (70 dB prepulse,  $p < 0.01$ ; 80 dB prepulse,  $p < 0.05$ ), and enhanced startle response at the 2,5 mg/kg dose ( $p < 0.01$ ). The data indicates that AM404 behaves like a psychotomimetics at high doses because disrupts normal sensorimotor gating, pointing to a possible “psychosis-like” state after AM404. The startle response was enhanced only following a moderate AM404 dose, indicating that AM404 induced hyperreactivity at a dose that did not affect PPI, further reinforcing a selective disruption of PPI after high AM404 dose.

*Study supported to EFE by Plan Andaluz de Investigacion (CVI-127), and Plan Nacional para las drogas.*

## P-27

### **MICE DEFICIENT IN CANNABINOID CB1 RECEPTORS DISPLAY A HIGH LEVEL OF ANXIETY AND DEPRESSION STATES**

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The role of cannabinoid receptor in the regulation of anxiety states is largely unknown. Here, we report that mice deficient in CB1 receptors display anxiogenic and depressogenic states. For that purpose, both wild type and mutant mice were exposed to different behavioral tests widely used to measure anxiety (open field, elevated plus maze, light-dark box and social interaction test) and depression states (forced swim test).

First we evaluated spontaneous locomotor activity in the open field for a period of 30 minutes. Distance and speed in peripheral and central areas were studied, by dividing the total time into periods of 10 minutes. The results revealed that distance in peripheral areas of the open field significantly increased at 10 and 20 min. whereas distance in central areas significantly decreased in the first 10 min period in CB1  $-/-$  mice when compared to CB1  $+/+$  mice. Speed in central areas significantly increased at 10 and 20 min. periods in CB1  $-/-$ . These results suggest that deletion of cannabinoid CB1 receptor produced altered responses in the open field that appear to be closer to anxiety-related behaviors than to impaired motor activity of these mice.

In the light/dark box test, CB1  $-/-$  spent significantly less time in the light area and displayed a significant reduction in the number of transitions between compartments, suggesting aversion for the light area. Similarly, in the elevated plus maze, mice CB1  $-/-$  spent less time in the open arms and showed a pronounced decrease in the number of transitions between closed and open arms. Also, in the social interaction test, the time in which mice from separate cages (unfamiliar situation) engaged in social interaction was significantly lower in CB1  $-/-$ .

In these four different types of experimental paradigms, deletion of CB1 receptors produces an increase of anxiety related behaviours, supporting the role of cannabinoid receptor function in the regulation of emotional responses.

In the forced swim test, a behavioral model of depression, CB1  $-/-$  mice exhibited a significant increase in time of immobility, compared to CB1  $+/+$  mice, suggesting a greater tendency in these animals to helplessness and depressive-like behaviour.

Overall, our findings revealed that CB1 receptors play a pivotal role in the regulation of emotional responses, although further investigations are needed to determine the neurobiological mechanisms underlying the role of endocannabinoid system in control of emotional states.

**P-28**

**ATTENUATION OF SPONTANEOUS OPIATE WITHDRAWAL IN MICE BY THE ANANDAMIDE TRANSPORT INHIBITOR AM404**

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The endogenous cannabinoid, anandamide, has been shown to attenuate naloxone-precipitated opiate withdrawal in rodents. Here we show that the spontaneous, but not the naloxone-precipitated withdrawal syndrome in morphine-dependent mice is attenuated by the inhibitor of carrier-mediated anandamide transport N-(4-hydroxyphenyl) arachidonylethanolamide (AM404) (2 and 10 mg/kg, intraperitoneal). These results suggest that spontaneous but not opioid antagonist-precipitated withdrawal is associated with dynamic changes in endogenous cannabinoid signaling, suggesting that along spontaneous, but not precipitated abstinence, there are dynamic changes in anandamide production/metabolism.

**P-29**

**CB1 RECEPTOR ACTIVATION BY THE CANNABINOID AGONIST WIN 55,212-2 IS REDUCED IN THE CAUDATE-PUTAMEN OF MU OPIOID KNOCKOUT MICE**

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Although the existence of functional links between the endogenous cannabinoid and opioid systems has already been demonstrated in numerous studies, extensive research is still needed to elucidate the biochemical mechanisms involved in this interaction. The absence of  $\mu$ -opioid receptors has recently been shown to abolish THC conditioned place preference in mice, suggesting that  $\mu$ -opioid receptors activity can modulate the pathways involved in the rewarding effects of cannabinoids. To test the possible existence of changes in the expression and/or functional activity of cannabinoid receptors in  $\mu$ -opioid receptors knockout mice, we have performed quantitative receptor autoradiography of CB1 cannabinoid receptors and activation of GTP-binding proteins by CB1 agonists in the brains of wild-type and homozygous knockout mice. No significant differences were obtained in the levels of CB1 receptors in the brains of  $\mu$ -opioid receptor mutant mice. In contrast, the activation of CB1 receptor by the cannabinoid agonist WIN 55,212-2 was dramatically reduced in the caudate-putamen of  $\mu$  knockout animals when compared to wild-type controls. Since co-expression of CB1 receptors and  $\mu$ -opioid receptors in the same patch neurons of the rat caudate-putamen nucleus has recently been reported, the present results suggest that deletion of  $\mu$ -opioid receptors uncouples CB1 receptors located in these striatal neurons.

**THE CB<sub>1</sub> CANNABINOID RECEPTOR ANTAGONIST, SR 141716A, IS SELF-ADMINISTERED I.C.V. BY RATS:INTERACTION WITH OPIOID SYSTEM**

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[N-piperidino-5-(4-chlorophenyl) 1-(2, 4-dichloro-phenyl)-4-methyl pyrazole-3-carboxamide hydro chloride] (SR 141716A) is a particularly interesting drug. First described as a selective CB<sub>1</sub> receptor antagonist, SR 141716A can block the effects of the cannabinoid agonists in a competitive manner (Rinaldi-Carmona et al., 1994). Besides the well known antagonistic effects on reward produced by natural and synthetic cannabinoid agonists (Maldonado & Rodriguez de Fonseca, 2002), contradictory results on rewarding effects of SR 141716A *per se* have been obtained. SR 141716A has been shown to establish place preference for the drug-paired compartment (Sañudo-Peña et al., 1997; Cheer et al., 2000), to have no effect using the same test or nose-poke self-administration (Chaperon et al., 1998; Hutcheson et al., 1998, Navarro et al., 2001) or to have low abuse liability in monkeys (Beardsley et al., 2002). The purpose of the present work was to examine the effect of i.c.v. self-administration of SR 141716A (0.03-6 µg per infusion) on an operant responding procedure as previously described (Braidà et al., 2001). On the basis of individual preference for one of two levers, developed during training, male Wistar rats were allowed to self-administer vehicle from the preferred lever and SR 141716A from the other. There was a progressive increase in the number of the bar pressings of the less preferred lever when SR 141716A was delivered at concentrations from 0.03 to 0.12 µg per infusion. Starting from the unit dose of 1.5 µg per infusion a gradual decrease in the number of pressings delivering the drug and an increase of those delivering vehicle, was obtained. The maximal reinforcing unit dose was 0.12 µg per infusion. Daily pretreatment with the CB<sub>1</sub> cannabinoid receptor agonist CP 55,490 (0.01 mg/kg) or naloxone (0.1-2 mg/kg) produced a significant decrease of SR 141716A self-administration. These findings provide the first evidence that SR 141716A is self-administered i.c.v. in rats via CB<sub>1</sub> and opioid receptor interaction.

**P-31**

**CHRONIC AND INTERMITTENT ALCOHOL CONSUMPTION DIFFERENTIALLY ALTER CANNABINOID RECEPTOR FUNCTION IN THE CAUDATE-PUTAMEN OF NON-ALCOHOL PREFERRING RATS**

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This study was designed to examine the effects of voluntary, chronic and intermittent forced alcohol intake on alcohol consumption and cannabinoid receptor function in the caudate-putamen (CPu) of alcohol non-preferring rats. To this purpose, male Wistar rats (275-300 gr) were exposed to free water/ethanol (10%) choice during 9 weeks to determine their preference for ethanol intake. After this period of time rats were considered as non-preferring if the mean ethanol intake was lower than 0.5 gr ethanol/kg/day. These non-preferring rats were divided into three groups: (NP) having free access to water and alcohol (2 bottles paradigm) during 13 weeks; a second group (7D), exposed to forced ethanol (10%) intake during 9 weeks; and a third group (4D) which was exposed to forced ethanol for 4 days a week and free water the rest of the week, during 16 weeks. All three groups, were exposed to 3 different types of stressors (cold, noise and restraint stress) in alternated days: 8 weeks (NP group), 4 weeks (7D group) and 11 weeks (4D). After the appropriated treatments, rats of each group were killed by decapitation and brain were quickly removed and frozen over dry ice. Coronal brain sections (12 µm) at the level of CPu were mounted onto gelatin-coated slides and cannabinoid receptor function was determined using WIN-55,212-2-stimulated [<sup>35</sup>S]GTPγS binding receptor autoradiography.

The results show that rats from continuous forced alcohol group (7D) and those from the intermittent group (4D) drink approximately 3.5 g/kg/day whereas the group with free access to water and ethanol did not reach the mean average of 2 g/kg/day. WIN-55,212-2-stimulated [<sup>35</sup>S]GTPγS binding increases 32% in the (4D) group and decreases 28% in (7D) group but was without effect in the NP group. Alternated stress cycles increased alcohol consumption (approximately 1 g/kg/day) in 7D and 3D groups reaching up to 3 g/kg/day only in the NP group. Stress markedly increased (57%) WIN-55,212-2-stimulated [<sup>35</sup>S]GTPγS binding in CPu of the 7D group and was without effect in NP and 3D groups.

In conclusion, the results revealed that: 1) the alteration of the pattern of alcohol consumption increased the intake of alcohol in alcohol non-preferring rats, 2) the pattern of alcohol intake differentially affects cannabinoid receptor function in CPu of these rats and 3) Stress increased the intake of alcohol compared to its respective non-stress group, however it appears that there is not a correlation between the degree of increase of alcohol intake and the change in cannabinoid receptor function in CPu.

Supported by FIS (01/1438) to J. Manzanares

P-32

**DIFFERENCES IN BASAL CANNABINOID AND OPIOID FUNCTIONAL ACTIVITY IN SELECTIVE BRAIN AREAS AND VULNERABILITY TO ALCOHOL CONSUMPTION BETWEEN WISTAR AND FAWN HOODED RATS**

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A number of studies suggests that alterations in gene expression of opioid peptides may alter the vulnerability to alcohol dependence and the therapeutic efficacy of alcohol anticraving drugs. Considering that administration of cannabinoid receptor agonists enhance endogenous opioid activity, differences in endogenous cannabinoid and opioid function may suggest distinct vulnerability and/or dependence to alcohol consumption.

This study examined the functional activity of opioid and cannabinoid systems in genetic alcohol-preferring (Fawn-Hooded) rats and alcohol non-preferring (Wistar) rats under basal conditions. To this purpose,  $\mu$ -opioid (DAMGO)- or cannabinoid CB1 (WIN-55,212)-stimulated [<sup>35</sup>S]-GTP $\gamma$  binding autoradiography, and opioid (proenkephalin (PENK) and proopiomelanocortin (POMC)) and cannabinoid (cannabinoid CB1 receptor) gene expression by *in situ* hybridization histochemistry were measured.

The results revealed that  $\mu$ -opioid [<sup>35</sup>S]-GTP $\gamma$  binding is approximately 25% lower in caudate-putamen (CPu), nucleus accumbens (Acc) and cingulate cortex (Cgx) in Fawn Hooded compared to Wistar rats. Similarly, PENK (CPu) and POMC (ARC) gene expression was also reduced in Fawn Hooded rats. Taken together, these results suggest a lower endogenous opioid function in the alcohol-preferring strain of rats.

CB1-cannabinoid receptor stimulated [<sup>35</sup>S]-GTP $\gamma$  binding autoradiography in the CPu was a 50% lower in Fawn Hooded rats compared to Wistar, whereas no differences between both strains were found in the substantia nigra. In addition, CB1 receptor mRNA levels were also lower in the CPu (25%), area CA3 (20%) of the hippocampus and ventromedial nucleus (60%) of the hypothalamus in Fawn Hooded compared to Wistar rats.

Overall, our findings led us to speculate that lower opioid and cannabinoid function appear to be related to a greater vulnerability to alcohol consumption. Furthermore, these results suggest that both systems may represent key targets in the treatment of alcohol dependence.

(supported by grant from Plan Nacional sobre Drogas to J. Manzanares)

**P-33**

**CANNABIS PSYCHOSIS: A FIVE YEARS FOLLOW-UP STUDY**

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The debate about the existence of cannabis psychosis as an independent entity remains unsolved. Thornicroft (1990) and Thomas (1993) supports the hypothesis that cannabis psychosis is just the first step to schizophrenia in predisposed subjects. Núñez Domínguez and Gurpegui (2002) describe it as a real disorder, different from schizophrenia.

We present the results of a five years follow-up study of a sample with the diagnosis of cannabis-induced psychotic disorder and we show the results concerning current diagnosis and the discussions about them.

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## P-34

### EXAMINATION OF BEHAVIORAL AND MOLECULAR CHANGES ELICITED BY AN ACUTE ADMINISTRATION OF SR141716 TO $\Delta^9$ -TETRAHYDROCANNABINOL-TOLERANT RATS, AN EXPERIMENTAL MODEL OF CANNABINOID ABSTINENCE

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Whether chronic cannabinoid consumption produces a dependence state comparable to those occurring with other drugs, with appearance of withdrawal signs when the consumption is interrupted, and whether chronic cannabinoid consumption increases the risk to consume other drugs of greater addictive power, are probably the two aspects of cannabinoid addiction that provoke more controversy. The present study was designed to explore these two questions in laboratory animals. First, we examined, by analyzing a variety of behavioral, endocrine and molecular parameters, the effects of an acute challenge with SR141716, a selective antagonist for the cannabinoid CB<sub>1</sub> receptor, in  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC)-tolerant rats, which has been reported that precipitates a pharmacological cannabinoid withdrawal syndrome. Second, we evaluated whether  $\Delta^9$ -THC-tolerant rats were more vulnerable to morphine in a self-administration paradigm for this opioid.  $\Delta^9$ -THC-tolerant rats, as expected, exhibited a marked reduction in CB<sub>1</sub> receptor binding sites in specific brain nuclei indicating pharmacodynamic tolerance. The administration of SR141716 tended to increase ambulation and stereotypic activities, and reduced the inactivity in  $\Delta^9$ -THC-tolerant rats, whereas this did not occur in controls, where, even, SR141716 reduced ambulatory activity. The administration of SR141716 to  $\Delta^9$ -THC-tolerant rats also enhanced responses such as tremor, turning and retropulsion, responses that were only slightly enhanced in controls. The administration of SR141716 increased plasma prolactin and corticosterone levels in controls, but these increases were much lower in  $\Delta^9$ -THC-tolerant rats. The analysis of proenkephalin-mRNA levels in several brain nuclei did not reveal any relevant differences between controls and  $\Delta^9$ -THC-tolerant rats after SR141716 challenge, and the same happened with the levels of c-fos mRNA. However, CRF-mRNA levels, whereas reduced in SR141716-treated controls, were significantly increased in  $\Delta^9$ -THC-tolerant rats. The analysis of endocannabinoid contents also revealed that the administration of SR141716, which was mostly inactive in controls rats, was, however, able to reverse the changes, found in  $\Delta^9$ -THC-tolerant rats, in anandamide or 2-arachidonoylglycerol levels in the striatum, diencephalon, cerebellum and brainstem, but not in other areas such as the midbrain and hippocampus. Finally,  $\Delta^9$ -THC-tolerant rats self-administered morphine to a similar extent than control rats, indicating that morphine is equally reinforcing for both animal groups. Accordingly, we did not observe any differences in dopaminergic activity measured in limbic and motor regions, before or after the morphine challenge, between  $\Delta^9$ -THC-tolerant and control rats. In summary, our data indicate that  $\Delta^9$ -THC-tolerant rats were not more vulnerable to reinforcing properties of morphine. However, they responded to the blockade of CB<sub>1</sub> receptors exhibiting slightly but possibly relevant differences compared to the response in non-tolerant rats. Therefore, this is indicative of the existence of a pharmacological withdrawal in cannabinoid-tolerant rats, that would be, however, mild compared to the extent of abstinence in morphine- or cocaine-dependent rats.

## **P-35**

### **ACTIVATION OF MULTIPLE TRANSCRIPTION FACTORS BY ACUTE AND CHRONIC EXPOSURE TO THC**

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The stability of the behavioral abnormalities that characterize addiction indicates that drug-induced changes in gene expression may be involved. In fact long-lasting changes in neuronal efficacy depend critically on gene regulation and protein synthesis. In considering how these events come about, it is important to understand the relationship between membrane events and nuclear events. A key intermediate in this process is the induction of signaling pathways that lead to phosphorylation of constitutive transcription factors (i.e. CREB) interacting with promoter regulatory elements. In this way, drug-receptor interaction rapidly activates expression of a class of genes called immediate early genes, encoding for inducible transcription factors that in turn will regulate expression of a later wave of genes coding for proteins directly involved in changes in synaptic efficacy or cellular morphology.

At this regard a single dose of THC was found to upregulate mRNA levels of c-fos, c-jun and zif-268 in the rat forebrain (Mailleux et al., *Neuroreport* 5, 1265-68, 1994). It was also reported that THC acutely increases AP-1 DNA-binding and Fos-related antigen activity in different areas of the rat brain (Porcella et al., *Eur. J. Neurosci.* 10, 1743-51, 1998). However, modulation of transcription factors activation after in vivo chronic exposure to THC remains to be established.

In the present work we surveyed the activation of both constitutive transcription factors, such as CREB, and inducible transcription factors, such as c-fos and fos B, in the CNS of rats acutely treated, tolerant and sensitized to THC.

The picture in the brain regions is quite different according to the considered transcription factor. CREB activation decreased in the caudate-putamen after the acute injection of THC, returned to control level after the chronic exposure and slightly decreased in sensitized rats. In the hippocampus there was a different trend: neither acute nor chronic exposure to THC altered CREB activation, however sensitized rats showed increased levels of pCREB. Regarding c-fos, in all the brain regions considered, it increased after the acute injection of THC, returned to control value in tolerant rats and increased again in sensitized animals. Finally, Fos B significantly increased in the caudate putamen of sensitized rats, gradually accumulated in the hippocampus of treated animals and slightly increased in the cerebellum of sensitized rats.

These results suggest that, as already shown for other drugs of abuse, chronic exposure to cannabinoids modulate the activation of some transcription factors previously implicated in drug addiction, in specific brain areas. The target genes of these factors remain to be established.

**P-36**

**BEHAVIOURAL AND GENE TRANSCRIPTION ALTERATIONS INDUCED BY SPONTANEOUS CANNABINOID WITHDRAWAL IN MICE**

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This study examined behavioral, hormonal and gene expression alterations that occur during tolerance and during cannabinoid withdrawal in mice. Tolerance was assessed by measuring rectal temperature and motor activity in the open-field test after CP-55,940 administration. Cannabinoid withdrawal symptoms were determined by measuring motor activity and behavioral signs. Corticosterone plasma concentrations were measured by radioimmunoassay. Cannabinoid receptor function was assessed by measuring cannabinoid receptor stimulated [<sup>35</sup>S]GTPγ binding autoradiography. Changes in tyrosine hydroxylase (TH), proenkephalin (PENK), pro-opiomelanocortin (POMC) and CB1 receptor gene expression induced by spontaneous cannabinoid withdrawal were determined by *in situ* hybridization.

Tolerance to CP-55,940 treatment developed for hypothermia, ambulatory and exploratory locomotor activity. Cessation of cannabinoid treatment resulted in a behavioral withdrawal syndrome characterized by a pronounced increase in ambulatory activity and rearings. Corticosterone plasma concentrations dramatically increased 24 and 72 h after cessation of cannabinoid treatment. Similarly, an increase (40%) in cannabinoid [<sup>35</sup>S]GTPγ binding was detected on days 1 and 3 of abstinence. Spontaneous cannabinoid withdrawal produced time related significant alterations in gene transcription :1) decreased (20%) TH mRNA levels in the ventral tegmental area and increased (50%) in substantia nigra, 2) increased PENK gene expression more than 100% in caudate-putamen, nucleus accumbens, olfactory tubercle and piriform cortex, 3) increased (20-40%) POMC gene expression in the arcuate nucleus of the hypothalamus and 4) increased in (20-30%) in CPu, Ce, and CA1 whereas in CA2 and CA3 of the hippocampus a significant decrease (15-20%) was detected.

These results suggest that spontaneous cannabinoid withdrawal occur after cessation of CP-55,940 treatment. This "syndrome" includes behavioral, hormonal and gene transcription alterations that may result relevant to understand cannabinoid addiction and/or vulnerability to other drugs of abuse.

Supported by FISS (01/1438) to J Manzanares.